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The Potential Productivity of Fresh Water Environments As Determined By An Algal Bioassay Technique

by

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ERRATA In a number of places in the text Selenastrum Gracile is written incorrectly as Selenastrum Gracile.

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FOREWORD

This bulletin is published in furtherance of the purposes of the Water Resources Research Act of 1964. The purpose of the Act is to stimulate, sponsor, provide for, and supplement present programs for the conduct of research, investigations, experiments, and the training of scientists in the field of water and resources which affect water. The Act is promoting a more adequate national program of water resources research by furnishing financial assistance to non-federal research.

The Act provides for establishment of Water Resources Research Institutes or Centers at Universities throughout the Nation. On September 1, 1964, a Water Resources Research Center was established in the Graduate School as an interdisciplinary component of the University of Minnesota. The Center has the responsibility for unifying and stimulating University water resources research through the administration of funds covered in the Act and made available by other sources; coordinating University research with water resources programs of local, State and Federal agencies and private organizations throughout the State; and assisting in training additional scientists for work in the field of water resources through research.

This report is number 20 in a series of publications designed to present information bearing on water resources research in Minnesota and the results of some of the research sponsored by the Center. The study with which this Bulletin is concerned was undertaken to develop a practical algal bioassay which could be used in the evaluation of the growth potential of water and therefore the state of its enrichment. An assay procedure involving the organism Selenastrum gracile was devised which could be used to evaluate the effects of chemicals on the aquatic environment as well as the detection of the ordinary enrichment associated with sewage and organic industrial wastes.

INTRODUCTION

Man has long been utilizing and depending upon surface waters of lakes, rivers, reservoirs and streams for a variety of needs and activities. These waters have been used as a source of drinking water, for domestic needs as bathing and washing, for recreation including swimming, fishing and boating, for industrial purposes and for agriculture (Klein 1962, Owens and Wood 1968). Surface waters have also been used to carry away wastes produced by man (Hynes 1963, Erichsen-Jones 1964, Klein 1962). When our population centers were small and our industries were modest, surface waters were able to accommodate the influx of human-related wastes without adverse effects (Hynes 1963, Klein 1962). However, with the coming of the industrial revolution in the nineteenth century and the industrial complex associated with an ever-increasing population, surface waters began to deteriorate and exhibit calamitous changes. This deterioration has become progressively more widespread and is now a matter for public concern (Beeton 1967, Hindle 1959, Hynes 1963, Erichsen-Jones 1964, Macon and Worthington 1951, Owens and Wood 1968, Gotaas et al. 1966).

During recent years considerable attention has been focused upon those materials produced by man and his activities which, upon being discharged into surface waters, subsequently enhance the fertility or alter the quality of these surface waters. Increased fertility or enrichment--termed eutrophication by some--usually stimulates an increase in the amount of plant growth or primary productivity characteristic of a given body of water. An increase in primary productivity can, in turn, stimulate an increase in the amount of animal growth or secondary productivity. Evaluation of this secondary productivity includes consideration of carnivorous animal populations as well as the herbivores which feed directly on plant material (Clarke 1954, Kendeigh 1964, Odum 1959, Oswald and Golueke 1966, Reid 1961, Ruttner 1964, Welch 1952). As indicated above, the process of surface water enrichment and the subsequent increase in productivity is generally referred to as eutrophication (Bardach 1964, Hasler 1947, Hynes 1963, Klein 1962, Owens and Wood 1968, Sawyer 1966).

Eutrophication can be good or bad, depending upon the extent to which changes have occurred in the aquatic habitat or upon the degree of interference with the intended or desired use of the water (Kendeigh 1964, Pentelow 1959, Sawyer 1966). For instance, an increase in the size of the game fish population brought about as a result of an increase in fertility would certainly delight the fisherman. However, the rapid enrichment of an aquatic system by discharges of certain industrial wastes, domestic sewage or nutrients leached from heavily fertilized farm lands frequently results in unwanted biological, chemical and physical changes. These deleterious changes are usually accompanied by algal blooms, unwanted growths of higher aquatic plants, odors and water treatment plant problems as well as the destruction of desirable aquatic species (Beeton 1967, Fruh et al. 1966, Hynes 1963, Kendeigh 1964, Klein 1962).

Productivity resulting from pollution may reach such a level that desirable aquatic species are unable to survive and are replaced by completely undesirable forms which proliferate into large nuisance

populations (Deak 1964, Cooke 1961, Copeland 1966, Dean 1964, Edmonson et al. 1956). Trout, whitefish and pike are examples of recreationally and commercially desirable gamefish which have disappeared or have been drastically diminished in many highly-enriched or eutrophic waters; they have been replaced by the rough fish such as carp, sheepshead and buffalo. The latter are not only able to tolerate these waters but actually thrive and develop into large, unwanted populations (Beeton 1967, Fruh et al. 1966, Hasler 1947, Hynes 1959). Similarly, certain species of blue-green algae responsive to high nitrogen and phosphorus and resistant to the stresses of the polluted environment have also been able to proliferate in highly enriched waters and produce nuisance growth (Edmonson et al. 1956, Hynes 1959, 1963, Oswald and Golueke 1966, Sawyer 1966). Among the flowering plants both the attached and free-floating forms tend to produce dense growths in enriched waters. These growths interfere with wading, swimming, boating and fishing as well as the aesthetic value of the environment (Hasler 1947, Prescott 1948). In situations such as those cited, the recreational use of surface waters has diminished and property values have decreased. Excessive algal growths also constitute a problem in water treatment plants because they clog water channel intakes and sand filters. Also, the decomposition of these algae produces taste and odor problems (Dean 1964, Hasler 1947, Prescott 1948). Furthermore, decomposition and--in some instances--the respiration of these algae can result in the removal of oxygen to the point where suffocation of desirable gamefish species occurs. This type of oxygen reduction occurs commonly in summer and is typical of winter periods when a heavy snow layer covers the ice and prevents light from reaching the photosynthetic organisms. Under such conditions, septic decomposition ordinarily leads to obnoxious odors, dark murky waters and the formation of gases which accumulate under the ice (Bardach 1964, Hasler 1947, Prescott 1948). Even where incident light is adequate, the turbidity produced by the accumulation of organic material in highly productive waters decreases the amount of light energy available to the aquatic organisms and can, therefore, adversely affect desirable aquatic organisms (Erichsen-Jones 1958, Klein 1962, Newell 1959). Upon settling, these materials form layers of debris over the bottom which can interfere with fish spawning and destroy or modify communities of bottom-dwelling organisms (Bullard 1966, Dean 1964, Fruh et al. 1966).

Another aspect of severe eutrophication is the fact that various species of blue-green algae are able to produce substances toxic to man and other animals. Among the reported poisonings have been cases of human gastro-enteritis in which the patients, while swimming, had accidentally swallowed large concentrations of toxic blue-green algae (Dillenberg and Dehnel 1960, Schwimmer and Schwimmer 1955). The deaths of thousands of mammals and birds--specifically horses, cattle, pigs, dogs, cats, shorebirds and waterfowl--from the toxic products of these algae is a matter of record. In every instance, lake water laden with blooms of blue-green algae had been ingested (Gorham 1964, Olson 1951, 1964). In addition, these algae have been reported to stimulate allergic responses; sensitive people who have swum or waded in waters containing blooms of blue-green algae soon after developed a severe dermatitis or demonstrated other reactions (Coen and Reif 1953, Heise 1949a, 1949b, Schwimmer and Schwimmer 1955).

If we wish to preserve our aquatic environment for future generations to utilize and enjoy, we will have to establish realistic water quality standards which can be used as a basis for controlling the amounts of enriching substances entering these waters. The establishment of such standards is not an easy task because of the large number of variables affecting the outcome of eutrophication. The interactions between the nutritional substances and the physical environment, the physical environment and the biotic community, and between the members of the biotic community all affect the level of productivity in aquatic habitats (Provasoli 1958). These differences are so variable in each aquatic habitat (Hutchinson 1957, Reid 1961, Welch 1952) that water quality standards for one aquatic system may not be applicable to another. It is evident, therefore, that a great deal of information about the biological, chemical and physical aspects of surface waters must be known before adequate criteria and, ultimately, satisfactory standards can be established. Whereas much research has been and is presently being conducted to provide us with this information, many gaps still exist in our knowledge. One of the areas which should be given special attention is the development of new techniques and the improvement of established methods for various water quality measurements.

Several methods are presently available for estimating the amount of productivity occurring in aquatic habitats. These include methods that provide an estimation of the standing crop or biomass of organisms present in the body of water at the time of sampling. For instance, water samples may be analyzed microscopically for the types and numbers of plankton organisms present (Putnam and Olson 1961, Standard Methods 1965). Other biomass estimations involve the analysis of residues obtained by passing the water sample through a suitable type of filter. From these residues dry weight, ash-free dry weight, pigment content, suspended proteinaceous material and total organic material of the contained organisms may be determined (Fuhs 1966, Reid 1961, Ruttner 1964, Welch 1952).

Another method of measuring productivity involves the rate of carbon-fixation within the aquatic environment and is based upon the measurement of oxygen production or carbon dioxide assimilation by the phytoplankton present in the water sample. Carbon-fixation may also be determined by Carbon^{14} techniques (Putnam and Olson 1961, Reid 1961, Ruttner 1964, Standard Methods 1965, Steeman-Nielsen 1963).

When satisfactory methods are available, chemical analyses can provide reliable information regarding concentrations of some of the myriads of chemicals which may be present in any given aquatic system. However, such analyses do not show the relationship of these substances to phytoplankton growth. Chemicals may be essential to the nutrition of algae or, even if non-essential, they may still stimulate algal growth, whereas other substances may have toxic effects. A complicating factor is that various chemical forms of an essential element, such as nitrogen, differ in their availability to the algae and that certain combinations of chemicals may react synergistically while others are antagonistic and inhibit productivity. Furthermore, slight changes in the concentrations of so-called "micronutrients" may have a dramatic effect on the growth of phytoplankton.

A relatively new technique for studying productivity is the algal bioassay method. Utilizing laboratory-adapted strains of algae, this technique provides a direct method for studying the relationship between phytoplankton growth and the chemical quality of the aquatic habitat. It is sufficiently sensitive to measure minute quantities of nutrients and to detect very slight fluctuations in their concentrations.

OBJECTIVES OF RESEARCH

In the past, various algal bioassay methods were developed without really satisfactory means for standardization. Thus, data obtained from one study were not necessarily comparable with those of another. The present study, initiated in the spring of 1966, was an attempt to improve the bioassay procedure, especially in terms of standardization and, for simplification, the scope was limited to a study of potential primary productivity as manifested in freshwater environments only. The ultimate objectives were as follows:

1. To select an algal species which would have most if not all of the characteristics needed by an ideal assay organism;

2. To develop a standardized procedure for the preparation of test samples as well as for the preparation of the seed algae inoculum;

3. To select the optimum incubation conditions for the water samples to be assayed;

4. To develop and evaluate a procedure for the assessment of algal growth which would be suitable for bioassay;

5. To test the final procedure by applying it to surface water samples collected from selected fresh water environments.

LITERATURE REVIEW

I. THEORY OF ALGAL BIOASSAY

Introduction

For an algal species to survive and grow in the laboratory, it is necessary that specific chemico-physical growth requirements be provided to the culture (Chu 1942, Fogg 1966, Skulberg 1966). These requirements include essential nutritional substances such as nitrogen and phosphorus along with suitable ranges of light and temperature intensities. By maintaining light and temperature at constant intensities suitable for optimum growth of the alga and by using the same size and age inoculum of good algae for all cultures, the amount of growth obtained from the cultures will depend upon the concentration of nutrients provided (Chu 1942, Pratt 1940, Rhode 1950, Skulberg 1966, Wurtz 1964). In nature, where nutrients are not always balanced, growth response reflects the concentration of the nutrient which is exhausted first (Belser 1963, Fogg 1966, Hutner et al. 1958, Kendeigh 1964, Odum 1959, Welch 1952). If one replaces the balanced and chemically defined nutrient medium with a sample of surface water, it will be found that the same relationships between algal growth, light, temperature, inoculum size and age of culture are still maintained although it is recognized that the water sample may contain, in some instances, factors which interfere or act as synergists with respect to the growth of the test alga. For example, various chemicals such as toxic metallic wastes or herbicides and certain algal metabolites can exert an inhibitory effect upon the growth of algae (Jorgensen 1956, Krauss 1962, Pratt 1943, Proctor 1957, Provasoli 1963, Skulberg 1966). On the other hand, growth-promoting substances such as glycollic acid and certain hormones may have a stimulating effect (Nomito and Fogg 1966, Provasoli 1957).

Algal Nutrition and Productivity

A good basic knowledge of algal nutrition is essential to an adequate understanding of the relationships between water fertility and phytoplankton productivity as manifested in surface waters. The inorganic and organic substances necessary for growth and reproduction of algae, the range of critical concentrations of nutrients needed for optimal growth and the chemical forms of nutrients which can be utilized by algae must be known before one can relate algal productivity to enrichment.

The science of algal nutrition began when scientists first isolated selected species of algae and successfully grew them in artificial media. Chu (1942) and, later, Rhode (1950) and Gerloff et al. (1950, 1952) were the first to initiate such comprehensive studies. They determined the importance of various elements to algal nutrition and the range of concentrations of each element which might be considered essential to optimum growth. Chu developed the first completely artificial nutrient medium, thus eliminating the previously undefinable soil-water extracts by substituting known elements including trace metals and chelating agents. This important milestone enabled algal nutritionists to proceed to the discovery that some species of algae

were unable to synthesize all substances essential to their nutritional requirements.

Several inorganic elements, designated as macronutrients because relatively large concentrations are required, have been found to be essential for the growth and reproduction of all algae (Eppley 1962, Eyster 1964, McElroy 1963, Odum 1959). In addition to carbon, hydrogen and oxygen, these elements are nitrogen, sulfur, potassium, iron, calcium and magnesium (Arnon 1958, Eppley 1962, Eyster 1964, Harvey 1937, O'Kelley 1968, Pirson 1955, Ryther and Kramer 1961, Trelease and Selsem 1939). Nitrogen and phosphorus are generally considered most important (Oswald and Golueke 1966, Owens and Wood 1966, Provasoli 1963, Ruttner 1964); for this reason, the role of nitrogen and phosphorus in algal metabolism has been studied more exhaustively than that of the other elements.

When inorganic sources of nitrogen are considered, it is generally believed that ammonia (NH_3) is preferentially absorbed from culture solutions. Nitrate nitrogen (NO_3) is also absorbed readily. nitrite nitrogen (NO_2), on the contrary, can only be assimilated by a limited number of species of algae (Chu 1942, 1943; Fogg 1953, Gerloff et al. 1952, Harvey 1940, 1955b; Kratz and Meyers 1955, Kylin 1945, Provasoli 1958, Rhode 1950, Ryther 1954). Elemental nitrogen (N_2) can only be used by certain nitrogen-fixing species of algae and apparently only the blue-green algae are able to accomplish this.

Organic nitrogen sources utilizable by algae include urea and uric acid which can be directly assimilated by some species and amides and amino acids which are absorbed by algal forms capable of reducing organic compounds to the ammonium form (Eppley 1962, Fogg 1953, Harvey 1940, Kylin 1945, Ryther 1954).

Among the various inorganic phosphorus compounds utilized by algae, orthophosphate phosphorus (PO_4) has been found to be the form preferentially absorbed. Pyrophosphate (P_2O_7) and hexaphosphate [$(PO_4)_6$] are also absorbed readily (Chu, 1942, Fogg 1953, Gerloff et al. 1952, Goldberg et al. 1951, Harvey 1940, 1955a; Ketchum 1939a, 1939b; Rhode 1950). According to some authorities, various organic phosphorus substances may be absorbed directly by some algae. These chemicals included caseinate, phytin and sodium nucleinate (Chu 1946, Harvey 1940, 1955a).

Certain inorganic elements have been referred to as "micronutrients" because they are utilized by algae in very minute quantities (Eyster 1964, McElroy 1963, Odum 1963, Wiessner 1962). Included with the micronutrients are the metallic constituents of enzymes and coenzymes (copper, manganese, molybdenum and zinc) and chlorine which is involved in the evolution of oxygen during photosynthesis (Arnon 1958, Arnon et al. 1955, Evens and Sorger 1966, Eyster 1958a, 1964; Eyster et al. 1956, 1958; Hopkins 1930, Ichioka and Arnon 1955, Kylin 1945, O'Kelley 1968, Pirson 1955, Pirson and Bergmann 1955, Price and Valley 1962, Walker 1953, Wolfe 1954). Other micronutrients such as boron, cobalt, iodine, silicon, sodium and vanadium have been found to be essential to the nutrition of some but not all species of algae (Arnon et al. 1955, Evens and Sorger 1966, Eyster 1964, Holm-Hansen et al. 1954, O'Kelley 1968,

Pirson 1955, Provasoli 1958, Walker 1954, Wiessner 1962). Rubidium, selenium and strontium can replace specific essential elements such as potassium, sulfur and calcium in the metabolic processes of certain species of algae (O'Kelley 1968, Walker 1954, Wiessner 1962).

Organic substances which have been found to be essential to the nutrition of algae include vitamins (cobalamine, biotin and thiamine), amino acids (histidine and aspartic acid) and hormones such as adenine, kinetin and indolacetic acid (Bentley 1958, Brown et al. 1955, Droop 1955a, 1957a, 1961, 1962; Fogg 1953, Ford 1958, Ford et al. 1955, Fries 1959, Gold 1964a, Guillard and Cassie 1963, Hutner et al. 1953, Lewin 1954, 1962; McLaughlin and Provasoli 1957, Provasoli 1957, 1958; Provasoli and Gold 1957, Provasoli and Pintner 1953b). Glycollic acid, an extracellular product of algae, may also be an important nutritional factor. This metabolite has been shown to decrease the length of the lag period of growth for Chlorella cultures (Nalewajko et al. 1963, Nomita and Fogg 1966).

II. THE APPLICATION OF ALGAL CULTURING METHODS TO THE STUDY OF WATER QUALITY

Measurement of Potential Primary Productivity and Relative Nutrient Concentrations

The first studies directed toward a determination of potential primary production were those of Allen and Nelson (1919), Atkins (1923), Schrieber (1927) and Naumann (1929). Using diatoms as test organisms, Allen and Nelson studied the differences in the growth potential of oceanic and aquaria waters. By suitable additions of phosphorus or nitrate salts, they further attempted to evaluate the relative concentrations of these growth-promoting substances in these waters. Schreiber conducted similar experiments with a more refined technique which included the control of light and temperature. Again, the limiting effects of nitrogen and phosphorus on primary productivity of marine waters were studied. Later, Strøm (1933) and Guseva (1937), utilizing the assay technique developed by Schrieber, expanded the scope of this bioassay to include other nutritional elements. Strøm, using lake water and lake mud samples from Lakes Furesø and Hulsø in Norway, determined the relative amounts of nitrogen, phosphorus, magnesium, iron and manganese in these lakes which would be available to selected species of algae. Guseva conducted similar experiments on the availability of nitrogen, phosphorus, iron and manganese in the Ulcho reservoir located on the Moscow-Volga canal in Russia. Having previously studied the nutritional requirements of three species of algae--Anabaena lemmermanni, Aphanizomenon flos-aquae and Asterionella formosa--he related the distribution and succession of these algae to assay results which indicated the availability of these four nutrients. Anabaena, having a high nutrient requirement, was replaced by Asterionella after the former had depleted the nutrients present in the reservoir; Asterionella has a relatively low nutrient requirement and was able to grow in profusion.

Since these early studies, a number of other algal ecologists have used algal bioassay methods to study potential productivity and to assess nutrient concentrations.

Using cultures of the green alga, Scenedesmus, Bringman and Kuhn (1956) studied the growth potential of various filtered water and mud samples taken from the Berlin watershed. From these experiments, which were conducted over a period of several months, the authors were able to determine the source, spread and radius of influence of fertilizing substances in the watershed area. These authors concluded that the algal bioassay method could be a valuable tool in the assessment of eutrophication.

Using a Kirchneriella sp. as his assay organism, Potash (1956) was able to detect a difference in the potential productivity of two interconnected ponds in Vermont. He also found that phosphorus, which in winter was the limiting nutrient in both ponds, became less limiting as spring progressed. By the time the spring phytoplankton appeared, nitrogen became the limiting nutrient and remained limiting throughout the summer. In a highly calcareous lake in Michigan, Eyster (1958a) found that phosphorus was the limiting nutrient factor for Nostoc muscorum; only in cultures where phosphorus was deleted did he detect a significant reduction in the growth response.

