

AN ABSTRACT OF THE THESIS OF

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EFFECT ON THE GROWTH OF ANABAENA CYLINDRICA
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An exploratory study was made of some ecological aspects of humic water of Klamath Marsh in southern Oregon. Samples of marsh water were taken in January and August, 1959. Physical and chemical investigations of the marsh water revealed that concentrations of mineral elements in the marsh water were lower than average freshwaters of the world. Color and pH of the water were stable in storage. The water was more highly colored in winter than in summer, but the summer collection contained more total and suspended residue, more mineral elements, and more organic matter than the winter collection. The carbon:nitrogen ratio was narrower in the summer collection. The study revealed inadequacies in standard methods of water analyses when applied to strongly humic waters. Suggestions were made for improving

methods of analysis and study of humic waters.

Two types of investigations were used to determine the ecological importance of marsh water in growth of freshwater algae: (1) physical-chemical studies and (2) cultural studies performed with pure cultures of Anabaena cylindrica Lemm. (strain number 629 from the culture collection of the University of Indiana) grown under controlled conditions of temperature and illumination. The following characteristics of the marsh water were studied for possible ecological significance: (1) the buffering capacity of the water, (2) the significance of the concentrations of essential elements in the water in growth of A. cylindrica, (3) effects of humic water upon absorption of iron by the experimental alga, and (4) effect of humic water upon the stability of iron and phosphorus in an alkaline culture medium.

The mineral composition of the synthetic medium used was derived empirically by averaging concentrations of mineral salts in media used by other workers successful in growing blue-green algae. Replacement of the distilled water in the medium with water from Klamath Marsh stimulated growth of the alga. Addition of extra quantities of calcium, sulfur, or potassium increased growth of the alga in the defined mineral medium. More calcium was required by A. cylindrica in a purely mineral medium than in a

medium containing water humus. Both collections of marsh water contained enough sulfur to increase growth of the alga, and the summer collection contained enough potassium to increase growth.

The presence of marsh water prevented precipitation of iron and phosphorus from an alkaline, aerated medium containing a relatively high concentration of calcium. Most iron and phosphorus were adsorbed or complexed on humic colloids and could be removed by passage through micropore filters with a pore size of 0.45 micron.

Stimulation of growth was not due to ability of marsh water to keep iron in solution under conditions of high pH, as has been claimed by other authors, nor was addition of the extra iron in the marsh water the cause of stimulation. The presence of marsh water greatly decreased absorption of iron by A. cylindrica as compared to its absorption of iron from a ferric citrate-citric acid complex or from ferric hydroxide.

Titration of culture media containing marsh water revealed that it was not an exceptional buffer substance, nor were changes in pH of media due to photosynthesis and respiration greatly lessened by the presence of marsh water.

The results suggest that addition of humic water to a culture medium may stimulate growth of algae by supplying essential

elements present at sub-optimal concentrations in unamended mineral media, by stabilizing mineral salts in the media, and by regulating absorption of minerals, all of which may provide beneficial conditions for growth of algae.

THE HUMIC WATER OF KLAMATH MARSH
AND ITS EFFECT ON THE GROWTH
OF ANABAENA CYLINDRICA LEMM. IN CULTURE

by

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THE HUMIC WATER OF KLAMATH MARSH
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INTRODUCTION

In the cycles of synthesis and degradation of organic matter on earth, the remains of plants and animals do not always undergo complete and rapid mineralization. Certain fractions of these residues are resistant to decomposition, and remain for some time in a modified state. Under the proper conditions, these residues may accumulate in the soil or in the bottom sediments of lakes. These resistant materials are light brown to black in color, possess characteristic physical and chemical properties, and are usually called "humus".

During the accumulation of humus, elements essential for life are removed from circulation for a time. They are released in a slow but continuous stream as the humus is subjected to gradual decomposition. Thus, humus serves as a reserve of essential elements and is a factor in regulating life on this planet. Humus possesses a variety of physical, chemical and biochemical properties that make its presence in a soil more beneficial to the growth of plants than when humus is absent (122, p. xi-xii).