In order to determine which nutrients were limiting to natural populations of phytoplankton in Alaskan and Californian lake waters, Goldman (1960a, 1960b) enriched samples, in turn, with phosphate, nitrate, carbonate, manganese, magnesium, and trace metals or vitamins. He found that molybdenum was a limiting factor in one lake and concluded that other trace metals as well could be limiting in freshwater. MacPhea (1961), using the green alga, Chlorella vulgaris, as his bioassay organism, studied limiting nutritional factors in Bear Lake, Idaho. After enriching the lake water samples with a variety of combinations and concentrations of nutrients, this author found that phytoplankton growth was limited by the availability of nutrients and not by the inhibiting effects of excessive nutrients. Lund (1959) investigated the growth potential of tributary streams and the importance of these waters and of basic slag, a material used by English farmers as a phosphorus fertilizer, as sources of enrichment of an English lake. Using Asterionella formosa as his culture alga, the author noted a tremendous increase in the growth potential of the streams after they had passed through a limestone belt. However, basic slag was the most important source of enrichment in the lake. Using Selenastrum capricornutum as the assay organism, Skulberg (1964) carried out several productivity studies. In a study of the Orre River in Rogaland, Norway, this author was able to demonstrate a radical change in the growth potential of the river water between sampling stations. These results, when combined with chemical data, provided a means of assessing the pollution of the river. In a second study, the potential productivity levels of lake inlets and outlets were compared. A drastic reduction was observed in the algal yields from water samples taken at the outlets of lakes when these were compared with growth obtained in water from lake inlets. These results again verified the well-known phenomenon that lakes act as efficient traps of nutrients. Skulberg also used the bioassay method to study the effectiveness of sewage treatment plants and especially their role in reducing the amounts of mineral nutrients present in raw sewage.

Algal ecologists have also undertaken algal bioassay studies of

marine environments. Ryther and Guillard (1959) examined the limiting effects of several nutrients including nitrogen, phosphorus, trace metals, silicon and vitamins on the growth of natural phytoplankton populations in oceanic waters. Later, Menzel and Ryther (1961) enriched Sargasso Sea water samples with nitrogen, phosphorus, trace metals and vitamins to determine which of the four major groups was limiting to natural phytoplankton populations. The individual nutrients were then deleted and added separately until all of the specific controlling nutrient factors had been determined. In 1963 and 1964, Johnston used sea water which was unable to support algal growth (apparently due to a deficiency of trace metals) and found that the samples would produce large yields of algae after certain chelating chemicals were added. Consequently, he concluded that, in oceanic waters, a deficiency in chelating agents might often be the limiting factor in phytoplankton growth; in the absence of chelators, trace metals could be converted into insoluble chemical forms which would be unavailable to the phytoplankton.

Quantitative Measurement of Nutrient Concentrations in Aquatic Habitats

Determinations of actual concentrations of nutrients present in aquatic habitats by algal culturing methods have been carried out during the past two decades by Belser (1953), Ryther and his associates (1949, 1952, 1961) and Provasoli (1953). The methodology is based on the principles that (1) the culture alga will not grow in a nutrient medium devoid of the nutrient to be assayed, and (2) the growth response of the alga is proportional to the amount of nutrient added. When these conditions are satisfied, a standard curve can be derived by determining the growth response of the alga over a wide range of concentrations of the nutrient under study. In the actual performance, the essential nutrients, minus the nutrient to be assayed, are added to the water sample to be tested. The yield of algae in this test portion is compared with the standard curve and, from such a comparison, the concentration of the nutrient can be extrapolated.

This technique has been applied to the study of vitamin B_{12} concentrations in marine waters. Impetus for these studies stemmed from the discovery by Ryther et al. in 1949 that a green alga, Euglena gracilis, exhibited a quantitative growth response to vitamin B_{12}. In 1955, Østergaard Kristensen also investigated this alga as a possible assay organism for vitamin B_{12}, using a method which was later adapted by Daisley (1958) and employed by Daisley and Fisher (1958) in studies of the distribution of vitamin B_{12} in oceanic waters. Another freshwater alga, Ochromonas malhamensis, has also demonstrated a quantitative growth response to vitamin B_{12} (Ford 1953, 1958; Ford et al. 1955, Ryther et al. 1955) and has been used for determinations of the distribution of this vitamin in oceanic and coastal waters (Cowey 1956). However, neither of these two algae gained popular acceptance in oceanographic work because marine water samples had to be desalinated, a process which was both complicated and time-consuming.

From the foregoing, it is evident that the discovery of a suitable marine algal species was at one time highly desirable. Droop (1955b, 1957b, 1961), in studying the marine alga Monochrysis Lutheri, found that this organism exhibited a linear growth response to different concentrations of vitamin B_{12}. However, the alga had an extremely slow growth rate which eliminated it as an assay organism for routine use. Guillard and Cassie (1963), Guillard and Ryther (1962) and Ryther and Guillard (1962) noted that a marine diatom, Cyclotella nana, had all the attributes necessary for a good vitamin B_{12} assay organism. Using this alga, Menzel and Spaeth (1962), Gold (1964b) and Carlucci and Silbernagel (1966a, 1966b) were able to develop specific routine procedures for assaying vitamin B_{12} in marine waters. A marine dinoflagellate, Glenodinium halli, has also been found to be satisfactory for vitamin B_{12} assays (Gold 1964a).

The use of algal cultures for quantitatively studying the distribution of nutrients in fresh waters has not been reported, although Provasoli and Pintner (1953) have suggested Euglena gracilis and Ochromonas malhamensis for such vitamin B_{12} analyses.

Measurement of Enriching Effects of Domestic Wastes on Aquatic Environments

Studying the effect of sewage effluent on two different mixed cultures of algae, one made up mostly of blue-greens and the other primarily of greens, Shapiro and Ribeiro (1965) noted that the addition of secondary sewage effluent to these algal cultures had a significant effect on their growth response. Where blue-greens were predominant they concluded that phosphate-phosphorus (PO_4-P) was the only nutritional factor responsible for the increased growth. Apparently, the blue-greens were able to fix sufficient nitrogen for growth requirements.

In the second mixed culture, in which species of green algae predominated, both phosphate-phosphorus and ammonium-nitrogen (NH_3-N) were needed. These algae are unable to fix nitrogen and, therefore, must rely on some outside source.

Using Chlorella pyrenoidosa as his culture alga, Maloney (1960) studied the effect of detergent phosphorus on growth responses. A series of cultures were prepared using a basal medium alone or the basal medium supplemented with a synthetic detergent or various components of that detergent. Comparisons were made of the growth response in these cultures and it was found that the detergent and one of its ingredients, sodium triphosphate, stimulated growth. No other component of the detergent except alkyl benzyl sulfonate (ABS) had an effect on the growth response. This component had an inhibitory effect which was evident at concentrations above 3.6 ppm. At lower concentrations, no inhibition was observed and Maloney concluded that synthetic detergents could be a factor in eutrophication.

The Selection and Evaluation of Chemicals with Algicidal Properties

Algal culturing techniques can also be adapted and applied to studies of the algicidal properties of chemicals (Allen and Skoog 1951, Fitzgerald 1959, 1962, 1963, 1964; Fitzgerald et al. 1952, Galloway and Krauss 1959, Maloney 1958, Maloney and Palmer 1956, Palmer and Maloney

1955). In these studies, chemicals were added in a series of concentrations to algal cultures, all of which contained the same concentrations of nutrients and algal cells. After the cultures had been incubated at a constant temperature and light intensity for a predetermined period of time, the effect of the chemicals on the algae was determined. Studies of this kind provide information on the selective toxicity and necessary dosages of chemicals needed to kill or control a given species of algae. Furthermore, this method provides a means for detecting and measuring any developing resistance to an algicide on the part of a specific alga.

MATERIALS AND METHODS

I. CULTURE ALGA

There are certain specific attributes which an algal species must possess in order to qualify as an assay organism. For example, the organism should grow readily in laboratory culture and exhibit a rapid growth response (an extended incubation time would severely limit the practical use of the method). The alga should remain in suspension and solitary except during times of cell division; otherwise error will be introduced into the counting procedure. If cells are to be counted microscopically, it is desirable that they should be at least 10u in their longest dimension. Smaller organisms are extremely difficult to count and for routine purposes such counts would be too time-consuming. Another preferable attribute of an assay organism is a relatively non-complex nutritional requirement; e.g., the organism should be able to grow in a completely inorganic growth medium. A final requirement is an absence of sticky secretions. Organisms producing such substances have a tendency to adhere to the walls of the culture vessels and to each other. Errors will be incorporated into the counting procedure due to loss of cells on flasks and to the need for estimation of the cell numbers in the algal clumps. In addition, non-adhering cells can be more easily suspended in solution, thus facilitating the mixing of cultures for an even distribution of cells during incubation and counting.

Several species of algae were cultured during the summer and fall of 1966 for the purpose of evaluating their potential as assay organisms. Species studied included Ankistrodesmus braunii and Golenkinia bibrainum provided by the Botany Department of the University of Minnesota; Chlorella vulgaris and Coccochloris sp. isolated from a pond located near Duluth, Minnesota; and Selenastrum gracile which was obtained from the Zoology Department. Of these species, Selenastrum gracile was selected as the most suitable organism. This species exhibited all of the essential characteristics whereas the other species had some but not all of the necessary attributes.

S. gracile is a species of algae which is widely distributed in the phytoplankton of lakes and ponds, although never in large concentrations (Forest 1954, Prescott 1951, Smith 1950). This green alga (Phylum Chlorophyta) is a member of the family Oocystaceae, a family characterized by the lack of motile reproductive units (Prescott 1951, Smith 1950). Other genera which belong to the same family are the well-known and widely-studied Chlorella and Scenedesmus. The cells of S. gracile (Plates 1 and 2) are crescent-shaped with sharply pointed apices. They are approximately 10-30u in cell length and 3-5u in cell diameter. The chloroplast or pigment-containing body is plate-like and lies along the convex side of young cells. However, in older cells, the chloroplast fills the entire cell. Reproduction is asexual with the mother cell dividing into four smaller daughter cells called autospores. The daughter cells, upon liberation from the mother cell wall, remain temporarily united along their convex surfaces; however, they soon separate, remaining solitary in the culture solution (Cooke 1890, Forest 1954, Prescott 1951, Smith 1920, 1950).

Plate 1. Selenastrum gracile (200X)

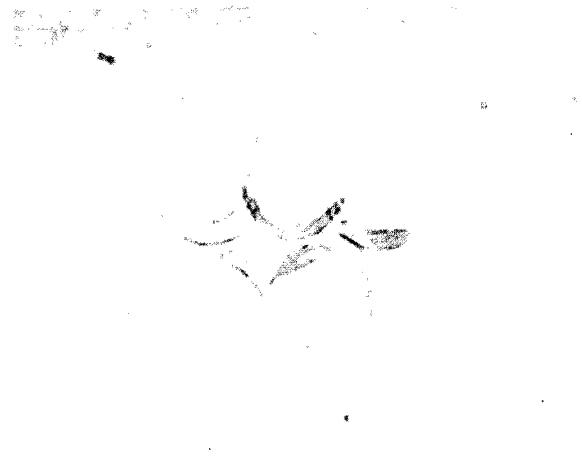

Plate 2. Selenastrum gracile (800X)

II. STANDARD CULTURE MEDIUM

The chemically defined nutrient medium used with this assay method was Bold's Basal Medium (Table I). This medium was originally developed by Bristol (1919a, 1919b) and later modified by Bold (1949). It is highly mineralized and, containing both mono- and di-potassium phosphate salts, has limited buffering ability. The medium also contains EDTA (Ethylenediaminotetraacetic acid), a chelating agent which complexes the metallic ions and keeps them in a chemical form which can be utilized by the alga. The main advantage of this medium over other nutrient media is its relative simplicity and the ease with which it can be prepared. Individual stock solutions of each of the first nine chemical salts (see Table I) plus a trace metal mixture containing the zinc, manganese, molybdenum, copper and cobalt salts were prepared and sterilized for fifteen minutes at 100° C. and 15 psi. Aliquots of these stock solutions were removed, as needed, and combined into the final solution which contained the salt and elemental concentrations listed in the table. The pH of this combined solution was approximately 7.0. Preparing and sterilizing the chemical solutions separately facilitated the maintenance of a sterile medium and eliminated precipitation problems which occurred when the medium was sterilized in the final combined form. The stock solutions and the final combined solution were stored in a dark cool cabinet when they were not being used.

An important consideration to be mentioned here is the necessity of a pure, uncontaminated distilled water source. Many water distillation systems utilize tin-plated copper holding tanks for storing the water after it passes through the distillation apparatus. These tanks should be regularly inspected for breaks in the tin coating and periodically replated; otherwise copper ions may be entering the stored water. Other sources of contamination include dirty faucets and connecting hoses or contaminated containers in which the water may be temporarily stored or transported. Valuable research time can be lost trying to identify the cause of erratic growth results which may occur from such sources of contamination. For this reason, an all-glass distillation apparatus and a glass-lined holding tank can be considered highly desirable if not essential. An additional protection is found in a deionizing column, but it must be inspected frequently and checked for efficiency and freedom from contamination. If the distilled water has to be transported, it should be removed directly from the holding tank and carried in carefully cleaned glass or Teflon-lined containers which are used for no other purposes.

III. SAMPLING PROCEDURE

This study was conducted at the University of Minnesota Limnological Research Center in Duluth, Minnesota. The laboratory is located on the shore of Lake Superior (Figure 1), the world's largest freshwater lake, according to surface area (31,820 sq. miles).* Lake Superior is 350 miles long, has a maximum breadth of 160 miles and a maximum depth

* Lake Baikal, located in the Siberian province of Russia is the world's largest freshwater lake according to volume (Hutchinson, 1957).

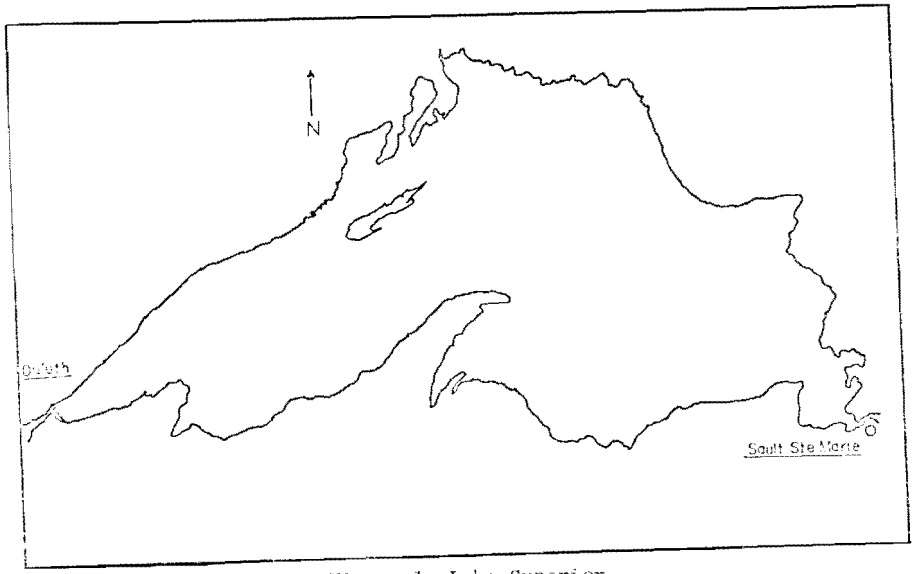

Figure 1. Lake Superior

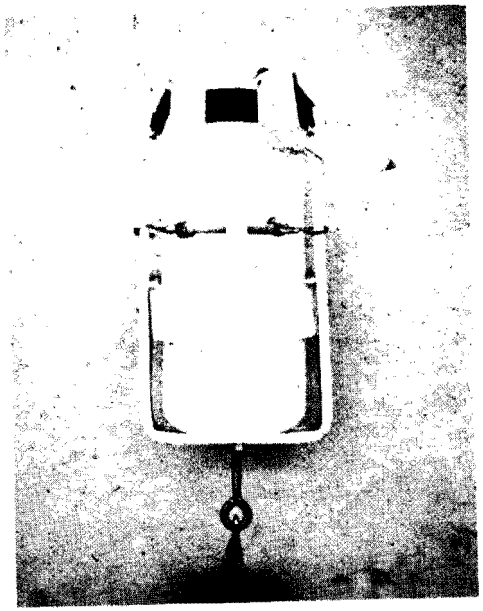

Plate 3. Sampling Apparatus

Table I.

COMPOSITION OF BOLD'S BASAL MEDIUM

Salt	Concentration (ppm)	Element	Concentration (ppm)
$NaNO_3$	250.00	Na	77.48
$CaCl_2 \cdot 2H_2O$	25.00	Ca	6.82
$K_2HPO_4 \cdot 3H_2O$	75.00	K	75.98
$NH_4H_2PO_4$	175.00	N*	7.40
		P	41.23
$MgSO_4 \cdot 7H_2O$	75.00	S	50.06
NaCl	25.00	Cl	11.50
EDTA	50.00		
$FeSO_4 \cdot 7H_2O$	5.00	Fe	21.46
H_3BO_3	11.42	B	1.00
$ZnSO_4 \cdot 7H_2O$	8.82	Zn	2.00
$MnCl_2 \cdot 4H_2O$	1.44	Mn	2.00
MoO_3	0.71	Mo	0.40
$CuSO_4 \cdot 5H_2O$	1.57	Cu	0.47
$Co(NO_3)_2 \cdot 6H_2O$	0.49	Co	0.10

* Does not include nitrogen present in EDTA.

in excess of 1300 feet. The major river flowing into the lake is the St. Louis River which enters the lake at its far western end. Lake Superior drains into Lake Huron (Hough 1958) via the St. Mary's River, essentially a short channel located near the city of Sault Ste. Marie, Michigan.

Water samples were collected from tributary streams and bays as well as from the main body of the lake at sampling stations located along or within the western arm of Lake Superior. These samples were collected in a two-liter plastic bottle harnessed to an aluminum frame which was weighted with a five-pound lead weight (Plate 3). This home-made sampling apparatus was convenient in that water samples of adequate size could be collected and stored in the collecting bottles until processing at the laboratory. In addition, the sturdiness of the sampler permitted the collection of water samples under a variety of different sampling conditions.

Lake water samples were collected from the Duluth entry into Superior Bay (Figure 2) and from bays located along the north shore of Lake Superior from Duluth to Grand Marais, Minnesota (Figure 3). These bays included Lester River Bay, Darlington Bay, Beaver Bay, Lutsen Bay and Grand Marais Bay. The Duluth entry samples were collected by lowering the sampler over the seawall at a location approximately midway between the lake and Superior Bay. The bay water samples were collected approximately 1,000 yards out from shore by using a 14-foot aluminum boat equipped with an outboard motor. To minimize the effects of river effluents, samples collected from bays with tributary streams were taken, in each instance, approximately 1,000 yards north of the river's mouth.

Water samples from the north shore river sampling stations--on the Lester, French, Knife, Gooseberry, Split Rock and Baptism Rivers (Figure 4)--were taken from bridges located along Highway 61. The south shore stream samples were taken along Highway 13 where bridges spanned the Nemadji, Amnicon, Brule, Iron, Cranberry and Sisikiwit Rivers (Figure 4). The sampler was lowered from the upstream side of the bridge except during winter months when ice cover prevented such collection; in winter, a twelve-inch Swedish ice auger was used to cut sampling holes at points approximately thirty yards upstream from the bridges.

Water samples were also taken from the lower St. Louis River from Cloquet to the entry of the river into Superior Bay (Figure 5). The uppermost sampling station was situated at the Highway 33 bridge, located well upstream from the industrial and domestic waste effluents discharged from Cloquet. Below Cloquet, samples were taken at the western or upstream span of the Highway 61 bridge near Scanlon; from the Highway 39 bridge approximately two miles southeast of Carlton; from the bridge at Fond Du Lac; the bridge connecting Duluth, Minnesota and Oliver, Wisconsin; and the Highbridge (Highway 39) at the entrance of the St. Louis River into Superior Bay. The sampling procedure was the same as that used for the other river sampling stations. The stations at Arrowhead Bridge and the Highbridge were sampled only during the winter months and only during the period of ice cover. Samples were taken approximately 100 yards from the Minnesota side.