There is no reason to suppose that cycles of synthesis and degradation of organic matter in natural waters are fundamentally

different from those on land, yet there is a tendency in much limnological work to regard unpolluted waters as solutions of mineral salts. Often the organic matter in natural waters receives only token recognition, sometimes none at all. This problem was recognized ten years ago by Ruttner (96, p. 84) who said:

It was long assumed in limnological investigations that 'pure' waters of lakes, streams, and rivers could be considered as inorganic solutions as long as they were not contaminated by human animal excreta or by certain industrial wastes (for example, from sugar refineries, distilleries, rayon factories), and that the organic matter contained in them consisted predominantly of organisms and organic detritus, that is, of particulate material.

Many lakes and streams contain considerable quantities of organic matter in solution and suspension. Even in relatively "clean" lake waters the dissolved and colloidal organic matter may exceed the particulate organic matter (including plankton) by a factor of five to ten (120). Some lakes receive from their drainage basins various amounts of organic material in waters stained yellow to brown by extractives from peat bogs or marshes. The humic nature of these stained waters has been recognized since the beginning of their systematic study. Richards and Ellms (93, p. 68) recognized that the source of color in certain streams of New England was "humus matter" draining from hillsides and swamps, and they recognized that the color was due to a mixture of substances.

Aschan (8, p. 172) called the colloidal substances of the colored waters of Finland "Humussole," a contraction of "humus hydrosols," and recognized that they contained a mixture of substances. It was Waksman's opinion (122, p. 306) that the organic materials in natural waters could be considered as members of the general group of organic complexes described as "humus". The writer agrees with this practice and, for convenience (plus other reasons to be explained later), will use the expression "water humus" in this thesis to designate all non-living organic matter in natural waters. Although this thesis is concerned primarily with colored humic waters, it should be noted that water need not be highly colored to contain organic matter.

Physical and chemical studies of colored, humic lakes and streams have been reported from Finland (8), Denmark (11), Sweden (1; 46; 69; 79; 81), Germany (35; 76; 77), northeastern Wisconsin (59; 60), Connecticut (107), and Massachusetts (93). Although these studies cover a span of 70 years, little attention has been paid to humic waters as ecological factors in the growth of freshwater algae. Some of the authors of the aforementioned studies have postulated that these waters should be of ecological significance (8, p. 176; 46, p. 153; 77, p. 597; 80, p. 471-474).

An exceptional example of a lake that receives much organic

matter from its drainage basin is Upper Klamath Lake in southern Oregon. A three year study of the phytoplankton problems in this lake (86) failed to produce any evidence that the dense algal bloom produced every summer could be attributed to exceptional concentrations of mineral nutrients. The most remarkable limnological feature of the lake was the amount of humic water draining into it. It receives drainage from approximately 136,000 acres of marsh land as well as the run-off from about 92,000 acres of "muck" soil under cultivation. Most of the water entering this lake is highly colored and contains large quantities of dissolved and colloidal humus material. Naturally, a possible connection between the humic water and the great algal "blooms" was envisioned. Accordingly, a study of the ecological significance of humic marsh water was begun. The effluent of Klamath Marsh was chosen for its ease of access.

Klamath Marsh occupies approximately 85,000 acres centered 15 miles east of Crater Lake National Park. The marsh lies in the course of the Williamson River which is the major source of water for Upper Klamath Lake. The marsh appears to be formed in the basin of a lake in the final stages of senescence. The principal vegetation of the marsh at present consists of sedges, bulrushes and cattails. There is a considerable accumulation of peat around

the margins and on the bottom. On passing through the marsh, the Williamson River picks up a great quantity of humic matter in solution and suspension and becomes amber colored.

During the spring and summer of 1957, a brief study of the effluent of Klamath Marsh was made. As a part of the study, some of the water was added to an algal culture medium (Rodhe's No. VIII) known to be effective in supporting growth of algae. This was used to grow a species of Scenedesmus. Even when diluted (replacing only one-fourth of the distilled water in the medium), the marsh water had a stimulatory effect on the growth rate and total growth of the alga as compared to the unamended medium. This result led to the studies reported in this thesis. The investigations reported here were based on the assumption that the brown water of Klamath Marsh contained a humus complex.