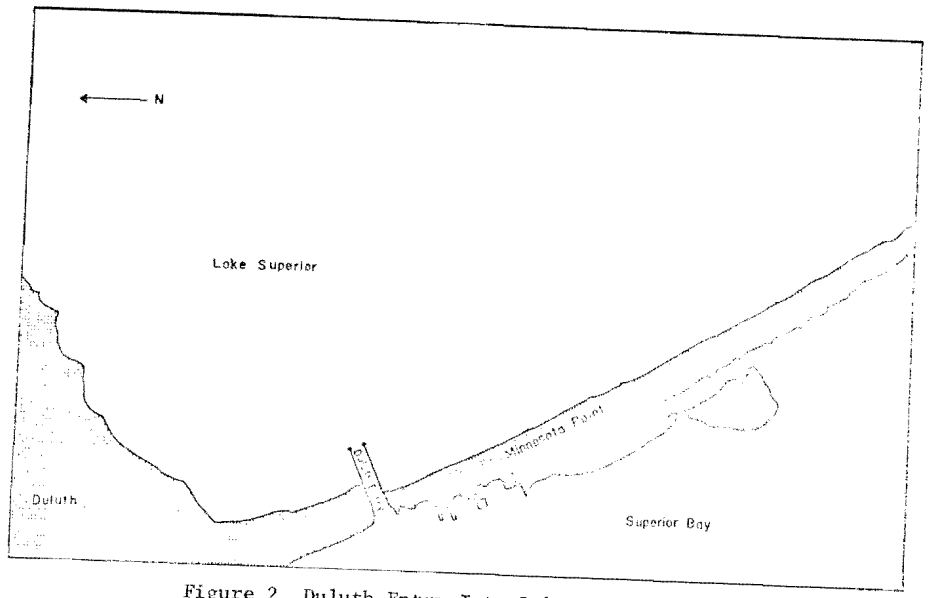

Figure 2. Duluth Entry Into Lake Superior

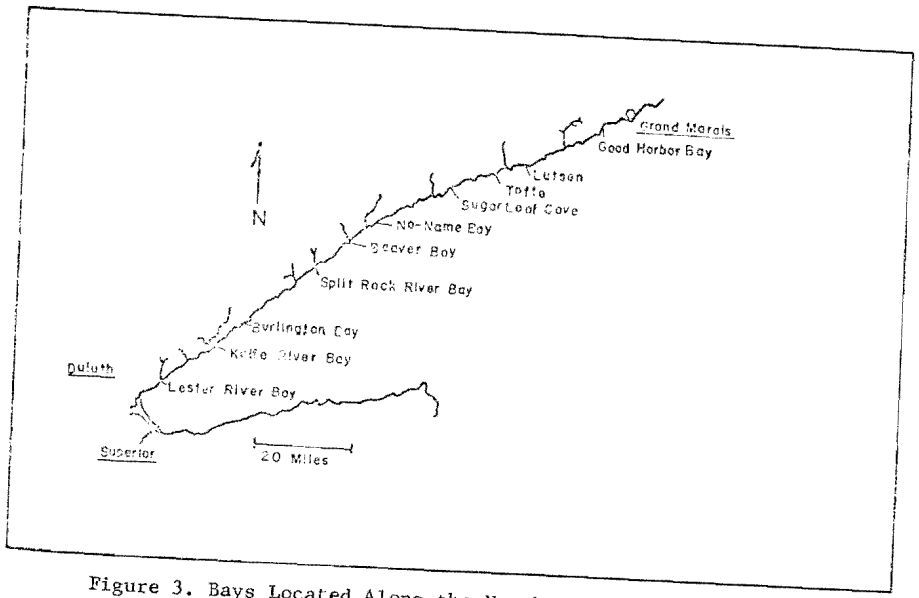

Figure 3. Bays Located Along the North Shore of Lake Superior

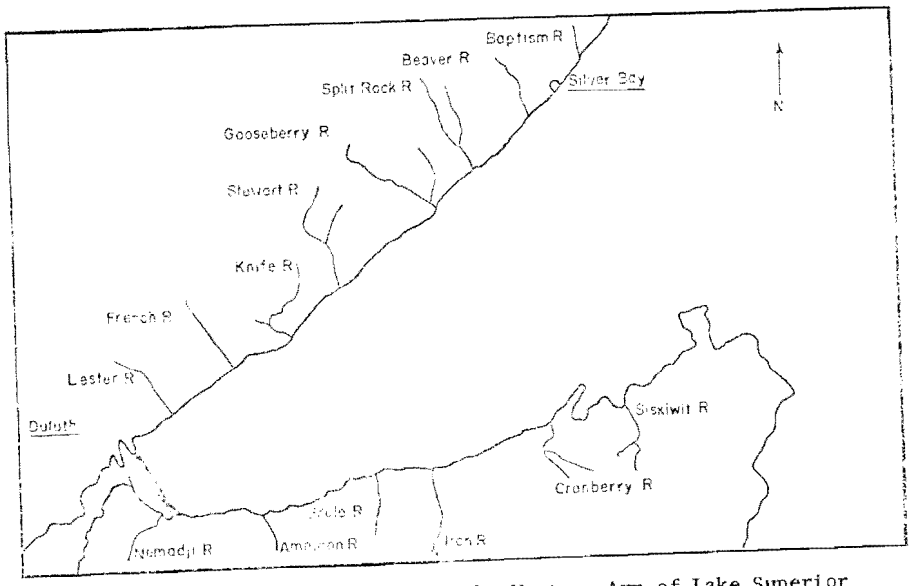

Figure 4. Tributary Streams of the Western Arm of Lake Superior

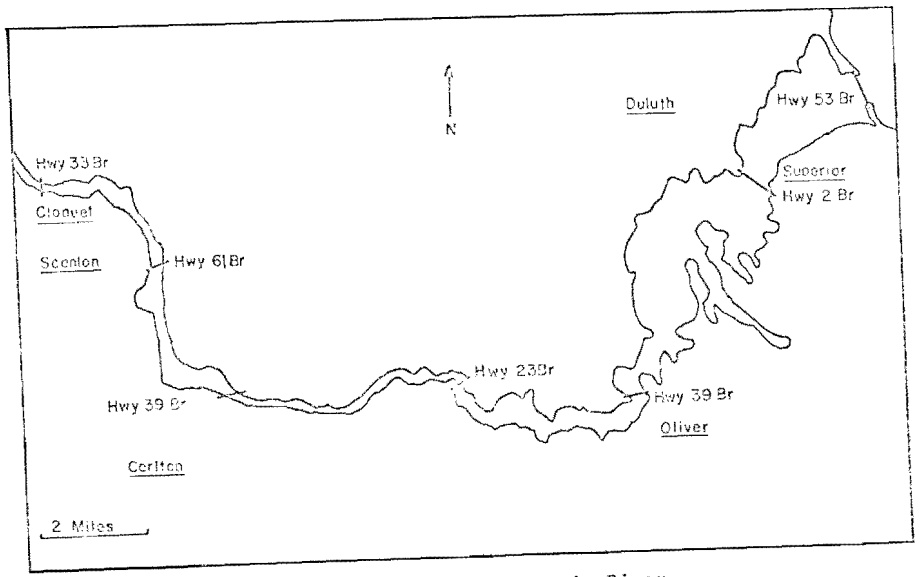

Figure 5. Lower St. Louis River

IV. PREPARATION OF CULTURES

In the laboratory all samples were filtered through a 0.45u Millipore filter to remove bacteria and planktonic organisms. Although some nutrients possibly may have been lost during this filtration process, the removal of all plankton organisms was necessary before inoculation with the assay organism to prevent competition and predation. High temperature sterilization of the water samples would have been another way of destroying interfering organisms but unaccountable changes may be expected in the physical and chemical properties of the water as a result of such heat treatment (Lund 1959, Skulberg 1964). For this reason, the filtration described was thought to be the preferable method for preparing the samples. In addition, by using the same size and type of filter for all water samples, the same sizes of suspended or colloidal materials should be removed, thus leading to a practical standardization of this procedure. Triplicate 200 ml aliquots of these filtered samples were placed in 500 ml Bellco Erlenmeyer flasks which were then ready for inoculation.

Control Cultures

The presence of "bad waters" (waters which contain inhibitory chemicals or lack essential nutritional substances) can be detected by growing control cultures simultaneously with the test cultures (Carlucci and Silbernagel 1966b, Gold 1964b, Provasoli 1963). In this procedure, two types of control cultures, the external and internal standard cultures, are utilized.

The external standard culture consists of the alga grown in a known concentration of the chemically defined medium in distilled water (Carlucci and Silbernagel 1966b, Daisley 1958, Gold 1964a). The yield of algae from these external standard cultures should be statistically equal to one another for all experiments performed. Significant deviations from the usual yield will indicate a failure or departure from the optimum within the culturing system in terms of light or heat, defects in the culture medium, improperly washed or contaminated glassware, impure distilled water or the like.

The internal standard culture consists of the alga grown in the test surface water sample to which is added the same concentration of chemically defined medium as utilized in the external standard culture (Carlucci and Silbernagel 1966b, Daisley 1958, Gold 1964a). The yield of algae from the internal standard culture should be greater than that of either the external standard culture or the water sample culture since the internal standard culture contains the combined nutrients of both. If the yield of algae from the internal standard culture is less than that from the external standard culture, then some inhibitory substance or substances must be present in the water sample. If the yield of algae from the internal standard culture is equal to or only slightly greater than that of the external standard culture, then one or more nutritional substances may be deficient in the water sample culture or some substance or substances having a slightly inhibitory effect on the algal growth may be present. This substance would partially suppress the growth response but not below the response observed in the external

standard culture.

V. INCUBATION EQUIPMENT

Algal cultures were incubated on a 24" x 60" x 52" aluminum shaking table (Plate 4) powered by a 3/4 h.p., variable speed electric motor which was designed for continuous operation and which could be adjusted from 76.7 to 230 r.p.m. For this study, the motor speed was adjusted so as to produce a gentle swirling motion within the culture flasks. The rectangular table was mounted on a 29" x 64" x 1/4" aluminum pan provided with, one-inch, hardened steel cylindrical bearing shafts at each of the four corners. The table's maximum displacement was approximately 5 inches in either direction.

The shaking table had three adjustable plexiglass shelves. The upper two shelves provided adequate space for one hundred 500-ml Erlenmeyer flasks. The third shelf was used for assorted stock algal cultures and pertinent controls. The flasks were held on the shelves by a flexible 2 inch "mesh" of 1/4 inch surgical tubing. The tubing was interwoven and anchored in an aluminum frame bolted around the shaking table four inches above each shelf (Plate 4).

The table was operated in an insulated room 7-3/4 feet long, 6-3/4 feet wide, and slightly over 6-1/2 feet high. Access to this room and the table was through a 30" x 74" door built into the front panel of the box. Each of the removable panels which comprised the sides, floor and roof were constructed from two 1/2 inch plywood sheets, which were separated by a 1-5/8" insulating layer of styrofoam. The room was designed to be portable so that it could be moved to a different location if desired.

Although the temperature within the box could be regulated by thermostatically controlled cooling and heating units, the motor which powered the shaking table generated sufficient heat, even during cold winter months, so that a heating unit was unnecessary.

VI. METHODS FOR ESTIMATING ALGAL CONCENTRATIONS

Three methods were employed for estimating the concentration of algae within the cultures: (1) microscopic cell counts, (2) chlorophyll determinations and (3) dry weight determinations.

Microscopic Cell Count

A compound microscope, at a magnification of 200X, in conjunction with a whipple disc was used for making the cell counts (Plate 5). At the magnification and with the microscope used the grid on the whipple disc when calibrated against a stage micrometer was found to have a surface area of 0.23 mm^2. One ml of the culture was first pipetted into a Sedgewick-Rafter counting chamber, a cell fifty mm long, twenty mm wide, and one mm deep. This chamber was then covered with its glass cover slip and placed on the stage of the microscope. Since the surface area of the grid was 0.23 mm^2 and the depth of the counting chamber was one mm, the volume of culture below the grid was exactly 0.23 mm^3.

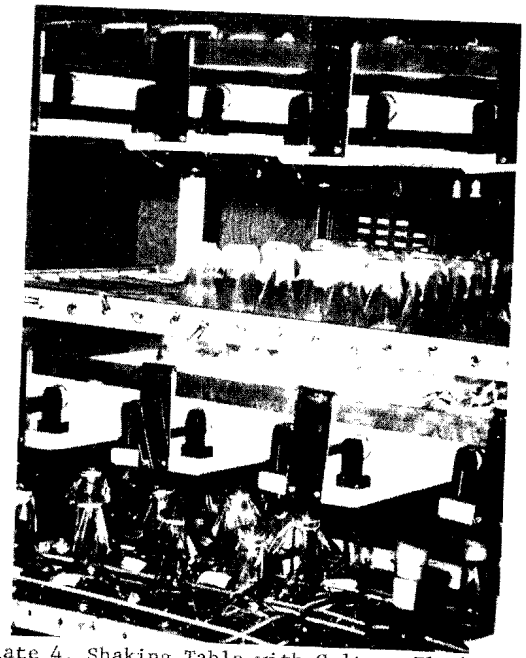

Plate 4. Shaking Table with Culture Flasks

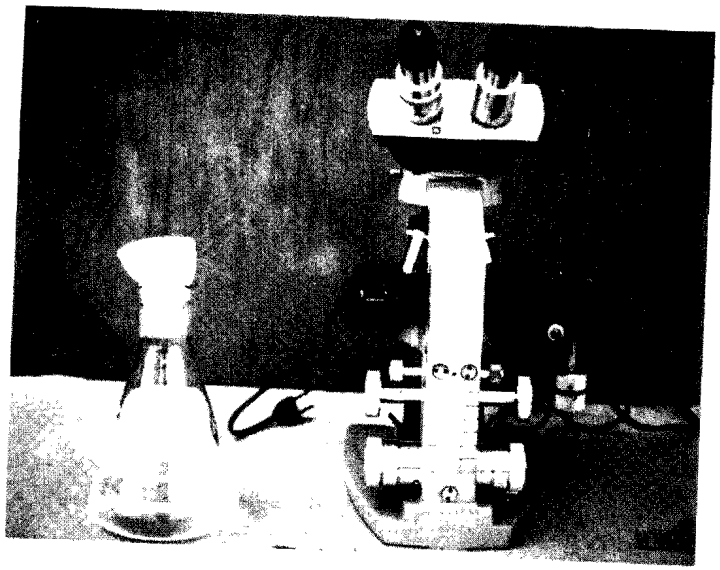

Plate 5. Equipment Used for Microscope Cell Counts

Cells touching the top and bottom edges of the grid were counted while cells touching the two sides of the grid were not. To convert the ten field count to cells per liter, the count was divided by 2.3 and multiplied by 10^6. In cases where cultures are diluted with distilled water the ten field count must also be multiplied by the dilution factor.

Chlorophyll Determination

As a second means of estimating algal growth a modification of the Creitz-Richards (1965) method was used for the measurement of the chlorophyll concentration of cultures.

A 180 ml aliquot of the culture was filtered through a Gelman type filter into a four-liter suction flask (Plate 6). This glass-fiber filter was used in place of the Millipore filter employed by Creitz-Richards in the original method, which when ground had the advantage of breaking into tiny glass fragments which acted as cutting agents in the disintegration of algal cells. After the liquid portion of the aliquot had passed through the filter, the sides of the filterhead were rinsed with distilled water. The algal filtrate was then fixed with fifteen ml of a saturated magnesium carbonate solution to prevent the degradation of chlorophyll to pheophytin. The filtrate was removed from the filter assembly and placed in a Kimax 19 x 150 mm Pyrex mortar (tissue grinder) along with five ml of 90% acetone. A Teflon pestle, powered by a 1/4-horsepower electric drill equipped with a speed reducer (Plate 6), was used to thoroughly grind the filter and the algal suspension was then decanted into a fifteen ml centrifuge tube. The tube was then centrifuged for ten minutes at 5,000 r.p.m. After the ten minute period, the liquid portion was carefully removed with a Pasteur pipette, transferred to a Klett-Summerson colorimeter tube and adjusted to ten ml with 90% acetone. A gentle agitation of the tube insured a uniform mixture of the pigment solution within the acetone. The Klett tube was carefully wiped clean and placed in the Klett-Summerson colorimeter (Plate 6) for the absorbance measurement. Two readings were taken with a number 66 filter which is sensitive to wave lengths ranging from 640 to 700 mu.* Ninety per cent acetone was used as the zero absorbancy standard. The raw data was converted into absolute units (ug chlorophyll per liter) by the following formula:

$$\text{ug chl/liter} = 0.65 \text{ Klett reading } \frac{(1)}{(L)}$$

where L represents the amount of culture analysed expressed in liters (see Klett-Summerson Operation Manual).

* The linear relationship between the Klett-units of the colorimeter and the concentration of chlorophyll dissolved in the acetone solution holds to a reading of approximately 400 Klett-units (See Klett-Summerson Instruction Manual). Therefore, when readings of 400 units or higher were observed, the sample was diluted to twenty ml with ten ml of additional 90% acetone. Ten ml of this diluted sample were then pipetted into a Klett tube and the two readings were taken.

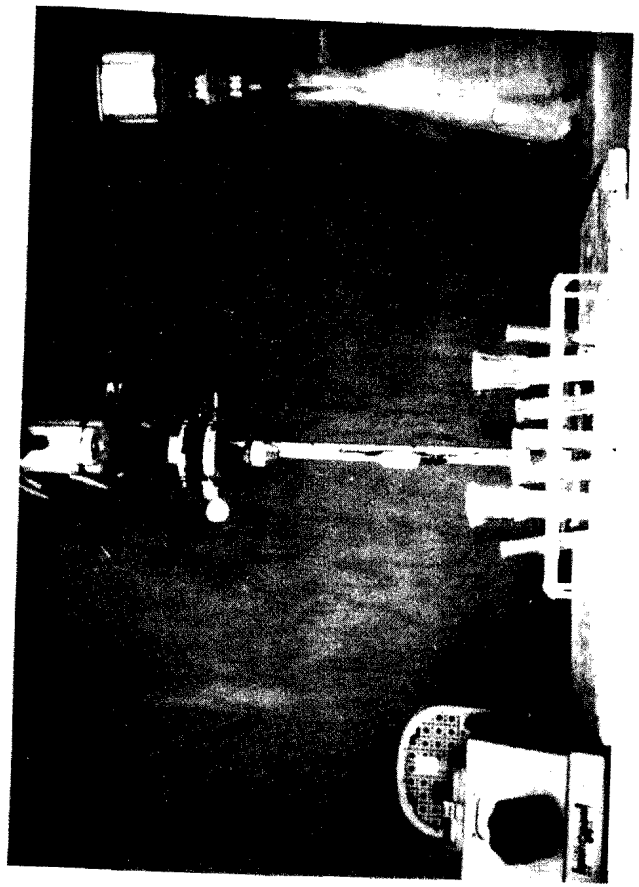

Plate 6. Equipment Used for Chlorophyll Analyses

Dry Weight Determination

Dry weight determination constituted the third method for estimating the concentration of S. gracile in the cultures. Millipore filters (0.45u pore diameter) were dried for twenty-four hours in a desiccating chamber containing anhydrous calcium chloride ($CaCl_2$) before they were weighed on an analytical balance. The algae cells from ten-ml aliquots of the cultures were collected on these pre-weighed filters using the Millipore hydrosol funnel and the four-liter suction flask apparatus, shown in Plate 6. The filters were then redried for twenty-four hours before the final weights were determined.

VII. CHEMICAL DETERMINATIONS

Total Solids

For total solids determinations, 250-ml porcelain casseroles were washed and then dried to constant weight in an oven at approximately 100° C. The casseroles were then allowed to cool in a desiccating chamber for twenty-four hours before the initial weights were measured on a Sartorius analytical balance. Five hundred ml aliquots of the water samples were evaporated in the casseroles over a hot water bath. The water bath was situated within a hood, which had a safety glass sash window, to prevent dust or other material from falling into the samples. After the water sample aliquots had evaporated, the casseroles were placed in the desiccating chamber for twenty-four hours after which final weights were determined.

Dissolved Oxygen

Two modifications of the Winkler method were used in ascertaining the dissolved oxygen concentration of water samples. The Alsterberg-Azide modification was used for all water samples except those collected from the Lower St. Louis River sampling stations from Scanlon to Fond Du Lac. High concentrations of sulfite waste liquor, originating from the paper mills in Cloquet, necessitated use of the alkali-hypochlorite modification for water samples from these stations. All water samples were collected in 250-ml dissolved oxygen bottles using a D.O. sampler designed especially for this purpose. The samples were immediately processed through all steps of the determination until free iodine had been liberated. The final titration with sodium thiosulfate ($Na_2S_2O_3$) was performed upon the return to the Lakeside Laboratory.

pH

Upon reaching the laboratory, the pH of the water samples were immediately measured using a Beckman expanded scale pH meter with hydrogen and calomel electrodes.

RESULTS AND DISCUSSION

I. DEVELOPMENT OF BIOASSAY TECHNIQUES

Cleaning of Glassware

For washing glassware used with this bioassay procedure 0.6N aqua regia acid solution was selected. Commonly used wash solutions such as dichromate-sulfuric acid solutions and detergents were not used because of the possibility of persistent toxic or enriching chemical residuals remaining in the glassware. Such residuals could affect the growth of the culture algae. In order to determine the effectiveness of the acid wash in removing organic and inorganic residuals and also the minimum number of rinses which would be necessary to remove the acidic influences of the acid wash, two kinds of experiments were initiated.

In the first set of experiments, the pH of the rinse water was measured using the Beckman expanded scale electric pH meter. The pH of each of the first six rinses, was measured and then compared with the pH of the original acid wash and the unused distilled rinse water. Figure (6) shows the average pH readings of triplicate samples from 250 ml Erlenmeyer flasks. Five rinses appeared adequate for removal of the residual acidic influences of the wash solution since the effluent of the sixth rinse had the same pH as the unused rinse water. At fewer rinses, however, the effects of the acid wash on the pH of the rinse water effluent were apparent. The rinse water effluent of the fifth rinse had a slightly lower pH reading than the 7.1 reading of the unused rinse water, and the effluent of the fourth and third rinses respectively elicited succeedingly lower pH readings. At two rinses and at one rinse, a very drastic reduction in the pH was observed. By using six rinses, a safety factor of one extra rinse could be added to the procedure. Since the additional time necessary would be small, the addition of the extra rinse seemed justified and this procedure was followed throughout. All six rinses were carried out with distilled and deionized water since the use of a less pure water source could have re-contaminated the glassware.

In the second set of experiments initiated to study the effectiveness of the acid wash on cell growth 250 and 500 ml Bellco Erlenmeyer flasks were rinsed in 10.0% BBM followed by raw sewage as sources of inorganic and organic residuals. The flasks were then washed in the 0.6N aqua regia solution. Triplicate sets of both the 250 and 500 ml flasks were rinsed either two, four, six, eight or ten times in distilled water. One hundred and two hundred ml of a 1.0% BBM solution were placed in these rinsed 250 and 500 ml flasks, respectively. Then an inoculum of S. gracile was added, which provided an initial cell concentration of approximately one million cells per liter. The flasks, plugged with styrofoam stoppers to keep out dust and other foreign material, were placed on the shaking table where they were incubated for seven days at approximately 700 ft-cd light intensity and 20° C. temperature. At the end of the incubation period, final microscopic cell counts were taken on the cultures. The growth results of these cultures are shown in Figure (7). After only two rinses, a complete suppression of growth occurred and after four rinses, significant fluctuation still

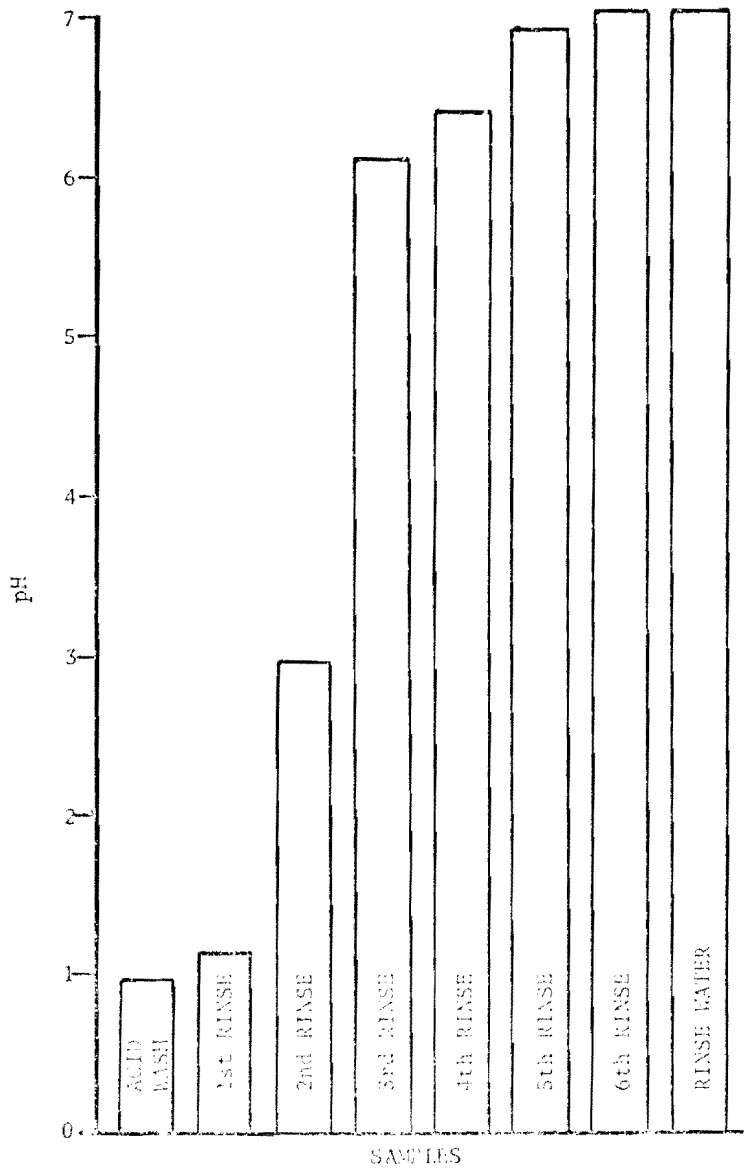

Figure 6

Average pH Readings of Acid Wash, Unused Distilled Rinse Water and Rinse Water Effluent

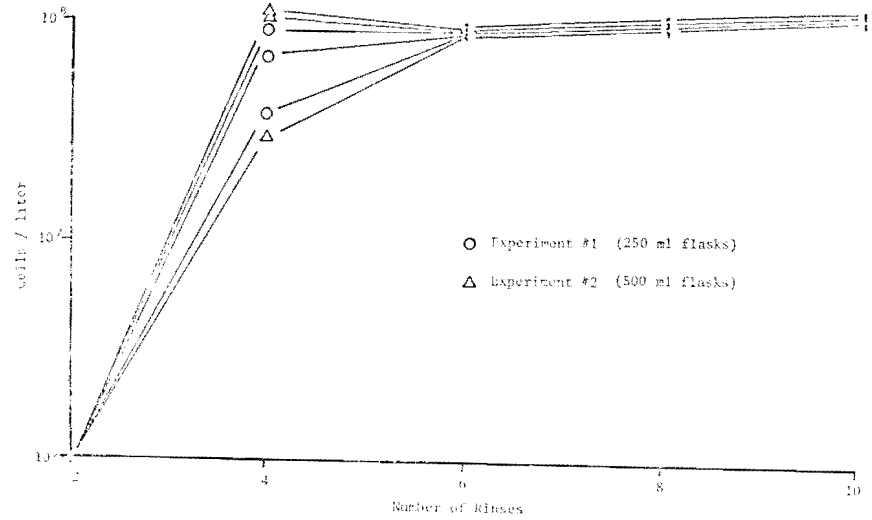

Figure 7

Seven Day Growth Yields of S. Gracile in 1.0% BBM (Flasks Washed Either 2, 4, 6, 8, or 10 Times in Distilled Water)

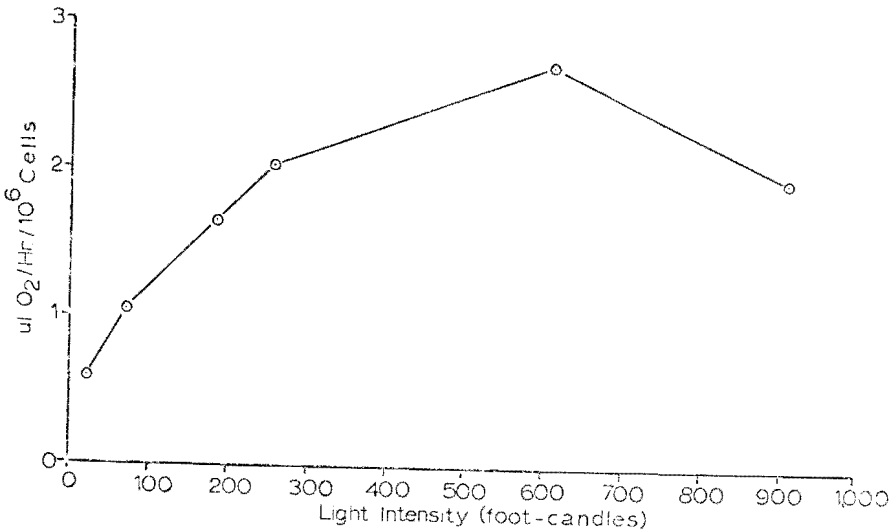

Figure 8

Average Rate of O_2 Production By S. Gracile Incubated at 6 Different Light Intensities

occurred between the algal yields of the triplicate cultures in both the 250 and 500 ml flasks. Six rinses, however, were sufficient to remove the effects of the residual chemicals on the growth of the alga.

The results of the two preceding experiments showed that the washing of glassware with a 0.6N aqua regia solution followed by six rinses in distilled water was a satisfactory method for removing significant effects of residual cleaning chemicals on the growth of S. gracile.

Between periods of use, culture flasks were presoaked in the wash solution by sealing the aqua regia solution within the flasks with parafilm. The flasks were then placed on the shaking table until needed for the next experiment. After the washing and rinsing procedure had been completed, the flasks were dried at approximately 100° C. in an oven. They were then ready for the next experiment. Pipettes were also presoaked in the wash solution but within a Teflon pipette washer, where they were also washed and rinsed. After the six rinses had been completed, the pipettes were removed from the washer, within the Teflon holder, and allowed to air dry. All other glassware, except the previously mentioned culture flasks, were also air dried.

Incubation of Cultures

Illumination was provided from 12 pairs of alternate white and daylight forty watt fluorescent tubes suspended below the plexiglass shelves of the shaking table (Plate 4). Two types of fluorescent tubes were used, because a wider range of photosynthetic pigment sensitive light emissions was obtained. Thus the alga would metabolize and grow with a higher degree of efficiency than when just one type of fluorescent tube was used. This illumination system produced a relatively uniform light intensity of approximately 700 foot candles across the upper surface of the plexiglass shelves as measured with a Weston light meter. Experimental evidence showed that a near maximum rate of metabolic activity was stimulated in S. gracile at light intensities ranging fro approximately 500 to 700 ft-cd. See Figure 8. Allowing for the reduction in light intensity within the culture flasks, the algal cells would be exposed to a light intensity well within this range when using the above mentioned lighting system. Therefore, the length of time which S. gracile would have to be incubated for the production of maximum growth, using this illumination system, would be minimized. In the experiment from which the data for Figure (8) were taken, the rate of oxygen production (ul of O_2/hour/million cells) of S. gracile was measured, at 20° C. in a Gilson differential respirometer. Thirty minute readings were taken at six different light intensities ranging from 60 to 900 ft-cd. The average photosynthetic rates from six algal samples at each light intensity are shown in the graph.

The Gilson differential respirometer also was used to determine the optimum temperature for incubation of the algal cultures. Thirty minute oxygen production studies (ul of O_2/hour/million cells) were made at six different temperatures ranging from 10° C. to 45° C. Figure (9) shows that near maximum photosynthetic rates were stimulated at temperatures ranging from approximately 20° C. to 35° C. The 20° C. temperature was selected for use with this assay method because it could be easily

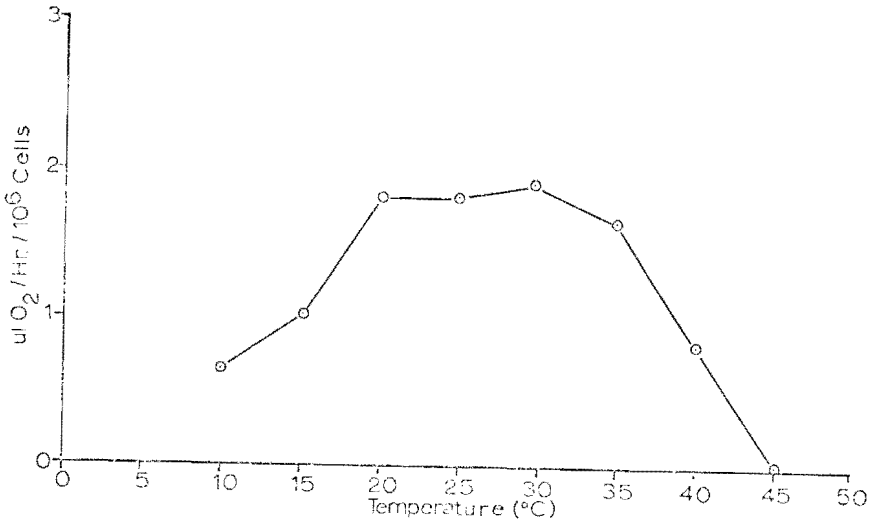

Figure 9

Ave. Rate of O_2 Production by S. gracile Incubated At 8 Different Temperatures

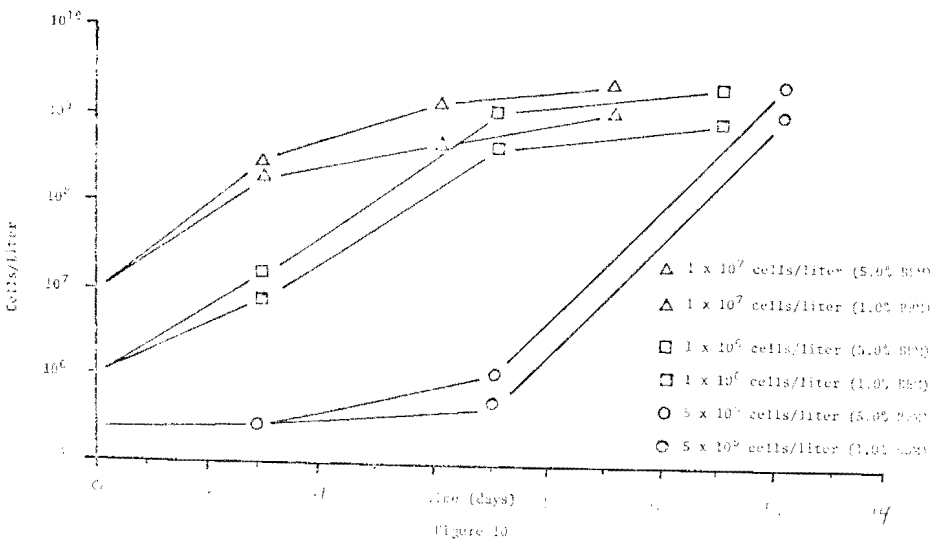

Figure 10

Growth Curves Of Selenastrum Gracile Cultured in 1.0 and 5.0% BBM; Initial Inoculum Concentrations 5.0 X 10^6, 1.0 X 10^6, 1.0 x 10^7 Cells Per Liter

maintained in the constant temperature room. Thermistors, connected to an ARA (Applied Research Association of Texas) electronic thermometer equipped with a strip chart recorder, were placed in the constant temperature room for a period of forty eight hours. One thermister was placed in a one liter Florence flask containing approximately 500 ml of distilled water and the other was directly exposed to the air in the constant temperature room. A temperature fluctuation of no more than 1° C. was observed for the thermister immersed in distilled water and a fluctuation of no more than 3° C. was recorded by the thermister exposed to the atmosphere of the room. For continuous monitoring, a mercury thermometer was placed on the third shelf of the shaker in a Florence flask containing distilled water. Over a sixteen month period, except for three temperature failures related to mechanical difficulties associated with the air conditioner, the temperature of the water within the flask was never observed to fluctuate by more than 1° C.

In order to check the stability of the incubation system, seven experiments were conducted over a two month period. Each consisted of triplicate cultures of S. gracile in both 1.0% and 5.0% BBM solutions. These cultures, containing initial cell concentrations of approximately one million cells per liter were grown for seven days under the previously described incubation conditions. The final cell counts of these twenty-one cultures, at each BBM concentration, were compared statistically using the Chi-square test for randomness (Bancroft 1963, Fisher 1958, Gilbert 1963). At the 0.05 confidence level, no significant deviations from the expected random sampling errors were observed at either concentration (Table II). Thus it appears that the incubation system is completely satisfactory.

Inoculation of Samples

Before each day's experiments were inoculated with the culture algae, the concentration of the algal cells in the continuous stock culture was determined microscopically. One ml of this culture was diluted using nine ml of distilled water so that a countable concentration of cells would be obtained. This concentration was approximately sixty cells per random field. After the concentration of the algae in the continuous culture had been estimated, an aliquot of five ml was removed and diluted, quantitatively, with sufficient distilled water to produce a cell concentration of approximately 2.0×10^{7} cells per liter. One ml of this diluted algae was then inoculated into the cultures in a total volume of 200 ml which gave an initial cell inoculum concentration of 1×10^{6} cells per liter. Initial cell counts were not taken on these individual cultures because the very low concentration of cells, approximately two cells per ten fields counted, was insufficient for accurate counting. However, this technique of estimating the concentration of cells in the continuous culture and then quantitatively diluting and inoculating, provided a practical method for inoculating all of the cultures. In use it proved to be a very satisfactory procedure for arriving at a uniform inoculum.

The one million cells per liter initial inoculum concentration selected for use with this assay method was based on experiments which determined the length of time needed to reach the stationary phase of

TABLE II

Ten Random Field Counts And Klett Readings Obtained From Seven Experiments. Each Consisting of Triplicate Cultures Of Selenastrum Gracile Grown In Both 1.0 and 5.0% Bold's Basal Medium For Seven Days

Experiment Number						
1	2	3	4	5	6	7
(1.0% BBM - Cell Counts)						
198	215	175	203	198	200	198
223	205	163	198	188	170	193
203	213	193	173	205	183	170
$\bar{X}$ = 194						
X^2 = 27.8						
0.95 confidence limits = 168-224 (cells per ten random fields) = 7.3-9.7 x 10^8 (cells per liter)						
(1.0% BBM - Klett Readings)						
32.8	20.0	21.6	23.9	19.6	22.5	14.7
26.2	20.3	19.1	21.9	23.0	27.5	15.3
31.9	21.5	19.1	20.5	20.7	25.3	14.8
$\bar{X}$ = 22.0						
X^2 = 25.5						
(5.0% BBM - Cell Counts)						
665	675	720	715	738	725	683
703	745	743	688	788	700	705
690	730	703	763	705	738	738
$\bar{X}$ = 714						
X^2 = 26.8						
(5.0% BBM - Klett Readings)						
77.8	61.2	63.6	83.1	---	75.0	81.9
77.5	62.3	61.6	81.6	73.8	72.3	75.7
64.6	---	---	81.5	83.1	69.8	68.6
$\bar{X}$ = 73.0						
X^2 = 14.5						

growth when different initial cell concentrations were used. The growth curves for cultures, with initial S. gracile concentrations of 5.0×10^5, 1.0×10^6 and 1.0×10^7 cells per liter in both 1.0 and 5.0% BBM solutions, are shown in Figure (10). An incubation period of approximately twelve days was necessary for the 5.0×10^5 cells per liter cultures, in both the 1.0 and 5.0% BBM solutions, to reach the stationary phase of growth. This period of time was considered to be excessively long for incubation of cultures because aquatic habitats could not be sampled on a weekly basis. At the 1.0×10^6 cells per liter initial concentration, the stationary phase of growth was reached in approximately seven days in both BBM solutions whereas five days appeared sufficient for maximum growth in both BBM concentrations using the 1.0×10^7 cells per liter initial concentration. However, the differences between the initial and final cell concentrations would be one order of magnitude less, when using the larger of these two initial cell concentrations (1.0×10^7 cells per liter). Thus the smaller initial concentration (1.0×10^6 cells per liter) would be more sensitive to low concentrations of nutrients such as found in highly oligotrophic waters.

Additional experiments were initiated to see if a seven day incubation period, using the 1.0×10^6 cells per liter initial S. gracile concentration, would be adequate for differentiation of aquatic habitats according to their potential productivity levels. A variety of surface water samples were collected for this evaluation. Figure (11) shows the growth curves when S. gracile was cultured in surface water samples taken from the Lester, Knife, Gooseberry and Baptism Rivers on September 20, 1967. A five day incubation period was sufficient for the culture alga to reach maximum growth in all four river water samples, since no further growth occurred after that time. Water samples were collected from the same four rivers on November 15, 1967 (Figure 12). The growth response of S. gracile in the Lester, Knife and Gooseberry Rivers was identical to that of the earlier samples. The growth response in the Baptism River, however, was somewhat greater than that of the September sample. In addition, a slightly greater growth response was observed in this river for the eleven day cell count than for the seven day count.