The importance of humus in soils is well-known and was summarized admirably by Waksman (122, p. xii-xiv, 293, 307-331). In addition to serving as a "storehouse" of elements essential for plant growth, humus compounds have a strong solvent action on soil minerals and, for this reason, humic waters flowing through marshes or bogs may become rich in various minerals. The physical and physicochemical properties of humus, for example its high content of electrically charged colloids, its cation exchange

capacity, buffer capacity, etc., should make it capable of "stabilizing" the environment considerably.

Another reason for these investigations was provided by the well-known research of Pringsheim (89, p. 40-43) in which soil and peat extracts were used to cultivate algae that were unable to grow in defined mineral media. Pringsheim claimed that such extracts had considerable buffering effect and prevented some substances, especially ferric compounds, from being precipitated, keeping them more readily available to the algae. Pringsheim offered no evidence to support these claims.

The study reported in this thesis was based on two hypotheses: (1) the humic water of Klamath Marsh might contain physiologically significant amounts of cations in soluble, complexed, or adsorbed form and therefore serve as a supply of essential elements; (2) the marsh water might serve as a "stabilizer" of a culture medium either by a buffering action or by complexing or chelating certain essential elements and keeping them in a form more readily available to plants under conditions where they might ordinarily precipitate from a completely mineral medium.

The objectives of the study were to obtain a brief physical and chemical description of the humic water of Klamath Marsh and to determine the ecological effects of this water on the growth of a

blue-green alga in culture. The experiments were exploratory in nature and were intended (in combination with the review of literature) to indicate the directions for further, more detailed studies of water humus.

REVIEW OF LITERATURE

1. Introductory remarks.

The scope of this review has necessarily been limited because of the great volume of literature relevant to the subject of humus. The primary objective has been to assemble the evidence indicating the ecological importance of humus in the growth of plants, especially in relation to the frequently observed stimulatory effect of humus on the growth of plants and the effect of humus on mineral nutrition of plants. It will be necessary to consider briefly the origin and nature of humus, and brief consideration must be given the physical and chemical properties of humic waters because the chief concern of this thesis is water humus and its ecological importance. Information from the literature of soil science must be used because soil scientists have studied humus more actively and for a longer time than have the limnologists.

Perusal of Oden's monograph on humic acids (75) and the first three chapters of Waksman's treatise (122, p. 3-64) will provide excellent accounts of the history of the study of humus and the changing concepts of the nature of humus, its formation, and its role in plant nutrition. The term "humus" is approximately 2000 years old and was originally used to designate the soil as a whole.

Systematic study of soil humus has been in progress for about 200 years. The first definition of humus as decomposed organic matter has been attributed to J. G. Wallerius in 1761 (122, p. 4).

The systematic study of water humus began 70 years ago with a report on the coloring matter of certain streams in New England (93). For the first 40 years, studies of water humus were primarily concerned with its physical and chemical properties. Within the past 30 years, limnologists have begun to investigate the ecological significance of water humus in the growth of aquatic plants. During this time, much attention has been directed to the role of water humus in supplying organic metabolites to auxotrophic algae (algae with small but definite requirements for certain preformed organic materials, such as vitamins). Among the reviews of the present state of our knowledge of water humus, only that of Vallentyne indicated the essential relationship between limnology and soil science in regard to the study of organic matter (121, p. 35). Apparently some limnologists have an erroneous notion that humus is a definite chemical entity or a closely related group of compounds. Humus is not static. It is a dynamic state of organic matter - a "complex" or "system" involving both organic and inorganic compounds. Humus changes constantly in time and space.