On September 26, 1967, other surface water samples from the Nemadji, Brule, Cranberry and Siskiwit Rivers were inoculated with Selenastrum and cultured. Results are shown in Figure (13). Again seven days appeared adequate for the attainment of maximum growth in all samples. However, water samples collected from the same four rivers on November 14, 1967 elicited a somewhat different growth response (Figure 14). A slight increase in growth of the Cranberry and Siskiwit River samples occurred after the seven day incubation period. The Nemadji River culture showed an increase of greater magnitude than any of the others at the end of 11 days. The relationship between the responses of the three cultures after seven and eleven days of incubation was the same, however. The Brule River sample elicited maximum growth of the culture alga within the seven day incubation period.

The growth responses of S. gracile in water samples collected September 1, 1967 from Sugar Loaf Cove, Tofte Bay, Good Harbor Bay and Grand Marais Bay are shown in Figure (15). The culture alga reached maximum growth in the Tofte Bay, Good Harbor Bay and Grand Marais Bay

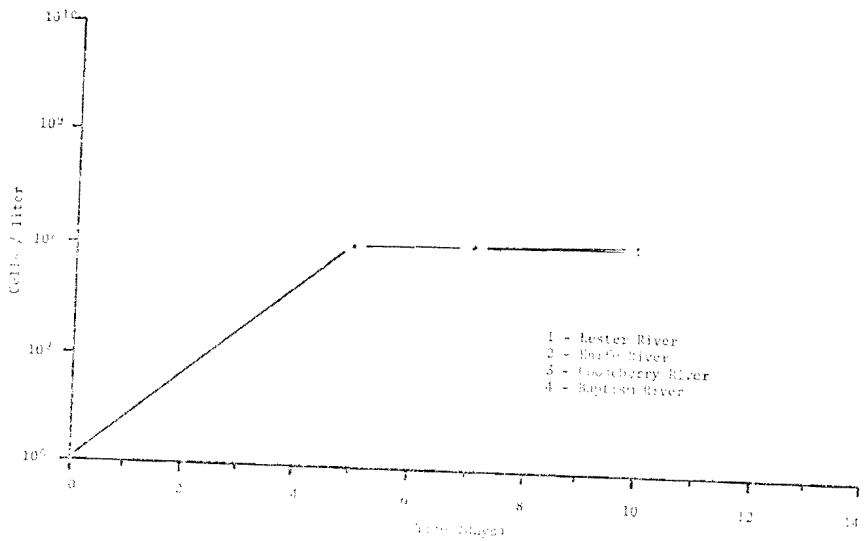

Figure 11

Growth Curves Of Selenastrum Gracile Cultured In Waters From North Shore Rivers.
Samples Collected September 20, 1967.

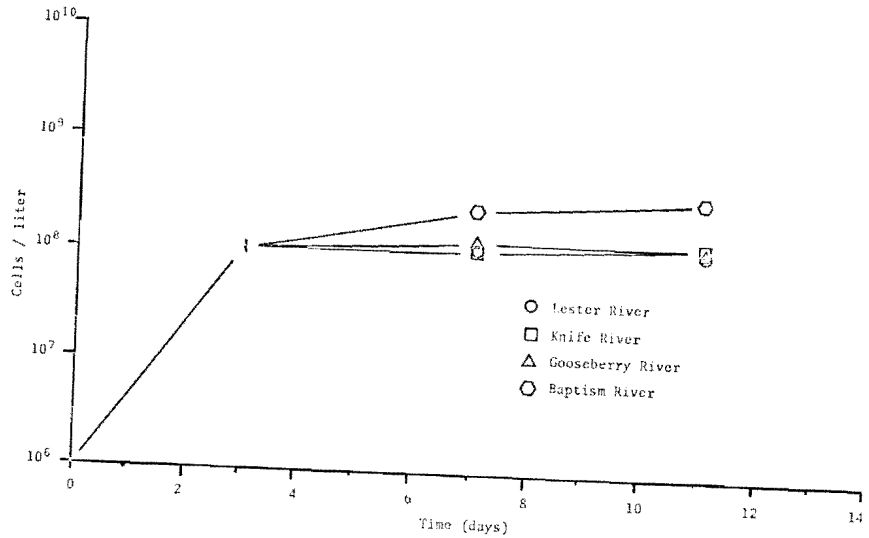

Figure 12

Growth Curves Of Selenastrum Gracile cultured in waters from North Shore Rivers.
Samples Collected November 15, 1967.

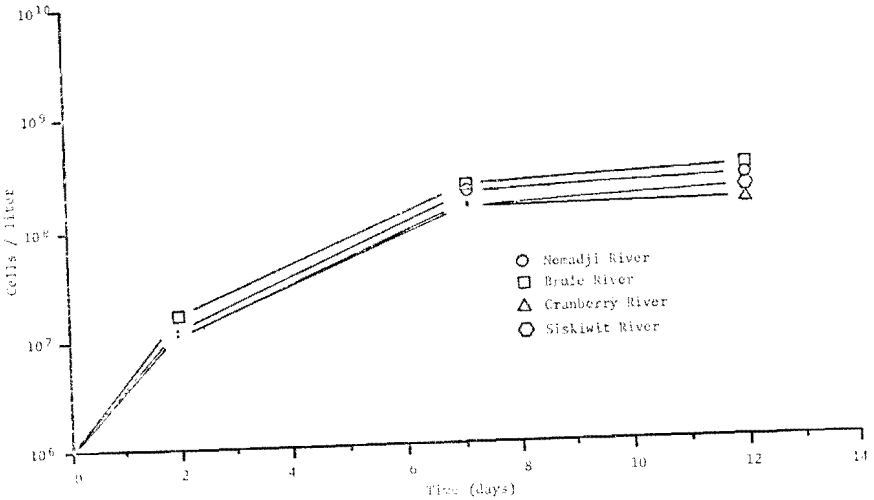

Figure 13

Growth Curves Of Selenastrum gracile Cultured in Waters From South Shore Rivers.
Samples Collected September 2e, 1967

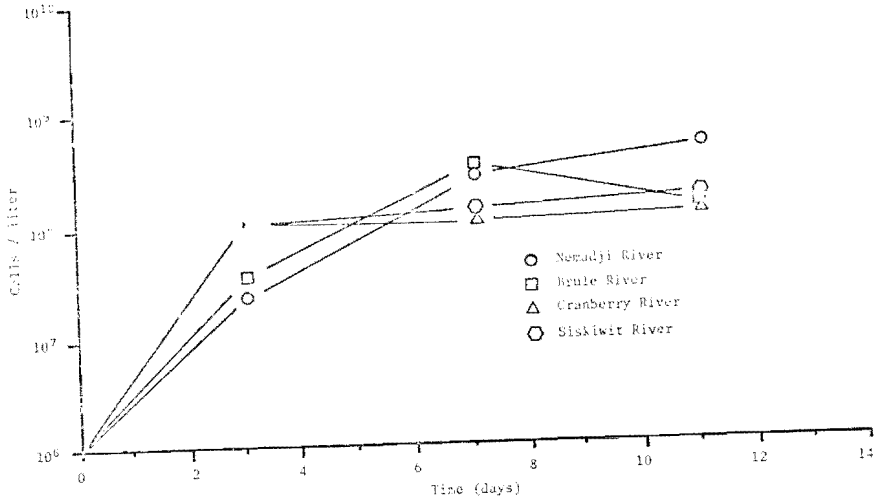

Figure 14

Growth Curves Of Selenastrum gracile Cultured In South Shore River Water.
Samples Collected November 14, 1967

samples within the seven day incubation period. A slight increase in the cell concentration occurred in the Sugar Loaf Cove sample after the seven day period. Figure (16) shows the growth response of S. gracile in four additional Lake Superior water samples also collected from bays located along the north shore of the western arm of the lake. A seven day incubation period was adequate for the culture algae to reach maximum growth in all four bay samples, including Lester River Bay, Silver Bay, Beaver Bay and Burlington Bay.

From the growth results of these several different experiments, it can be seen that the incubation time necessary for maximum growth to occur varies with different water samples. However, the seven day growth period was sufficient for the attainment of maximum growth in most of the water samples studied and for those samples which needed extra incubation time, the seven day period provided a near maximum growth rate. Therefore, because of the several advantages in using the seven day period, which have been previously discussed in this section, this period of time has been chosen as the most satisfactory incubation period for assay procedure. An additional advantage of the fixed incubation period is the standardization of procedure which results.

Control Cultures

The external standard culture, consisting of triplicate 200 ml cultures of S. gracile grown in 1.0% BBM, was prepared when an experiment was initiated. Only one triplicate set was used per day even though sewage experiments may have been initiated. Since the control was prepared as a means of monitoring the preparation technique and the adequacy of incubation, it was necessary to find out what range of variation could be expected in the final cell counts. The seven day cell counts from the seven 1.0% BBM experiments initiated for studying the stability of the incubation system (Table II) were utilized for this purpose. The 0.95 confidence limits, using the statistical procedure suggested by Lund et al. (1958) and Picher (1957)* were calculated from the mean of these cell counts. The 95% confidence limits showed that the average seven day cell concentrations of the triplicate external control cultures could vary from 7.3×10^6 to 9.7×10^6 cells per liter without significant statistical variation occurring. In one out of every twenty experiments the average cell concentration could be expected to fall outside this range due solely to random sampling errors. Such a variation observed in successive cultures would necessitate a careful investigation to find the cause.

The internal standard culture, like the external control consisted of 200 ml of 1.0% BBM in the filtered surface water sample. It too was prepared in triplicate for each water sample to be assayed.

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* Confidence Coefficient 0.95

upper limit = $X + 2.42 + 1.960\sqrt{(X + 1.5)}$

lower limit = $X + 1.42 - 1.960\sqrt{(X + 0.5)}$

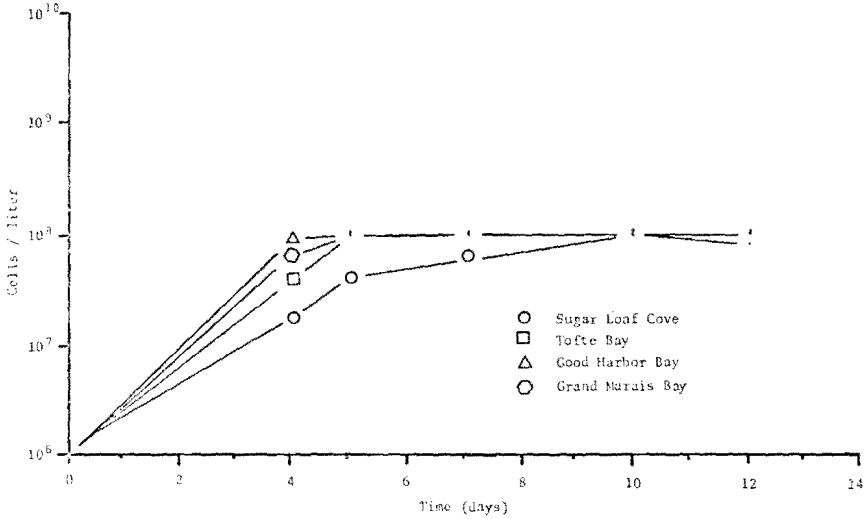

Figure 15

Growth Curves Of Selenastrum gracile Cultured in Waters From Lake Superior.
Samples Collected September 1, 1967.

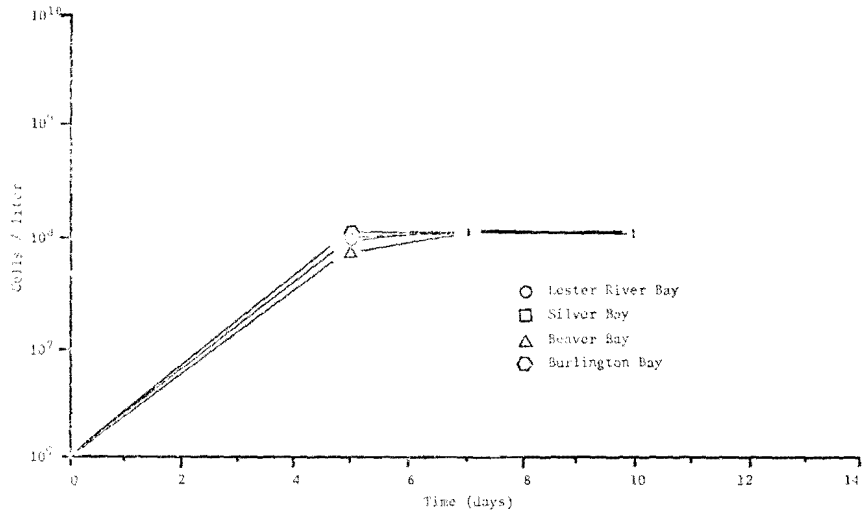

Figure 16

Growth Curves of Selenastrum gracile cultured in Waters From Lake Superior.
Samples Collected September 20, 1967

Evaluation of Methods for Estimating Algal Concentrations

Microscopic Cell Count Technique

The precision of the microscopic cell count technique depends upon the magnitude of errors inherent in the methodology of the technique. These errors include those of sampling, and counting (Gilbert 1942, Lund et al. 1958, Whipple 1927).

Sampling and subsampling errors arise from the uneven distribution of the alga in the cultures and in diluted aliquots of them. Since S. gracile cells remain solitary in the nutrient solution and do not readily stick to the culture flasks or to each other, the alga for all practical purposes could be considered as being uniformly suspended in its medium. As a special precaution each flask was swirled 200 times in a counterclockwise direction.

Counting errors like sampling errors may arise from the uneven distribution of algae (in the counting cell). Other factors are interfering food organisms, extraneous material and errors in filling the chamber. Studies conducted by Gilbert (1942) and Lund et al. (1958) have shown that errors arising from the counting of algae in a counting chamber are insignificant in comparison to the random sampling errors. These studies were concerned with natural communities of algae. However, since the present study involves the counting of one species of algae these errors should be even smaller in magnitude. Extraneous material would not interfere with the counting procedure because the sample was filtered before seeding with the test algae. Dead algal cells would not interfere since the incubation terminated before large numbers of dead cells were in evidence.

Two experiments designed to determine if the sampling, subsampling and counting errors would significantly affect the precision of the counting technique were carried out.

In the first, a one ml aliquot of the culture was pipetted out and diluted to twenty-five ml with distilled water. Eight replicate one ml aliquots of this dilution were then removed and individually counted. A similar aliquot was removed from the stock culture and diluted to fifty ml. Eight replicate one ml subsamples were removed and counted. A third and fourth aliquot removed from the stock culture were diluted to 100 and 200 ml respectively. These were counted in the same manner. Application of the Chi-square test to each of these series of counts showed that at the 0.95 probability level there were no significant fluctuations. See Table III. It was concluded that cells were randomly distributed in the diluted samples and in the counting chamber.

In the second experiment, twenty replicate one ml subsamples were removed from a culture of S. gracile and individually counted. Next, a one ml aliquot of the same culture was pipetted out and diluted to ten ml with distilled water. Twenty replicate one ml aliquots of this suspension were then individually counted. A one to 50 and a one to 100 dilution of the stock culture were treated in the same manner. When the Chi-square test was applied to the four series of counts it was found

that, based on a 95% confidence limit, no random sampling errors occurred. See Table III. Thus the algal cells were found to be randomly distributed in the diluted as well as the undiluted sample.

To study the effects of pipetting and dilution of cultures, each series of twenty cell counts taken from the diluted subsamples were multiplied by their respective dilution factor and compared, statistically, with the twenty cell counts from the undiluted sample (Table III). No significant differences were found between the twenty undiluted cell counts and the twenty counts from the 1:10 dilution. But, comparison of the twenty cell counts from the undiluted sample with those of the next two dilutions (1:50, 1:100) showed that significant differences existed. Therefore it appears that dilution of algal cultures beyond the 1:10 level can significantly affect the precision of the cell counts. For that reason the 1:10 dilution was the one selected for this study.

When the distribution of algae to be counted is random, as appears to be true in this instance, the number of algal cells which must be counted to obtain any desired level of accuracy can be calculated (Bancroft 1963, Lund et al. 1958). The approximate number of organisms which must be counted to obtain various levels of accuracy at the 95% confidence limit is shown in Table IV. For example, when 100 organisms are counted (ten organisms per each of the ten fields counted), an accuracy of ± 20% will be achieved. The level of accuracy varies inversely with the square root of the number of cells counted. Therefore, to double the level of accuracy, four times as many organisms must be counted. The question arises as to what level of accuracy should be used since a point will finally be reached where the time spent in counting does not justify the increase in accuracy achieved. According to Lund et al. (1958) an accuracy of ± 50% should be adequate for most algal work. Although this level of accuracy may be satisfactory for routine algal studies, a higher level of accuracy was thought desirable for this research in which comparative levels of potential primary productivity from different waters were to be measured.

Experience has shown that, at 100 or more organisms per random field, the large number of overlaying cells interfere with the counting procedure. A ten-fold dilution of such a culture would reduce the concentration of cells to 10 or more per field.

Chlorophyll Determination

The chlorophyll technique in comparison to cell counts has the advantage of dealing with large sized aliquots. Observation of various

TABLE III

Replicate Subsamples*

Dilution	Mean Count	Number of Subsamples	X^2
	Experiment 1		
1:25	209	8	1.6
1:50	107	8	2.5
1:100	53	8	2.3
1:200	27	8	2.0
	Experiment 2		
1:1	1402	20	8.4
1:10	142	20	4.2
1:50	28	20	7.0
1:100	15	20	6.4
1:1, 1:10	1412	40	52.3
1:1, 1:50	1411	40	361.0 **
1:1, 1:100	1460	40	769.0 **

* Data in Appendix I.
** Denotes significant difference, at the 0.95 confidence level, from random distribution.

TABLE IV

Accuracy of Counts

Number Of Organisms Counted	Cells/liter	Approximate 0.95 C.L. Range	% Variation From Mean
4	2×10^6	0-8	± 100
8	3×10^6	2-14	75
16	7×10^6	8-24	50
25	1×10^7	15-35	40
50	2×10^7	36-64	28
100	4×10^7	80-120	20
200	8×10^7	172-228	14
500	2×10^8	455-545	9
1,000	4×10^8	937-1063	6
2,000	9×10^8	1911-2089	4

types of cultures, especially nitrogen deficient ones, has shown that the relationship between chlorophyll content of a culture and the cell population may vary with environmental and physiological factors, thus the technique could become a valuable tool for studying the physiological effects of substances or lack of substances on algae if it is combined with the cell count procedure.

To study the reliability of the technique, chlorophyll analyses were made on 100 ml aliquots of the cultures from the seven experiments which were designed to evaluate the reliability of the incubation system (Table II). In each instance chlorophyll was extracted and the resulting Klett readings compared with each other. Application of the Chi-square test to these Klett readings showed that no significant variation, at the 0.05% probability level, existed between the aliquots. Thus the technique appears to be sufficiently precise for use as a second method for expressing levels of productivity. Since an excessively long period of time, approximately 20 to 30 minutes, was found to be necessary to carry out each of the determinations the chlorophyll procedure is relatively slow and the number of samples which can be processed each day would be limited. Because of the time required for analysis the chlorophyll determination would appear to be applicable only when few algal cultures are to be utilized.

Dry Weight Determination

Under the conditions of this study, the dry weight determination was found to be unsatisfactory as a reliable measure of productivity. Error caused by the rapid absorbance of moisture on the filter paper during the short period of time it was outside the desiccating chamber for weighing, was greater than the actual weight of the sample. Part of this weighing error was due to the small size of the aliquot used (50 ml or less). This aliquot could not be increased because the remaining portion of the culture was needed for cell counts and the chlorophyll determinations. The dry weight determination was therefore discarded.

II. SENSITIVITY OF TECHNIQUES

If this algal bioassay method is to be used for relating algal growth to the chemical quality of freshwater environments, then the culture alga must be sensitive to differences in nutrient concentrations of water. This sensitivity has to be of sufficient magnitude so that the differences in nutrient concentrations can be detected in the cell count and chlorophyll determinations. Several experiments were initiated in which distilled water and Lake Superior water were enriched with different concentrations of nutrients. The nutrients were added in the form of BBM or raw sewage. Triplicate samples of each enrichment solution were individually incubated and analyzed for cell and chlorophyll responses at the end of the seven day studies.

Enrichment Experiments In Distilled Water

In the first series of enrichment experiments, S. gracile was grown in distilled water in six different concentrations of BBM ranging from 0.1 to 10.0%. The average cell count results for three growth

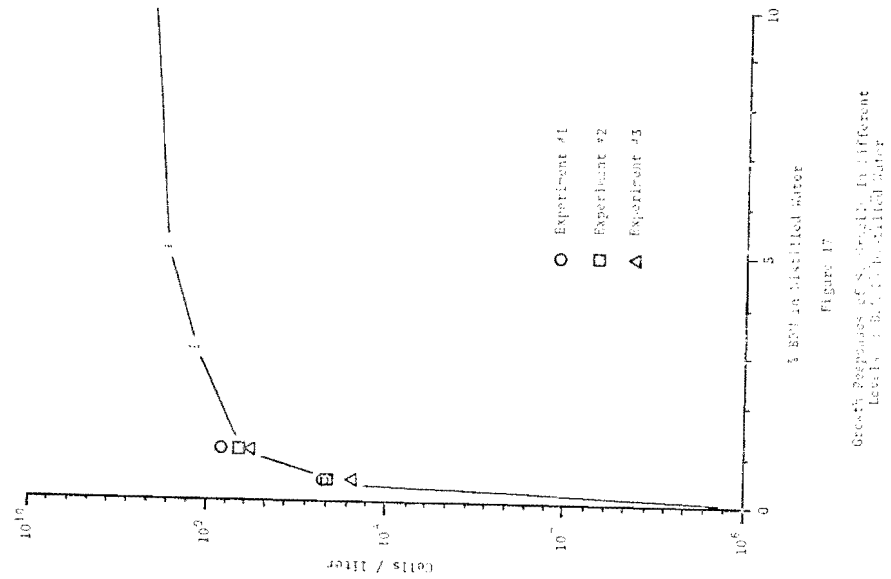

Figure 17
Growth Responses of S. gracile In Different Levels of BBM In Distilled Water

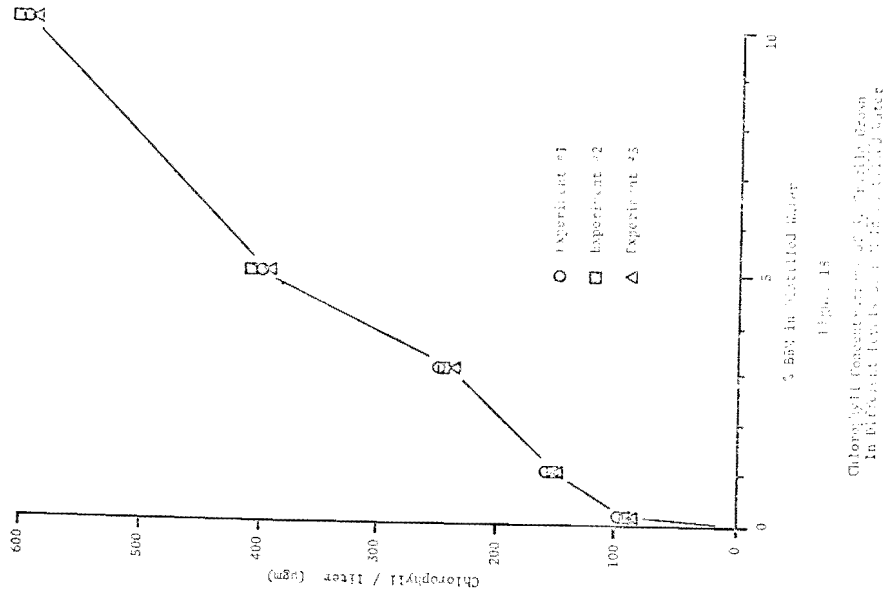

Figure 18
Chlorophyll Responses of S. gracile Grown In Different Levels of BBM In Distilled Water

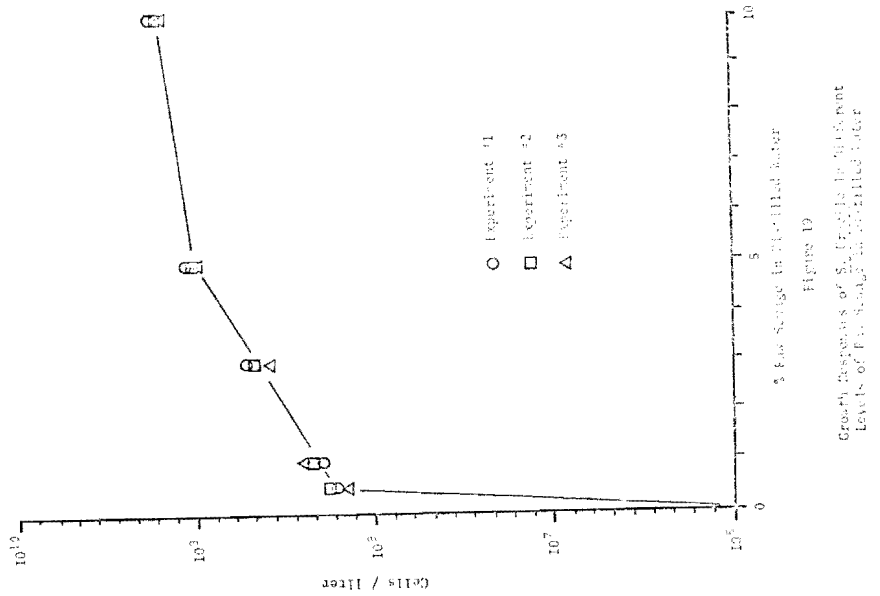

Figure 19

Growth Responses of S. gracile to Increasing Levels of Raw Sewage in Distilled Water

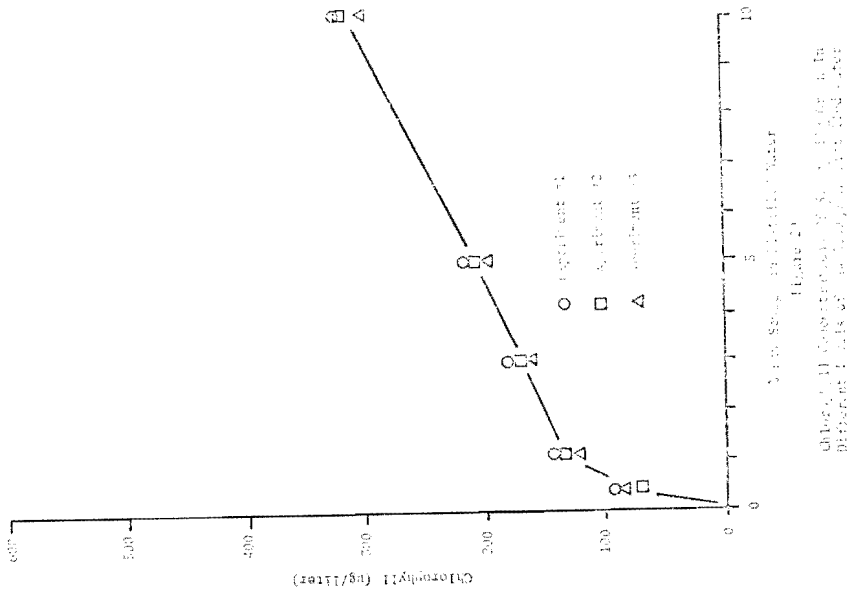

Figure 20

Chlorophyll Responses of S. gracile to Increasing Levels of Raw Sewage in Different Experiments

experiments are shown in (Figure 17). The alga grew in all BBM concentrations above 0.1% and differences in each of these responses could be readily detected. The average chlorophyll responses to different BBM concentrations (Figure 18) were in agreement with the preceding responses in terms of cell numbers. In the BBM concentrations above 0.1% a nearly linear response was obtained and differences in the amount of chlorophyll produced at these BBM concentrations could be readily detected.

The second series of experiments involved the enrichment of distilled water with different concentrations of raw sewage. Domestic sewage was selected as the source of essential nutrients for these experiments because it represents one of the major sources of eutrophication in aquatic environments. If the response of S. gracile, in terms of cell numbers and chlorophyll concentration, would not differ significantly with increasing concentrations of raw sewage in distilled water, then the alga could not be expected to respond to varying degrees of sewage enrichment in aquatic systems. Raw sewage utilized in this experiment was collected from the Duluth, Minnesota sewage treatment plant. Upon reaching the laboratory, the samples which had been collected in one liter polyethylene bottles were immediately filtered through a Celotex "A" glass-fiber filter to remove large suspended particles. The samples were then refiltered through a 0.45 Millipore filter to remove any remaining suspended material and to remove all plankton organisms. The final filtrates were placed in sterile 250 ml dissolved oxygen bottles and refrigerated until needed.

The average cell concentrations of S. gracile are shown in (Figure 19) for a series of three experiments in which sewage concentrations varying from 0.5 to 10.0% were used. The shape of the growth curve was similar to that obtained with the BBM cultures in distilled water. Significant differences in the growth responses could be detected for each added increment of raw sewage. The chlorophyll responses (Figure 20) were similar to those of the BBM cultures in that a linear relationship occurred between the chlorophyll concentrations and the amount of enrichment added. However, the magnitude of the responses was much smaller when sewage was used. Perhaps nitrogen was present in the sewage in insufficient concentrations for maximum production of chlorophyll.

A third series of experiments was undertaken in which the growth and chlorophyll responses of S. gracile to specific nutrients in distilled water were examined. Nitrogen and phosphorus, because of their importance in phytoplankton growth problems, were selected for these enrichment experiments. If S. gracile is to be useful as the culture alga for assessing the potential primary productivity levels of aquatic systems, it must be sensitive to varying amounts of these two extremely important nutrient elements.

For those experiments in which the nitrogen growth response was to be studied, a 10.0% modified BBM solution without its usual nitrogen source was prepared. The cobaltous nitrate salt was replaced with an equivalent amount of cobalt in the chemical form cobaltous chloride ($CoCl_2 \cdot 2H_2O$) and the sodium nitrate ($NaNO_3$) was left out. This change was made so that all sources of nitrogen could be removed from the

medium without also deleting an essential nutrient. After the media had been prepared, it was analyzed for nitrogen contamination. The phenol disulfonic acid method given in Standard Methods was used. It is sensitive to nitrate concentrations as low as 0.01 mg per liter. No nitrate-nitrogen was detected by this procedure. The media was also analyzed, by the Kjeldahl method (Standard Methods), which indicated that the medium was free of total nitrogen. The 10.0% BBM concentration was selected for use in these enrichment studies because it contained a concentration of nutrients which was found to produce maximum growth of the Selenastrum (Figure 17). Thus in the modified medium, nitrogen would be limiting in any culture which produced less than the maximum growth response.

Before the nitrogen experiments were undertaken, all glassware was rinsed in a 0.1N HCl solution, after washing in the aqua regia solution. This additional rinse was added to the washing procedure for the purpose of removal of any nitrogen residual left from the acid wash. The flasks were rinsed six times, as usual, in distilled water. To a series of triplicate 500 ml Bellco Erlenmeyer flasks containing the nitrogen-free 10.0% BBM solution, nitrate-nitrogen in the chemical form ($NaNO_3$) was added in concentrations ranging from 0.0001 to 10.0000 mg per liter. The volume of each culture was 200 ml.

A different source of inoculation algae had to be used for these experiments since algal cells in the stock culture could presumably accumulate sufficient nitrogen to have significant effects on the results. A twenty ml portion of 5.0% modified BBM (without nitrogen) in a 100 ml Bellco Erlenmeyer flask was inoculated with 1.0 ml of the stock Selenastrum culture. This culture was sealed with a styrofoam stopper and incubated for seven days under the same conditions of light and temperature as used for the stock culture. Agitation of the cultures was produced by a shaking table. At the end of the seven day incubation period, a one ml inoculum of the nitrogen deficient culture was placed in a fresh twenty ml 5.0% BBM solution, also void of nitrogen, and incubated for an additional seven days. Cell counts taken on these cultures showed that little or no growth occurred during the second seven day period. In addition, the cultures had lost their characteristic grass green color. The suspension had assumed a yellowish to milky-white color. Microscopic examination of these cells showed that the chloroplasts had become shriveled. The cultures, now deficient in nitrogen, were ready for the bioassay test. As in all other experiments, an initial cell concentration of approximately 1.0×10^6 cells per liter was used.

(Figure 21) shows the average growth responses of S. gracile to the six different nitrate-nitrogen concentrations used based on three experiments. The culture alga grew in all of the nitrate-nitrogen concentrations studied. However, the growth response at the lowest concentration (0.1 ug nitrate-nitrogen per liter) could have been the result of nitrogen contamination in the growth medium since nitrogen in very small quantities would have been undetected by either the phenyldisulfonic acid method or the Kjeldahl total nitrogen analysis. The seemingly equal response for the 0.001 and 0.01 mg nitrate-nitrogen per liter concentrations may have been due to a reduction in cell size at

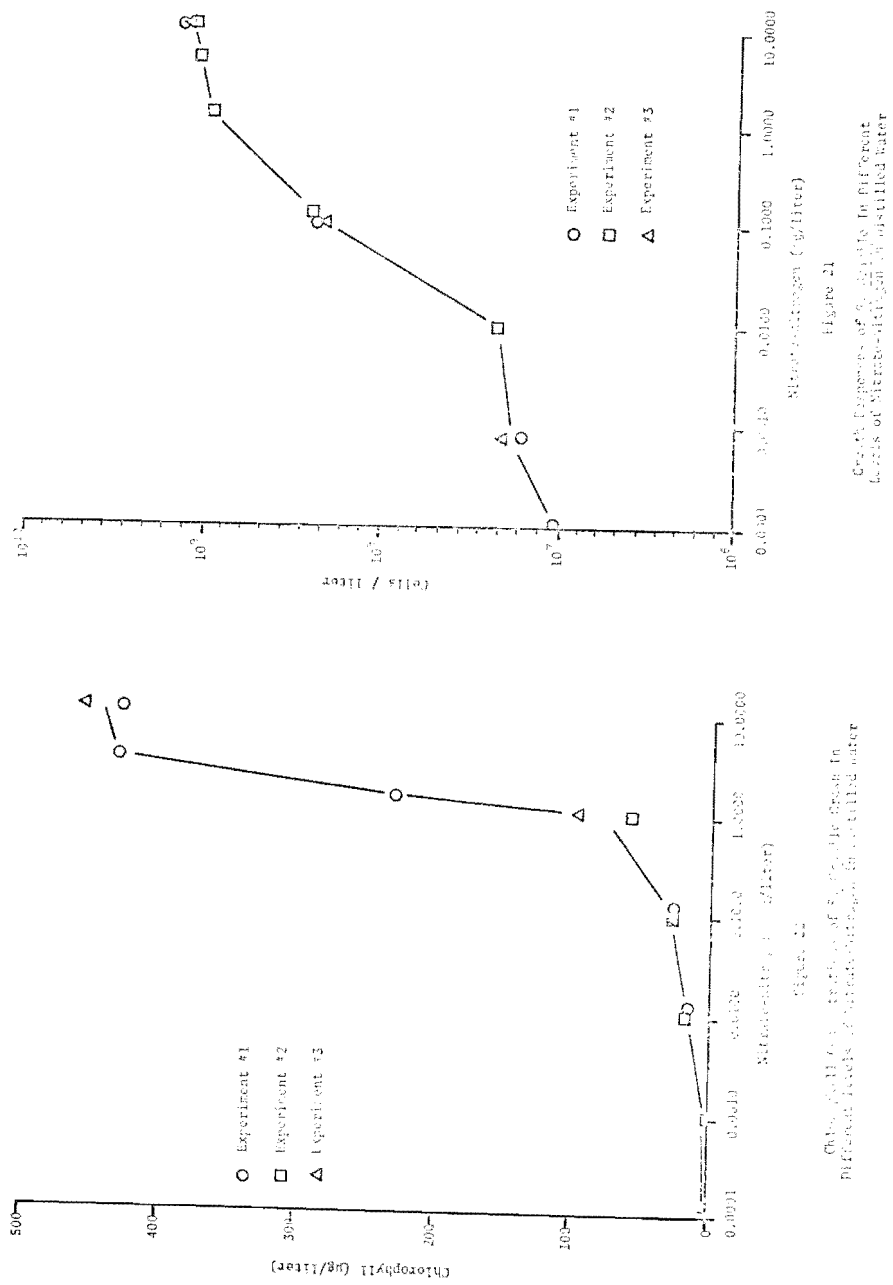

Figure 21

Growth Response of S. gracile to Different Levels of Nitrate-Nitrogen in Distilled Water

the lower concentration. This phenomenon of cell size reduction was quite evident in the cultures with the two lowest nitrogen concentrations. The magnitude of the growth responses, above 0.01 mg nitrate-nitrogen per liter, could be differentiated with the cell counting technique. The chlorophyll response at the three lowest nitrate-nitrogen concentrations (Figure 22) appears to substantiate the hypothesis of cell size reduction. No response was observed at either the 0.0001 or 0.001 mg nitrate-nitrogen per liter concentrations. At the 0.01 mg per liter concentration a chlorophyll response was evident, although the cell concentration was apparently equal to that of the 0.001 mg per liter cultures. The chlorophyll response showed distinct differences at each nitrate-nitrogen concentration above the 0.001 mg per liter concentration. As in the two previous enrichment studies, both the cell count and chlorophyll determinations were sensitive to a wide range of nutrient concentrations. The 0.01 to 10.0 mg nitrate-nitrogen per liter range of sensitivity should enable this algal assay method to detect nitrogen concentrations of the most oligotrophic situations. When water samples of extremely high enrichment are to be analyzed, they could be diluted with nitrogen-free water in much the same manner as water samples of high BOD are diluted before the analyses are performed. In this manner, samples of any nitrogen concentration could be analyzed.

For the phosphorus studies, a phosphorus-free 10.0% BBM solution was prepared. The modified medium was then analyzed for phosphorus contamination using the persulfate method originated by Gales et al. (1966) and Menzel and Corwin (1965). No phosphorus contamination was detected. To a series of triplicate flasks containing the phosphate-free 10.0% BBM, phosphate in the form of dipotassium hydrogen phosphate (K_2HPO_4) was added in concentrations ranging from 0.001 to 1.000 mg per liter.* The volume of each of these cultures was 200 ml. The average responses of S. gracile, as determined by three experiments and the cell count technique, are shown in(Figure 23). The alga responded strikingly to increasing increments of phosphate-phosphorus concentrations. At concentrations ranging from 0.01 to 1.0 mg per liter, the growth response was linear. The chlorophyll responses for two of these experiments are presented in (Figure 24). Again, differences in the magnitude of the responses, from 0.01 to 1.0 mg per liter phosphate-phosphorus, could be detected. It is evident that the algal bioassay method is sensitive to phosphorus as well as nitrogen in concentrations as low as those found in the most oligotrophic of waters. By diluting extremely eutrophic samples with phosphorus-free water, in the manner previously discussed in regards to nitrogen analyses, the method could also be used to study phosphorus levels of eutrophic waters.

Enrichment Experiments in Lake Superior Water

The final series of experiments initiated for studying the sensitivity of the S. gracile growth response to different levels of nutrient

* Phosphorus deficient cells were prepared in the same manner as the nitrogen-deficient cells. However, a 5.0% modified BBM solution with KNO_3 in place of the potassium phosphate salts was used.

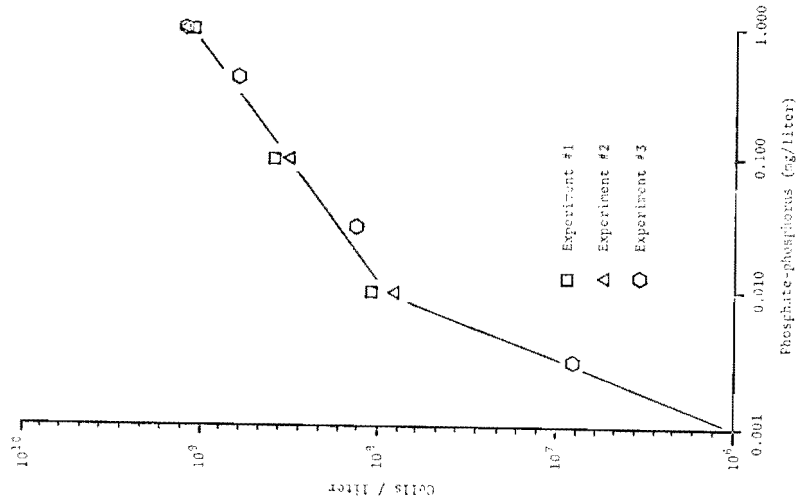

Figure 23

Growth Responses Of S. Gracile To Different Concentrations of Phosphate-Phosphorus

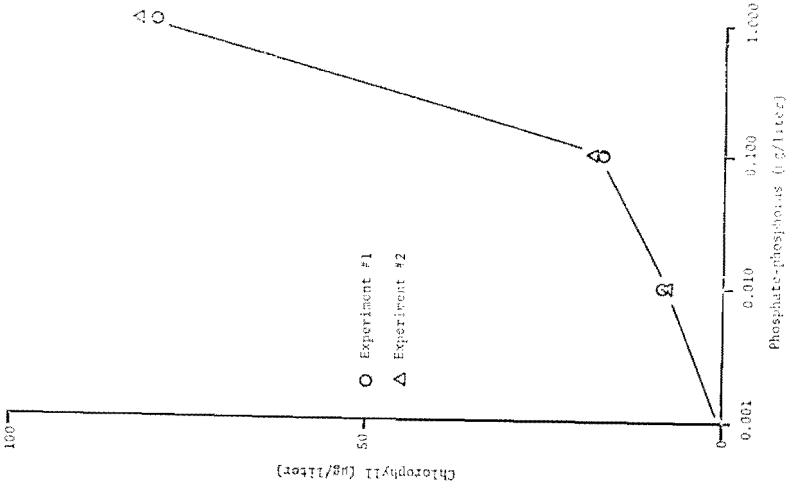

Figure 24

Chlorophyll Responses of S. Gracile To Different Concentrations of Phosphate-Phosphorus

concentrations were prepared with Lake Superior water. The lake water was collected September 23, 1968 from a location approximately 2,000 yards northeast of the mouth of Knife River (Figure 3). The raw sewage, which was used for enrichment, was obtained September 23, 1968 from the Silver Bay, Minnesota sewage treatment plant (Figure 4). This city has a population of approximately 4,000 persons and is located fifty miles northeast of Duluth. The waste coming to the plant is domestic sewage. No industrial effluent is involved. For this reason, the sewage from this source seemed ideal for enrichment studies since growth inhibiting industrial pollutants would not be present to complicate growth results. The samples were collected in 250 ml BOD bottles. Upon returning to the laboratory, the samples were filtered and stored in exactly the same manner as the previously described Duluth sewage samples.