2. The origin, nature, and transformations of humus.

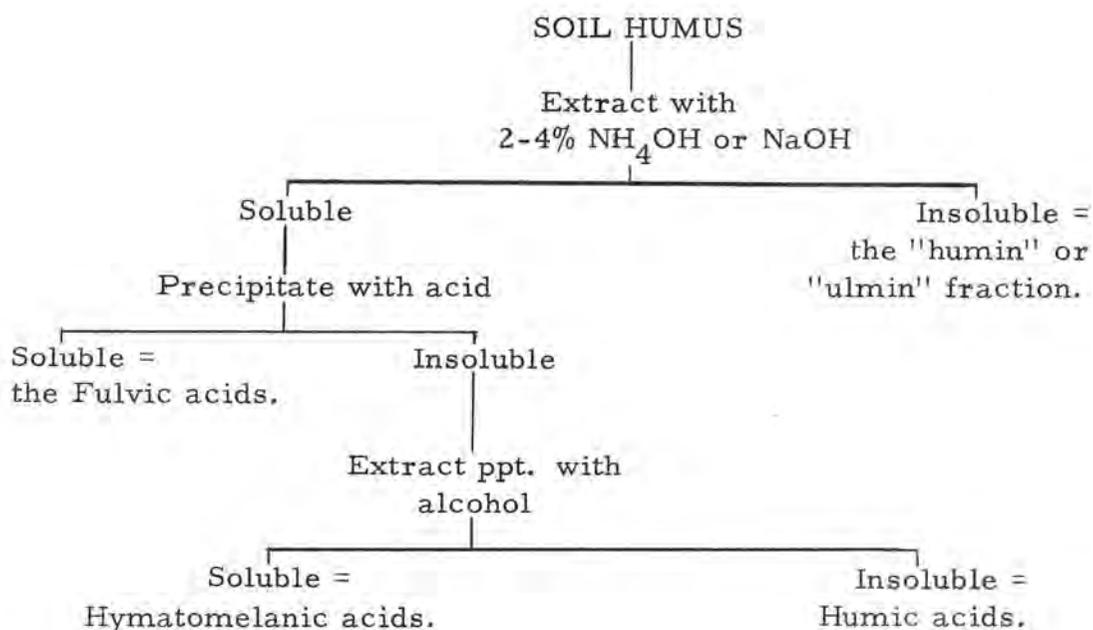
A great deal more is known of the origin, nature and reactions of humus in soils than in water. One might expect the processes to be fundamentally similar, possibly with some deviation in the humus of lakes and streams because of the presence of large quantities of water.

a. Humus in the soil.

Transformations of humus in the soil are extremely complex. Formation of humus begins, of course, with dead bodies of plants and animals. It was once believed that the easily decomposed constituents of this organic matter did not participate in humus formation because microorganisms can mineralize them completely. According to this view, the carbohydrates (including celluloses and hemicelluloses), proteins and fats of recently dead organisms were excluded from humus formation. These easily attacked compounds are the "nutrient" humus (Nährhumus) of Scheffer (100, p. 209). It is now known that as the microorganisms attack the non-colored nutrient humus, they form various metabolic by-products, e. g., uronic acid "slimes" and other compounds which appear to be slightly more resistant and can serve as precursor substances

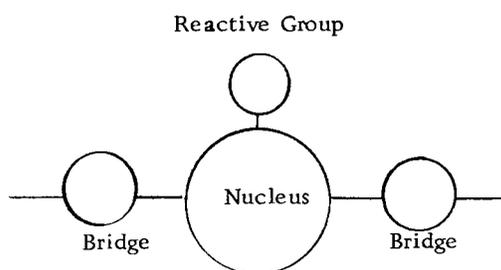
for the formation of "resistant" humus - the "Dauerhumus" of Scheffer (100, p. 208). The resistant humus is characteristically dark colored and is attacked more slowly by microorganisms. The term "humus", when used without qualification, usually means resistant humus.

Soil scientists have used various methods of extraction in attempts to determine the chemical nature of humus. An excellent history of the earlier work is presented in Oden's monograph (75). At present most students use a fairly standardized procedure, generally following that presented by Bertramson (12, p. 53):



The "humin", fulvic acid, and hymatomelanic acid fractions have received little study. Humic acids have been studied quite

extensively. One of the more illuminating of the recent works on the fractions of humus is that of Thiele and Kettner (118). They feel that fulvic, humatomelanic and humic acids are related groups of compounds with increasing complexity of structure in the order named. These "acids" are mixtures of related substances; none are distinct chemical entities. Humic acids are polymers of quinoid or phenolic substances; they are of colloidal size, hydrophilic, but completely insoluble in water. The basic building units consist of: (1) a nucleus with a ring structure; (2) bridges connecting the nuclei and (3) reactive groups attached to the nuclei. They may be represented diagrammatically as follows:



The nucleus may be a 6-carbon ring, a 5-carbon ring, or a condensed ring such as naphthalene, anthracene or tryptophane. If the original nucleus is provided by decomposition of lignin, the position of the reactive group may be occupied by a methoxyl group (-O-CH₃) which is not particularly reactive. During humification, it is replaced by hydroxyl groups (-OH), sulfonic groups (-SO₃H), or carboxyl groups (-COOH). The bridges can be -O-, -NH-, -CH₂-, -S-, or the nitrogen of amino acids. The reactive groups

are the main determinants of the behavior that a humic acid will show, i. e. , its acidity, hydrophily, ion exchange capacity, etc.

One can compare the humic acids to proteins in that a few building units can assemble in various ways, giving different compounds with a fundamental unity. Humic acids are spherical colloids (30, p. 191). One humic acid may be a colloidal micelle consisting of approximately 10,000 building units (118, p. 132). If one imagines these 10,000 building units in a three-dimensional network, one is reminded of the high polymer phenols used as ion exchangers. Thus, the humic acids may be envisioned as naturally occurring ion-exchange "resins".

Humic acids have been synthesized from hydroquinone and related compounds (30; 39; 100; 103; 104; 128). The synthetic humic acids resemble the natural ones, but comparative studies using ultra-violet and infra-red absorption spectra and chromatography show them to be less complex than the natural ones, as might be expected.

The fulvic acid fraction has been studied far less than the humic acid fraction, but significant studies of fulvic acids have been reported (61; 113; 115). The fulvic fraction is a mixture of substances including organic phosphorus and nitrogen compounds, and colored materials that show fluorescence in ultra-violet light.

Fulvic acids are water soluble. On standing in solution they become darker in color. For this reason, some authors believe that fulvic acids can be transformed into humic acids through condensation and polymerization (61, p. 145).

The little known "humin" fraction of humus may be a product of aging of the humic acids, accompanied by loss of the reactive side groups, whereby the acidic character becomes less and less. This, of course, is accompanied by decreasing solubility in alkalis (102, p. 31).

Because resistant humus contains aromatic rings, methoxyl groups, and sometimes considerable amounts of nitrogen, Waksman believed that it was formed by condensation of lignin with proteins synthesized by microorganisms. Lignin was regarded as the principal source of humus nuclei (122, p. 93-129). Later hypotheses were based on Waksman's, but differed in assuming that lignin, on being decomposed, furnishes phenolic nuclei that are coupled by means of ammonia liberated during decomposition of nutrient humus (63, p. 77-78).

The contemporary view of humus formation does not discount the importance of lignin as a precursor of humus, but adds the idea that humic compounds can also be formed by a two-step process including: (1) decomposition of plant and animal residues to

simpler compounds and (2) subsequent synthesis of these compounds into the building units of the high molecular humic substances (63, p. 77). The origin of humus is not limited to lignins, proteins and tannins or their degradation products. The origin of the building units of humic compounds, i. e., aromatic or quinoid compounds, may be any of the simpler, easily decomposed components of nutrient humus, e. g. cellulose, starch, sugars, etc., as well as decomposition of lignins, proteins and tannins. The amino acids and peptides used in building humic compounds probably are products of microbial resynthesis, because the nitrogen-containing components of the original nutrient humus are broken down fairly rapidly by fungi and bacteria. The condensation phase, when building units of humus are coupled together, proceeds with biocatalysis by enzymes of the phenoloxidase type, produced by many organisms, especially the fungi (66).

The importance of microorganisms in humus formation is great. Azotobacter in culture can form dark brown substances from inorganic nitrogen, using benzoic acid as the only source of carbon. These dark substances are quite similar to humic acids of soils (87). Aspergillus niger and Penicillium spp. are able to form

humic acids in a medium containing glucose as the only source of carbon and sodium nitrate as the only source of nitrogen. The fungi can manufacture both the phenolic building units and the peptide nitrogen and then condense them into typical humic acids that are almost identical with humic acids extracted from podzolic and Chernozem soils (63).

Possibly future research will reveal still other mechanisms of humus formation, but it seems likely that the basic structure of humic acids will vary only in details, while the fundamental structure - aromatic rings linked through bridges and bearing functional groups as side chains - will prove to be characteristic.

b. Humus in water.