Four series of triplicate enrichment cultures were prepared using the Lake Superior water as the water source and either or both the raw sewage and BBM as the source of enrichment. The volume of each culture was 200 ml and each was placed in the 500 ml Bellco Erlenmeyer culture flasks. The first series of cultures included unenriched Lake Superior water and Lake Superior water enriched with 0.1, 1.0 and 10.0% raw sewage. The second series included Lake Superior water enriched with 10.0% BBM and Lake Superior water enriched with 10.0% BBM plus either 0.1, 1.0 or 10.0% raw sewage. The third series of cultures included Lake Superior water enriched with 10.0% BBM minus nitrogen and Lake Superior water enriched with 10.0% BBM minus nitrogen plus either 0.1, 1.0 or 10.0% raw sewage. The fourth and final series of cultures included Lake Superior water enriched with 10.0% BBM minus phosphorus and Lake Superior water enriched with 10.0% BBM minus phosphorus and either 0.1, 1.0 or 10.0% raw sewage. Two experiments, each including the four series of enrichment cultures, were carried out. The nitrogen and phosphorus deficient cells were produced in the same manner as described in the preceding section.

(Figure 25) shows the growth responses of S. gracile in unenriched lake water and in water with 10.0% BBM and 10.0% BBM-N enrichments. As previously mentioned, each of these series of samples was also enriched, individually, with 0.0, 0.1, 1.0 and 10.0% raw sewage. The addition of 10.0% BBM to lake water was apparently sufficient for the alga to produce near-maximum growth as the addition of raw sewage had only a slight effect on the growth responses. Addition of 0.1 or 1.0% sewage had no effect on the growth responses of either the unenriched or 10.0% BBM-N enriched samples. Addition of 10.0% sewage, however, elicited near maximum growth responses in both of these lake water cultures. Apparently some nutrient, probably nitrogen, was limiting the growth of S. gracile in these two series of cultures. The 10.0% sewage addition provided sufficient amounts of this nutrient to raise the growth response up to the maximum level observed in the 10.0% BBM enriched cultures. The chlorophyll concentrations in the same three series of cultures are shown in (Figure 26). Addition of up to 1.0% sewage to the samples enriched with 10.0% BBM elicited a slight decrease in the chlorophyll responses of the alga. However, 10.0% sewage enrichment stimulated an increase in the chlorophyll concentrations of all the cultures. This seemingly paradoxical situation may have been caused by some substance present in the sewage which had an inhibitory effect on the culture alga at the two

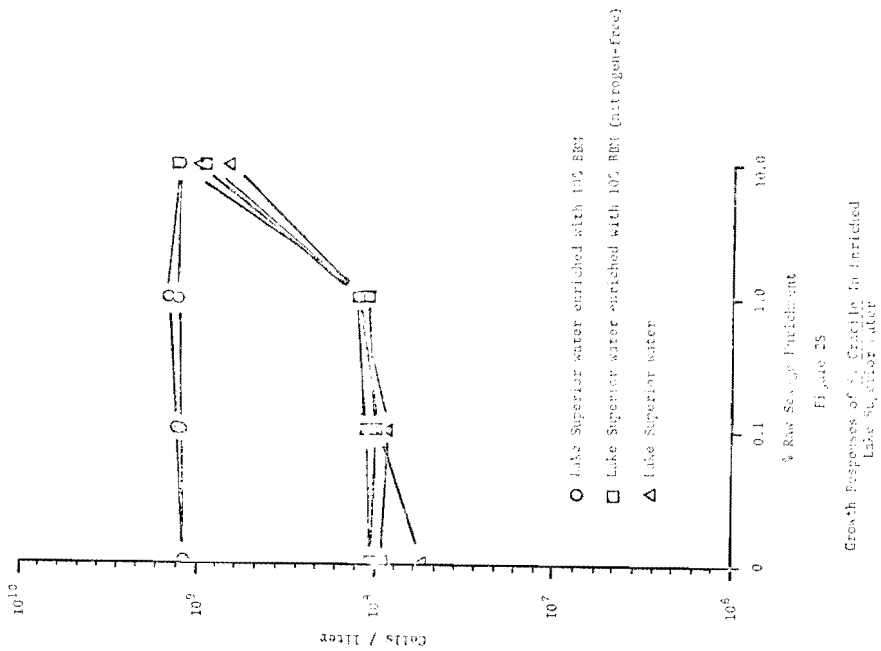

Figure 25

Growth Responses of S. Gracile in Enriched Lake Superior Water

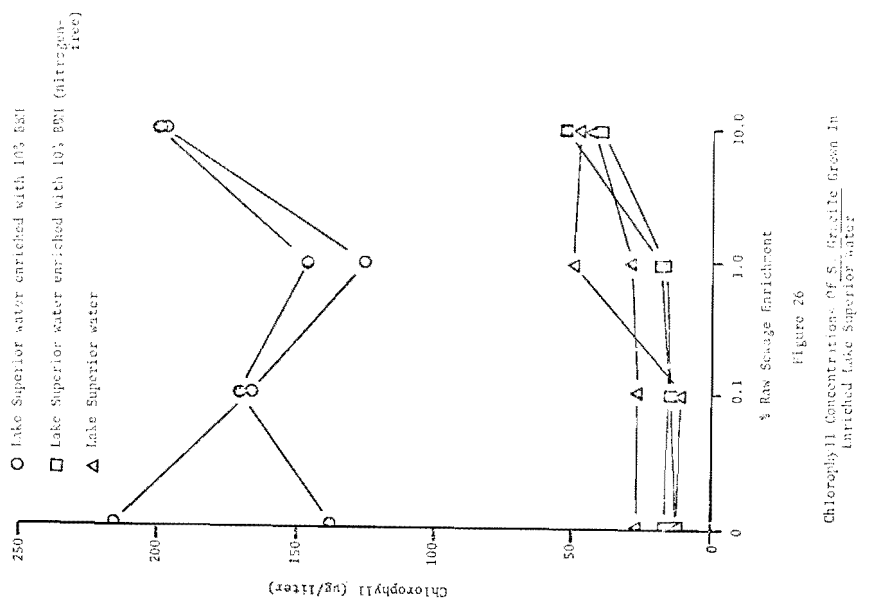

Figure 26

Chlorophyll Concentrations Of S. Gracile Grown In Enriched Lake Superior Water

lower sewage concentrations. At the 10.0% sewage concentration, the substance was somehow neutralized. The chlorophyll concentrations in the lake water samples without BBM and with 10.0% BBM-N were affected only slightly by sewage enrichment. In addition, the chlorophyll responses in these cultures never approached the level of the responses observed in the 10.0% BBM enriched samples.

Apparently sufficient nitrogen was present in the 10.0% raw sewage enriched cultures for metabolic processes of S. gracile including maximum cell production, but insufficient for a maximum production of chlorophyll.

The growth responses of S. gracile in the series of cultures containing unenriched lake water, lake water enriched with 10.0% BBM-P and with 10.0% BBM are shown in (Figure 27). The addition of 0.1 and 1.0% sewage to the cultures with BBM-P enrichment elicited large increases in the growth responses. Addition of 10.0% sewage stimulated only a slight additional increase, which raised the level of responses up to the maximum level observed with the 10.0% BBM enrichment. The chlorophyll concentrations of the samples enriched with BBM-P reflected the growth responses (Figure 28). Addition of 0.1% sewage increased the chlorophyll concentrations of the cultures only slightly. Addition of 10.0% sewage, however, stimulated a large increase in the chlorophyll level which was approximately equal to the responses in the 10.0% BBM enriched cultures. Nitrogen, as previously discussed, was apparently present in insufficient amounts for maximum chlorophyll production in the unenriched and BBM-N enriched samples (Figure 26). However, this nutrient was provided in adequate amounts in both the 10.0% BBM-P and 10.0% BBM enrichments (Figure 28) for maximum chlorophyll production. Phosphorus was present in the sewage in sufficient concentration to produce maximum algal growth and chlorophyll production at the 1.0% concentration.

The results of these several assay experiments show that S. gracile, as an assay organism, is very sensitive to changes in the chemical quality of waters. Therefore, this alga is well suited for use in an assay procedure.

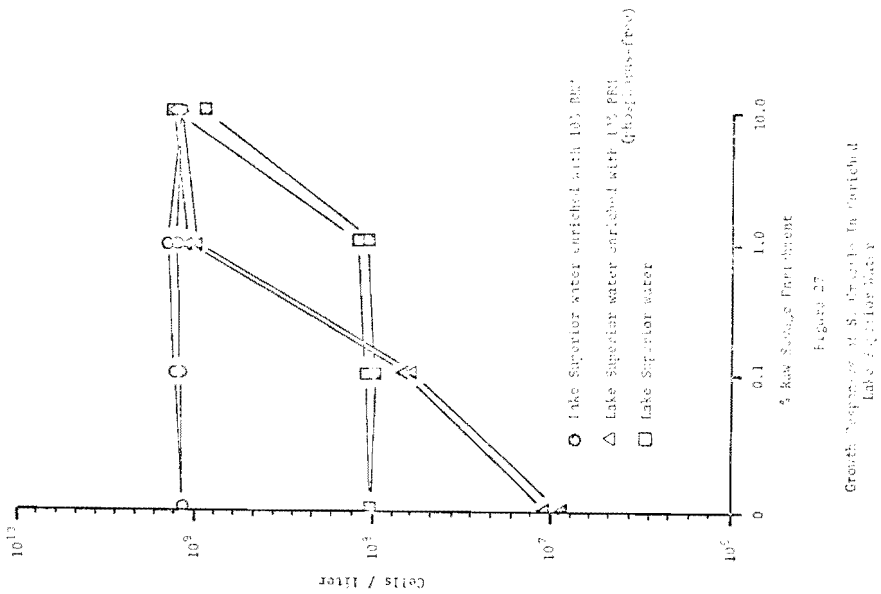

Figure 27

Growth Responses of S. Gracile in Enriched Lake Superior Water

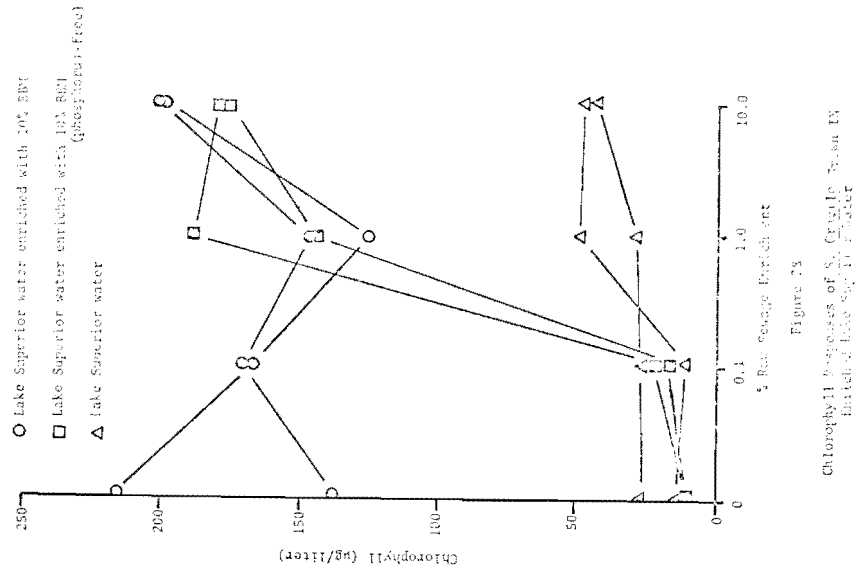

Figure 28

Chlorophyll Responses of S. Gracile [illegible] in Enriched Lake Superior Water

SUMMARY AND CONCLUSIONS

I. SUMMARY

This study was undertaken to develop a practical algal bioassay which could be used in the evaluation of the growth potential of water and therefore the state of its enrichment. It was hoped that such a test would also be able to detect the presence of inhibiting or toxic substances.

Selenastrum gracile was found to be a most satisfactory assay organism. This alga grew readily in culture and was very responsive to small changes in the nutrient composition of water. Where nitrate-nitrogen concentrations were concerned, differences in response were easily seen in culture concentrations of nitrogen ranging from 0.01 to 10.000 mg per liter. Phosphate-phosphorus produced similar responses to concentrations ranging from 0.005 to 1.000 mg per liter.

A routine procedure was developed for the preparation of test samples. A very important part of this activity was the washing of glassware in a 0.6N aqua regia solution followed by six rinses in distilled water. This was followed by the filtration of the test samples through a 0.45u Millipore membrane filter to remove plankton and debris. The test samples and control cultures were then prepared in 200 ml volumes and placed in clean 500 ml Bellco Erlenmeyer flasks. The controls, which were prepared for the detection of growth-inhibiting substances in the water samples, were the internal controls (aliquots of each water sample, enriched with 1.0% BBM) and the external control (1.0% BBM enriched distilled water). A sufficient amount of suitably prepared seed algae was added to the flasks to provide an initial cell inoculum of approximately one million cells per liter.

Optimal conditions for the incubation of cultures were determined by respirometry and incubation experiments. It was found that light at an intensity of 700 foot-candles and a temperature of 20° C. stimulated the best growth response of the culture alga. A period of seven days, while the cultures were gently swirled on a shaking table was found to be the optimum incubation period. Growth of the cultures was measured by the microscopic cell count technique and chlorophyll determinations. Both methods were excellent for the assessment of productivity.

The finalized assay procedure was evaluated by trials in which samples of distilled water or Lake Superior water enriched with Bold's Basal Medium or raw sewage were observed for growth responses. In these tests the assay procedure was found to be extremely sensitive in detecting small changes in the nutrient composition of these waters.

Further assay experiments were carried out using water samples from the lower St. Louis River and also on the growth inhibiting properties of sulfite waste liquor, an industrial waste product dumped into the St. Louis River below Cloquet, Minnesota. Growth inhibiting substances were detected in samples collected below Cloquet but not in waters collected above the waste liquor source. Furthermore, sulfite waste liquor was toxic to the assay organism in 5.0% BBM enriched distilled

water cultures, in Lake Superior water cultures and in waters collected from the St. Louis River above the liquor source. Waste liquor concentrations of 1.0% of the total volume of the cultures caused either complete growth suppression of the culture alga or almost complete suppression.

Because of the simplicity of the techniques, the sensitivity of the method to enrichment and the inexpensive nature of the equipment, it is envisioned that this assay procedure can have widespread application. For example, this assay could be used to evaluate the effects of chemicals on the aquatic environment as well as the detection of the ordinary enrichment associated with sewage and organic industrial wastes. Another use could be a comparative study of productivity levels of aquatic habitats; information which could be useful in the classification of lakes and the assessment of their respective eutrophication levels. Still another application of this bioassay procedure is the measurement of the growth inhibition. This information could be used to characterize industrial wastes, herbicides and other substances with regard to their potential effect on primary productivity.

II. CONCLUSIONS

1. Selenastrum gracile was found to be a most satisfactory assay organism. It grew readily in culture and was extremely sensitive to small changes in the nutrient composition of waters.

2. The routine procedure developed for the preparation and incubation of test samples and for the evaluation of the growth response of the assay organisms in these samples was also satisfactory.

3. The finalized assay procedure when evaluated for its sensitivity to changes in the nutrient composition of waters was found to be extremely sensitive to such changes.

4. It was found that the assay method when used to detect the presence of growth-inhibiting substances in the aquatic environment and the growth-inhibiting effects of such a substance on the culture alga was very effective and that the method can, therefore, be used to assess such materials in water.

5. Because of the simplicity of the techniques, the sensitivity both in the area of measuring eutrophication and of pollution evaluation and the inexpensive nature of the equipment, the procedure has a potential and widespread application.

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APPENDIX I

Cell Counts From Two Experiments Initiated For Studying The Precision Of The Microscopic Cell Count Technique

Experiment One							
			1:25 dilution				
219	217	211	205	203	211	205	199
			1:50 dilution				
103	96	115	110	103	108	106	113
			1:100 dilution				
54	52	50	57	49	46	55	58
			1:200 dilution				
26	26	30	24	22	30	28	26
Experiment Two							
			1:1 dilution				
1379	1387	1460	1394	1373	1405	1382	1436
1426	1377	1384	1383	1395	1401	1388	1419
1383	1408	1409	1450				
			1:10 dilution				
139	133	146	145	147	143	151	147
142	145	143	143	133	140	137	132
142	151	138	147				
			1:50 dilution				
27	27	29	27	31	32	31	32
25	29	27	25	25	28	31	29
24	23	30	36				
			1:100 dilution				
14	11	16	15	13	15	15	18
17	13	13	16	17	15	17	19
13	16	16	19				

APPENDIX II

Field Studies

These studies deal with the *Selenastrum* assay in actual operation. Included are all the steps in the procedure including sample collection, sample processing, sample incubation and determination of the growth response of the culture alga at the end of the incubation period. Only the microscopic cell count method was used for measuring the response of the assay organism.

Lower St. Louis River Study

Water samples were collected from six stations (Figure 4) along the lower St. Louis River and from the Duluth entry of Superior Bay into Lake Superior on May 2, 1968. The St. Louis River stations included Cloquet, Carlton, Fond Du Lac, Oliver, the Arrowhead Bridge and the Highbridge. Ice cover was present at all sampling points except at Carlton and at the Duluth entry. The largest growth response occurred in the Cloquet sample (1.0 billion cells per liter) whereas, going downstream, succeedingly lower growth responses occurred at the next three stations (Figure 29). These growth responses were 160 million cells per liter in the Carlton sample, 98 million cells per liter in the Fond Du Lac sample and 22 million cells per liter in the Oliver sample. An increase was observed in the growth responses of the final three stations. The Arrowhead Bridge sample elicited an algal yield of 110 million cells per liter and the Highbridge and Duluth entry samples had growth responses of 340 million and 140 million cells per liter, respectively. Some very interesting growth results were obtained from the internal standard cultures of these waters (Figure 30). Only in the enriched Cloquet sample (1.8 billion cells per liter), Highbridge sample (2.1 billion cells per liter) and the Duluth entry sample (1.5 billion cells per liter) did the algal yields exceed that of the external control (750 million cells per liter). The growth response in the enriched Fond Du Lac sample (730 million cells per liter) was approximately equal to that of the external control whereas the algal yields of the remaining three stations were all considerably lower. The enriched Carlton sample had a growth response of 130 million cells per liter and the enriched Oliver and Arrowhead Bridge samples had growth responses of 220 million and 430 million cells per liter, respectively. Since the internal standard control contained all the nutrients present in the external standard (1.0% BBM) and any additional nutrients originally present in the water samples, their algal yields would be expected to be at least as large as that of the external control. Furthermore, if all the essential plant nutrients had originally been present in the water samples, the growth responses would be expected to be somewhat larger than that of the external control. That is, unless some substance or substances were present in the water sample which had an inhibiting effect on the growth of the alga. The presence of such a growth inhibiting substance would explain the small algal yields of the Carlton, Oliver, and Arrowhead Bridge samples. Since a positive growth response occurred in all of the unenriched samples (Figure 32), the essential nutrients had to be present in them. Therefore, it appears that growth suppression must have occurred. The pH's of the Cloquet, Carlton,

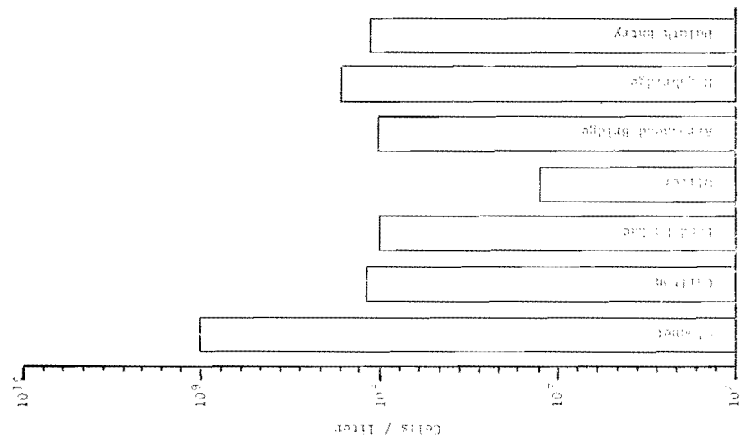

Figure 29

Growth Response of *St. Crucige* Cultured In 1.0% BBM Enriched Lower St. Louis River and Duluth Entry Water Samples, Collected, February 2, 1968.