The only reviews on the origin and transformations of humus in natural waters (122, p. 289-306; 123) are over 20 years old, so the need for study of humification processes in natural waters is obvious. Bacteria have been credited for most transformations of humus in water. Study of the role of aquatic fungi in the formation of water humus has been neglected. In view of the known importance of soil fungi in the formation of resistant humus (51; 63; 66), this is a serious lack in limnology. There can be little doubt that aquatic fungi are important in these processes, but at present

there is little knowledge of their activity.

There are two sources of organic matter in natural waters:

- (1) it may be produced in the water by synthetic activities of the aquatic plants and growth of aquatic animals and microorganisms;
- (2) it may be brought in from outside sources such as detritus blown in by wind or drainage from bogs and marshes. These latter contribute great quantities of water humus to lakes and streams (13, p. 453).

It is now known that certain algae, while still living and healthy, may excrete polysaccharides and polypeptides into culture media (14; 42; 44). If these substances are produced in nature, i. e., if they are not products of adaptive enzymes in laboratory strains, they should be excellent source materials for the synthesis of resistant humus. There is presumptive evidence that such compounds may be produced in nature because amino acids and free sugars have been isolated from lake waters (34; 85; 120; 121, p. 39). It is interesting in this connection to note the "correlation" between the population of Cyanophyceae and the content of organic matter (particularly albuminoid nitrogen) noted by Pearsall in certain English lakes (84). Pearsall felt that the appearance of blue-green algae "correlated" with a build-up of organic matter in the water. Quite the reverse could have been the case - the organic

matter could have been present because of the algae instead of vice versa.

Regardless of source, the organic matter undergoes continuous transformation, being utilized by aquatic animals and bacteria which, in turn, are utilized later by other microorganisms. Plant and animal residues are not completely destroyed; carbohydrates and proteins are oxidized first. Lignins, fats, proteins, chitins, and polyuronides may accumulate in the bottom sediments, forming resistant humus which is slowly decomposed by the microorganisms. This causes an exchange of organic and inorganic materials between the bottom sediments and lake water (123; 130). Organic materials arising from the bottom or from influent waters are partly colloidal (46, p. 151-154; 59, p. 45-47; 78, p. 253; 79; 80). Whether these colloids are the same as humic acids found in soils has not been determined.

As would be expected, transformations of humus in water are controlled by the available mineral nutrients, season of the year, pH, temperature, oxygen content, etc., in the water. These transformations probably are quite important in determining the productivity of a lake or a stream, but the magnitude of their importance is unknown.

3. Physical and chemical properties of humic waters.

In general, limnologists have studied humic waters from the "classical" approach, i. e., features such as color, acidity and alkalinity, specific conductivity, residue, mineral content and total organic content have been measured. Attempts to interrelate the results of these studies have been made, but these efforts have not been especially fruitful. The only generalizations that can be made with certainty are that humic waters differ greatly from place to place and time to time, and they may vary greatly within a relatively restricted geographic area.

A study of 530 lakes in northeastern Wisconsin (60, p. 258) revealed that the lakes varied in color from almost none to 340 platinum units, a great range in color, yet the lakes were located within a radius of 30 miles from the Trout Lake laboratory. The amount of color in the lakes showed pronounced seasonal variation related to precipitation and run-off. Specific conductivity of the water was correlated with the quantity of "fixed" or bound carbon dioxide, which is related in turn to the quantity of calcium and magnesium present, and these are regulated by the nature of the soil in the drainage basin of each lake.

Physical and chemical data from 12 humic lakes in two

adjacent counties in northern Michigan, given as ranges compiled from the records of several summers, showed striking differences in color, pH, alkalinity and specific conductivity, not only among the different lakes, but in the ranges found in individual lakes (127, p. 390). The 12 lakes exhibited reactions covering the whole range for normal, unpolluted waters (pH 4.0 - 9.0).

Some of the strongly colored (so-called "dystrophic") lakes in northern Germany are alkaline and rich in electrolytes while others are acid and poor in electrolytes (77, p. 589).

The problem of variability in humic lakes is better illustrated by intensive study of one lake, such as Berg and Peterson's investigation of Lake Gribsø in Denmark (11). Gribsø is a small seepage lake (no outlet) fed by a number of small brooks, some of which drain peat bogs. During the spring thaw, there was a rapid and strong increase in color, due to the addition of humus to the lake by influent waters. In August the color of the lake reached a minimum, although a heavy rain could bring it back. The mineral content varied widely with the seasons. The study of Gribsø throws doubt on all determinations of bicarbonate alkalinity in waters that contain weakly acidic substances other than bicarbonate. The usual titration method for bicarbonate alkalinity showed 4.3 milligrams per liter, but when the investigators distilled the carbon dioxide and bicarbonate into barium hydroxide and titrated with normal

hydrochloric acid, they found that the actual bicarbonate content ranged from 0.16 to 0.29 milligram per liter - far less than the "standard" method indicated.

Lake waters in the spruce forests of Halifax County, Nova Scotia, are strongly influenced by the nature of their geological substrata, the topographies of their drainage basins, and their proximity to the sea. Lakes on granite are acid; on carboniferous rocks the lake waters are circumneutral. Both acid and circumneutral waters show wide ranges of organic content. The proximity of the lakes to the sea is indicated in their relatively high content of sodium chloride as compared to the average freshwaters of the world (49).

The vegetation of a lake can have a marked effect on its chemical composition and pH (49, p. 19; 127, p. 391). Of course, the geological substratum will influence the type of vegetation that will develop in a lake or a peat bog (50, p. 160). The acid character of some lakes has been connected with the presence of Sphagnum mosses (76, p. 399). Humic "bog" waters are not necessarily (or even usually) acid. The cause of high acidity in such waters is the establishment and dominance of Sphagnum which is able to reduce the pH of a salt solution by adsorption of the basic ions, leaving the anions in solution (65, p. 63-64).

Because one of the most characteristic features of humic waters is their content of organic matter, limnologists have attempted to investigate the forms in which the organic matter occurs. An extensive list of organic compounds found in natural waters is accumulating. Amino acids have been found in the free state and in hydrolysates of residues, but proteins have not been isolated. Hydrolysis of the residues always yields far more amino acids than can be found in the free state. Carbohydrate compounds of various sorts have been isolated, including free sugars. Various fats, waxes, non-nitrogenous acids, alcohols and vitamins have been found (121).

Proximate analyses of the organic matter of 529 lakes in northeastern Wisconsin were reported by Birge and Juday (13) who attempted (and probably failed) to separate the organic matter into "planktonic" and "dissolved" forms by means of centrifugation. These two fractions were analyzed for organic carbon, organic nitrogen, and "fat" (ether extract). The study clearly showed the influence of drainage on the organic content of the waters. "Drainage" lakes (those with inlets and outlets) generally contained more organic matter and were more colored than "seepage" lakes. The study also showed that there was considerably more "dissolved" than particulate organic matter in the water. It seems doubtful that

their centrifuge could remove the smaller colloids.

Domogalla, Juday and Peterson (34) and Peterson, Fred and Domogalla (85) claimed to have established the presence of specific amino acids, purines, and various other forms of organic nitrogen in certain lakes in Wisconsin. This work is 40 years old and needs repeating.

These last three papers are well reviewed and discussed by Waksman (123, p. 95-100) and Hutchinson (57, p. 882-896), who presented the data in considerable detail.

The validity of these studies must be questioned because quantitative determination of amino nitrogen by the van Slyke method (used by the investigators in Wisconsin) depends on time of reaction, and phenolic substances (surely to be expected in brown waters) interfere with the van Slyke determination (25). The Kjeldahl determination of organic nitrogen has limitations. At least 30% of the soil nitrogen is resistant to acid or alkaline hydrolysis and, for this reason, the Kjeldahl method does not determine total organic nitrogen in the soil (22, p. 77-79). The practice of multiplying total organic nitrogen by 6.25 and naming the result as "protein" is questionable.

The highly colored extractives from bogs and marshes tend to widen the carbon:nitrogen ratio of lake waters receiving them,

whereas the organic matter produced within the lake basin is less colored and has a narrower carbon:nitrogen ratio (13, p. 455-469).

Birge and Juday (13, p. 453) have used methoxyl determinations to indicate lignin-like substances in lakes. Similar determinations by others are discussed by Hutchinson (57, p. 887). It is likely that in the process of humification, lignin loses methoxyl groups, these being replaced by carboxyls, phenolic hydroxyls or amino acids (23, p. 221-228; 47, p. 450). For this reason, a methoxyl determination is of little value in determining lignin unless the methoxyl content of the original material is known. This is impossible in studying natural waters.