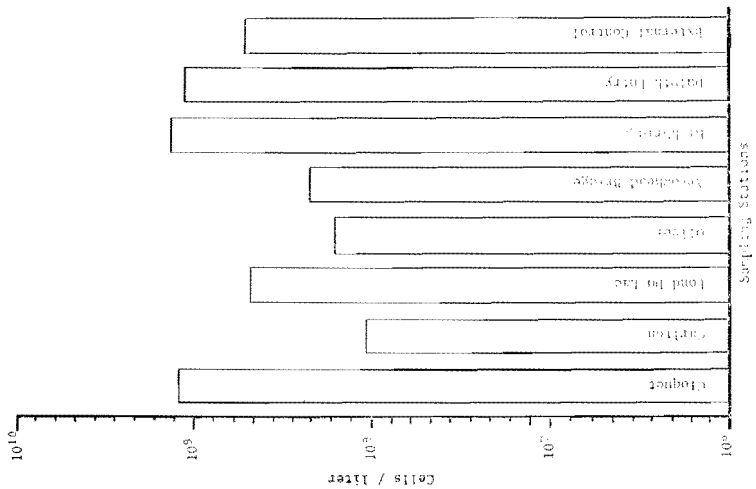

Figure 30

Growth Responses of *St. Crucige* Cultured In 1.0% BBM Enriched Lower St. Louis River and Duluth Entry Water Samples, and in 1.0% BBM In Distilled Water. Samples Collected, February 2, 1968.

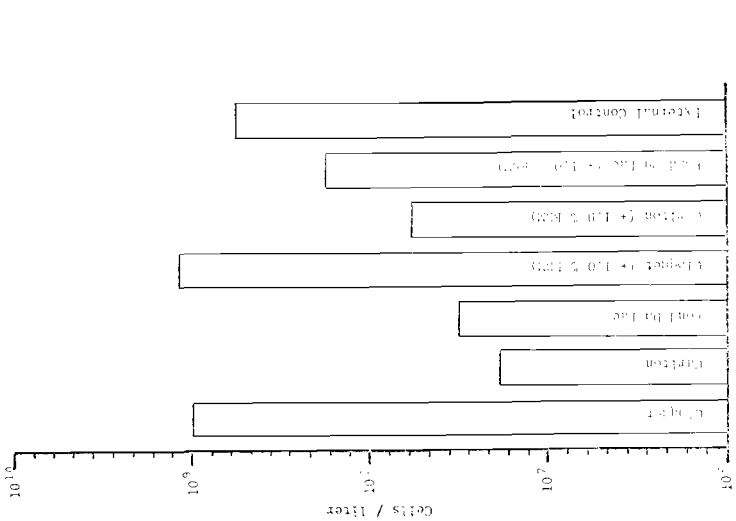

Figure 31

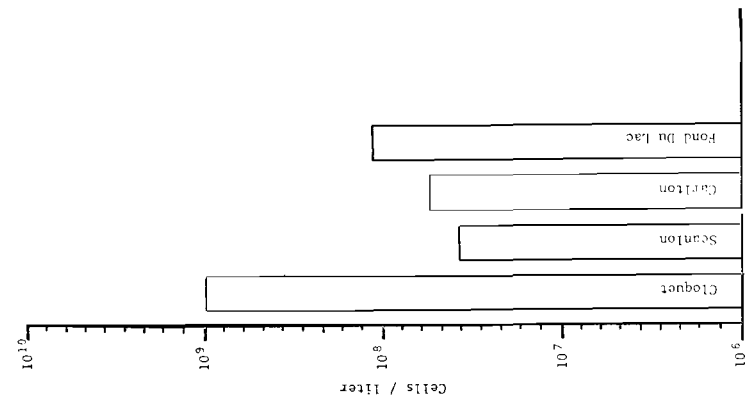

Figure 32

Growth Responses of S. Gracile Cultured In Lower St. Louis River Water. Samples Collected March 11, 1968

Fond Du Lac and Oliver water samples showed a similar trend to that observed for the unenriched growth results (Table V). At Cloquet, a pH of 7.1 was observed followed by successively lower pH's of 7.0, 6.8, and 6.7 at the next three stations. The Arrowhead Bridge and Highbridge samples also had pH's of 6.7 and at the Duluth entry the pH was equal to that of the Cloquet sample (7.1). Total solids determinations on aliquots of these water samples showed a sharp increase had occurred between the Cloquet sampling station (129 ppm) and the Carlton station (247 ppm). The high total solids concentration of the Carlton water samples was also observed in those from Fond Du Lac (236 ppm), Oliver (242 ppm) and the Arrowhead Bridge station (245 ppm). Then a slight decrease occurred at the Highbridge station (216 ppm) followed by a sharp reduction in the total solids concentration at the Duluth entry (83 ppm). This dramatic increase in total solids at Carlton may possibly be explained, at least in part, by the presence of a wood pulp industry in Cloquet, the Northwest Paper Company. Wood fibers, presumably emanating from this company could be visually observed in the river in large concentrations at Carlton and were observed as far south as the Fond Du Lac sampling station. The Cloquet station was located approximately 1/2 mile north of these wood pulp industries; consequently, wood fibers were never observed in the water samples taken from this location. Also evident in the Carlton and Fond Du Lac samples was the characteristic odor of sulfite liquor. This chemical is a bisulfite of a base, such as lime (Van Horn 1949, Vasseur 1966) which is used for digesting wood by various wood processing industries. Being economically unfeasible to recover, the sulfite liquor and the assorted waste products dissolved from the wood are discharged into river systems such as the St. Louis River.

Only the Cloquet, Carlton and Fond Du Lac stations were sampled on February 28, 1968. Because of the interesting growth results obtained from the February 2, 1968 samples with apparent growth suppression occurring in the Carlton sample and possible growth suppression occurring in the Fond Du Lac sample, it was decided to limit this second experiment to the three mentioned sampling stations. The growth results (Figure 31) in the Cloquet water sample (960 million cells per liter) was approximately equal to that of the earlier February 1, 1968 sample. The responses of S. gracile in the other two samples, however, were even lower than those which occurred in the earlier samples. These responses were 3.3 million and 54 million cells per liter, respectively for the Carlton and Fond Du Lac samples. The 1.0% BBM enriched samples again produced interesting results (Figure 4). The Cloquet growth response (1.6 billion cells per liter) was, again, considerably higher than that of the external control (820 million cells per liter). The other two enriched samples, however, had much lower growth responses than the external control and were also lower than those obtained in the February 2, 1968 enriched samples. They were 76 million and 370 million cells per liter respectively, for Carlton and Fond Du Lac. Thus even greater evidence of growth suppression was present in these two samples. This may have been due to the thicker ice layer present at the later sampling date which would have reduced the volume of water flowing under the ice. If growth inhibiting substances had been emitted into the river at some point between the Cloquet and Carlton sampling stations, they would have been more concentrated in the smaller volume of water.

Table V

CHEMICAL DATA FOR WATER SAMPLES COLLECTED FROM THE LOWER ST. LOUIS RIVER

Sampling Station	Date of Collection	pH	Total Solids (ppm)	D.O. (ppm)
Cloquet	February 2, 1968	7.1	129	
Carlton	February 2, 1968	7.0	247	
Fond Du Lac	February 2, 1968	6.8	236	
Oliver	February 2, 1968	6.7	242	
Arrowhead Bridge	February 2, 1968	6.7	245	
Highbridge	February 2, 1968	6.7	216	
Duluth Entry	February 2, 1968	7.1	83	
Cloquet	February 29, 1968	7.0	179	
Carlton	February 29, 1968	7.1	298	
Fond Du Lac	February 29, 1968	6.7	302	
Cloquet	March 11, 1968	7.3	142	8.9
Scanlon	March 11, 1968	6.8	231	7.7*
Carlton	March 11, 1968	7.2	262	15.0*
Fond Du Lac	March 11, 1968	6.7	220	6.7*

* Because of the presence of Sulfite waste liquor in these water samples, the alkali-hypochlorite modified winkler O_2 method was used.

No direct relationship could be ascertained between the pH's of the samples and the growth results (TABLE V). These values were 7.0, 7.1 and 6.7 respectively for Cloquet, Carlton and Fond Du Lac. Total solids concentrations, however, reflected the growth responses in both the unenriched and enriched water samples (TABLE V) and were larger than the concentrations of the preceding February 2, 1968 samples (TABLE V). The total solids concentrations were 179 ppm at Cloquet, 298 ppm at Carlton and 302 ppm at Fond Du Lac. These larger concentrations would be expected if pollutants emitted into the river bed had become more concentrated at the time of the later collection date.

The final lower St. Louis River samples were collected on March 11, 1968. Ice cover of approximately thirty inches was present at the Cloquet sampling station but all other stations were open. Besides the Cloquet, Carlton and Fond Du Lac stations, a fourth station, at Scanlon, Minnesota (Figure 4) was sampled. Since the Scanlon station was located between those at Cloquet and Carlton, any growth inhibiting pollutions emitted into the river below the Cloquet station should have been most evident at Scanlon. Growth responses of the culture alga in these river samples had a similar pattern to the earlier sample (Figure 32), but of a larger magnitude. These increased responses would be expected, however, because the melting ice and consequently higher water level would have caused greater dilution of any growth inhibiting pollutant present. A cell concentration of 1.0 billion cells per liter was obtained in the Cloquet sample whereas growth responses of much smaller magnitude were observed in the other three samples. They were 61 million, 76 million and 190 million cells per liter, respectively, in the Scanlon, Carlton and Fond Du Lac samples. Thus the closer to Cloquet that these three samples were collected, the greater was the reduction in the growth response. This is additional evidence for the presence of a growth inhibiting pollutant being emitted into the river at some point below the Cloquet sampling station but above Scanlon and Carlton. A growth response of 2.1 billion cells per liter was obtained in the enriched Cloquet river sample (Figure 33) which was considerably larger than that in the external standard culture (750 million cells per liter) and about the same as that of earlier enriched Cloquet samples. The enriched aliquots of the other three samples again produced growth responses of much smaller magnitude than that of the external control. They were 110 million cells per liter in both the Scanlon and Carlton samples and 200 million cells per liter in the Fond Du Lac water sample. Thus evidence of growth suppression was present in all three cultures. pH determinations carried out on the four river samples produced results similar to those of the previous samples. The Cloquet and Carlton samples had similar pH values of 7.3 and 7.2. For the Scanlon and Fond Du Lac samples they were 6.8 and 6.7, respectively. Total solids concentration were also similar to those obtained from the preceding February 28, 1968 samples (TABLE V). However, the magnitude of the concentrations was less in the present samples. The Cloquet sample was, again, considerably lower in total solids (142 ppm) than the other three samples. The concentrations of the other three river samples, 231 ppm at Scanlon, 212 ppm at Carlton, and 220 ppm at Fond Du Lac. Dissolved oxygen determinations carried out on the four river water samples produced varied results (TABLE V). At Carlton, a dissolved oxygen concentration of 15.0 ppm was obtained. This concentration was much larger than those of

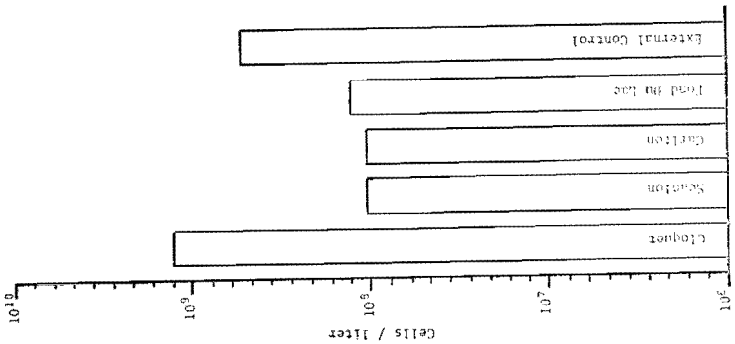

Figure 33

Growth Responses Of S. Gracile Cultured In 1.0% BBM Enriched Lower St. Louis River Water and In 1.0% BBM In Distilled Water. Samples Collected March 11, 1968

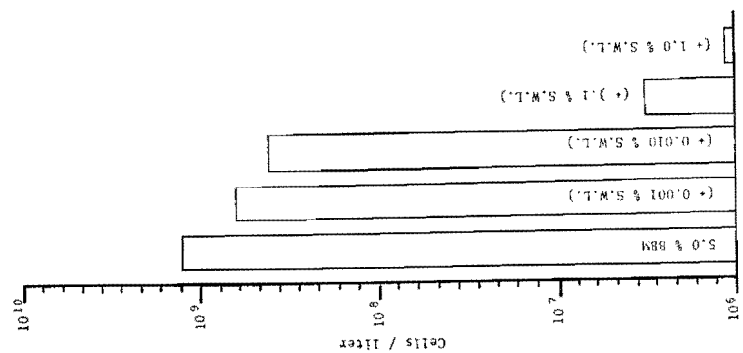

Figure 34

Growth Responses Of S. Gracile In 5.0% BBM In Distilled Water and In 5.0% BBM Enriched Distilled Water With 0.001, 0.01, 0.1 and 1.0% Sulfite Waste Liquor

the other three river samples. However, as this sample was collected just below the Carlton dam, the water at the sampling site could be expected to be well aerated. Thus an increase in the dissolved oxygen concentration of the sample would be expected. The concentrations obtained from the Cloquet, Scanlon and Fond Du Lac samples were 8.9, 7.7 and 6.7 ppm, respectively. Thus, except for the Carlton sample, a decreasing trend in dissolved oxygen concentrations occurred. The dumping into river at Cloquet of wood fibers and other waste products dissolved from the wood by the sulfite liquor processed would have placed a biological oxygen demand on the river. Since evidence of these waste products was visible as far south as Fond Du Lac, oxidation and biological decomposition have occurred at greater intensity at this southernmost sampling station. This would explain the largest reduction in the dissolved oxygen concentration having occurred at Fond Du Lac.

Sulfite Waste Liquor Study

The interesting growth results obtained from the lower St. Louis River study, in which apparent growth suppression of the culture alga had occurred, was the impetus for this final study. As was previously mentioned, the Northwest Paper Company of Cloquet, Minnesota was using the sulfite liquor process for digesting wood. At the end of the process, the spent liquor and waste products dissolved from the wood were discharged into the St. Louis River. This discharge occurred on a continuous basis with approximately 600 million gallons of diluted liquor and 300 million gallons of concentrated liquor being discharged into the river each day (Data furnished by Mr. Schroeder of the Northwest Paper Company).

The first experiment was carried out with 1.0% BBM in distilled water. The waste liquor was added to the cultures in concentrations of 0.001, 0.010, 0.100 and 1.000% of the 200 ml volume used. Triplicate cultures were prepared of each liquor concentration studied and the average growth results are shown in (Figure 34). A reduction in the growth response of *S. gracile* occurred even in the flask containing the smallest concentration of liquor. This reduced response was 820 million cells per liter as compared to 2.0 billion cells per liter in the control cultures. A somewhat smaller response of 650 million cells per liter was observed in the 0.010% liquor cultures. In the cultures containing the two largest concentrations of liquor, a dramatic reduction in the growth responses of the cultures alga was observed. They were only 5.4 million cells per liter in the 1.000% liquor cultures and 1.5 million cells per liter in the 1.000% liquor cultures.

A possible cause of the observed toxic effects of the sulfite waste liquor could have been the acidic nature of the waste substance (approximately 4.0). However, pH determinations made on the cultures did not indicate this. In the cultures containing the largest concentration of liquor, an average pH of 6.0 was observed and those of all the remaining cultures were approximately 7.0.

The second experiment in which the effects of sulfite waste liquor on *S. gracile* were studied were carried out in Lake Superior water. This water was collected March 12, 1968 through the approximately thirty

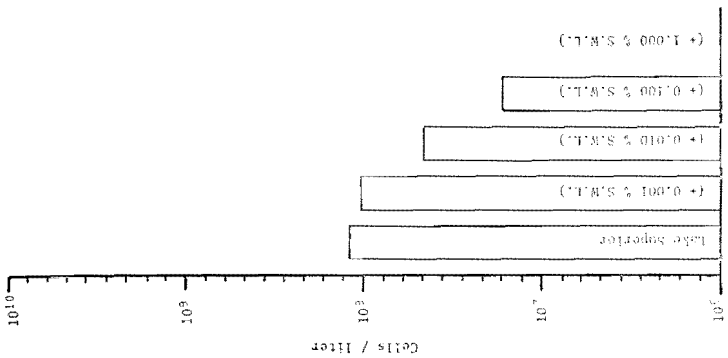

Figure 35

Growth response of S. Gracilis in Lake Superior Water and Lake Superior Water Enriched with 0.001, 0.010, 0.100 and 1.000% Sulfite Waste Liquor (Water Sample Collected March 8, 1968.)

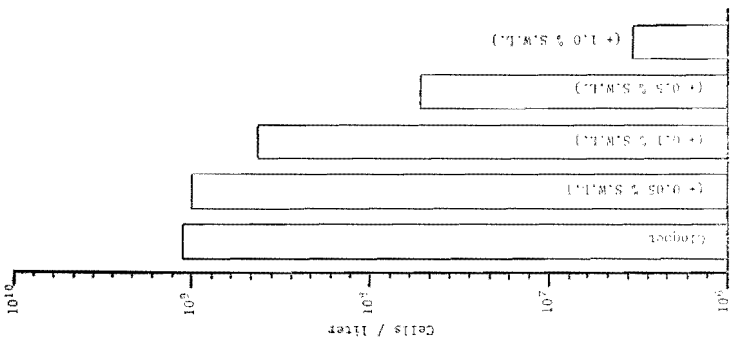

Figure 36

Growth Response of S. Gracilis in Lake St. Louis River Water (Cloquet, Minnesota) and in Mixtures of the Sample Water Enriched with 0.05, 0.1, 0.5, 1.0% Sulfite Waste Liquor (Water Sample Collected March 27, 1968.)

inch layer of ice which covered the Lester River Bay at that time (Figure 3). The sample was taken about 300 yards north of the Lester River and 100 yards out from shore. Waste liquor was added in concentrations of 0.001, 0.010, 0.100 and 1.000% (Figure 35). As in the previous experiment, the addition of this substance caused a reduction in the growth response of the culture alga. The response in the 0.001% liquor culture was 110 million cells per liter as compared to 170 million cells per liter in the control cultures. Further decreases of 70 million and 31 million cells per liter, respectively, were observed in the cultures containing 0.010 and 0.100% liquor. In the triplicate cultures containing 1.000% liquor, no algal cells could be found at the end of the seven day incubation period. However, the acidic nature of the last series of cultures (pH 5.1) could have caused, at least in part, this lack of growth response. A pH of approximately 7.0 was observed in the remaining cultures.

The final sulfite waste liquor experiment was carried out in water from the station above Cloquet. This station was selected because previous water samples taken from Cloquet had shown no signs of inhibiting the growth of the culture algal. In the experiment, sulfite liquor was added to the water sample cultures in concentrations of 0.05, 0.10, 0.50 and 1.00% of the total culture volume. The Cloquet samples were collected March 27, 1968. A slight decrease in the growth response of the 0.05% liquor cultures was observed. The average response was 970 million cells per liter as compared to 1.5 billion cells per liter in the control cultures. Further reductions of 670 million, 76 million and 5.3 million cells per liter were found in the 0.10, 0.50 and 1.00% liquors, respectively. pH measurements taken on these cultures showed that in none of them was low pH a factor in the growth inhibition observed. (The pH was nearly 7.0 for all cultures.) Therefore, as in the other two experiments, some other quality of the sulfite waste liquor was responsible for its toxic nature.