Relatively few conclusions can be drawn from the present state of knowledge of the organic compounds in natural waters. The data are varied, fragmentary, and derived by questionable methods. They serve only to reinforce the idea that water humus, like soil humus, is organic matter in a state of continuous transformation. No doubt modern methods will be developed in limnology, e. g. , microfiltration, the use of isotopes, chromatography, electrophoresis, etc. , all of which will add greater precision to studies of water humus, especially if the studies are performed under controlled conditions.

Since the beginning of the study of humic waters, a

characteristic feature has been stressed - their color. There is always a positive (but not linear) correlation between color and the total content of organic matter as measured by the reducing power of the water, i. e. , the use of oxygen from potassium permanganate to oxidize the organic matter (8, p. 174; 11, p. 113-115; 46, p. 134; 60, p. 230; 77, p. 589-590; 81, p. 147).

Ohle started a "classification" of humic waters based on their reducing power, color, and transparency (77, p. 602). This system was endorsed by certain other limnologists (1, p. 152; 11, p. 104). In the light of the discussion presented on the preceding pages, the futility of such a classification is obvious. A further problem is the unreliability of permanganate as an oxidizing agent. It is subject to autodecomposition and fails to oxidize a number of organic compounds (111). There is also the possibility that the waters will contain inorganic reducing compounds such as hydrogen sulfide or ferrous or manganous compounds (11, p. 114). There is a question as to whether the oxidation should be performed under acid or alkaline conditions. Ohle recommended that the oxidation be performed in an alkaline medium (81, p. 148), but both acid and alkaline oxidations have been used.

To date, the most significant work on the coloring matter of humic waters was that of Shapiro (107) who isolated a mixture of

yellow carboxylic acids from several lakes in Connecticut. Shapiro called them "humolimnic acids", but this may have been an unnecessary addition to the vocabulary of limnology. The solubility behavior of the acids (acid, alcohol, and water soluble), their relatively low molecular weight, phenolic structure, and fluorescence in ultra-violet light would seem to place them in the fulvic acid fraction of resistant humus.

In summary, it is probably more realistic to recognize that all waters, like all soils, contain a certain amount of humus, the content ranging from considerable amounts to almost none, and not attempt to "classify" humic waters. If the humus is mostly colorless nutrient humus, most of it will be mineralized in the lake water, relatively little resistant humus will accumulate on the bottom, and the lake will be comparatively clear. If the productivity of the lake increases to the point where the mineralization of organic matter falls behind the rate of synthesis, more resistant humus will accumulate on the bottom and the lake will become more colored. If the lake receives a great amount of resistant humus from peat bogs and marshes, the lake will be highly colored, and humus will accumulate rapidly on the bottom. The kind and relative amount of humus in each lake will be determined by factors external to the lake.

4. Humus as an ecological factor in the growth of plants.

Humus may affect the growth of plants in many ways. The effects may be inhibitory or stimulatory, depending upon the plant in question, the conditions under which it is growing, and the origin and age of the humus in question. Since this thesis is concerned primarily with the stimulation of plant growth by humus, only a few examples of the detrimental effects of humus on plants will be cited.

a. Color and turbidity of humic waters.

As mentioned earlier, color and turbidity probably indicate the presence of humus in waters, even though some of the humus may be colorless. Both colloidal and dissolved colored materials can have strong effects on the transparency of lake waters (59;60). Lake Mary, a "medium" colored lake (color of 123 platinum units) in northeastern Wisconsin, at a depth of one meter filtered out 96% of the total radiation striking the surface. In the more deeply colored Helmet Lake (color of 268 platinum units), the upper meter filtered out 99% of the incident radiation (60, p. 230). Suspensoids and colloids scatter light in a non-selective fashion, but the dissolved colored material is selective in its absorption, and

removes the shorter wave lengths (49, p. 19; 60, p. 229). The colored lakes of southern Sweden seldom transmit much light below one meter (69, p. 366), and the greater part of the plankton production in these lakes is in the surface layers (46, p. 144). Most of the colored material in lakes is soluble, and may belong to the group of acids isolated by Shapiro (107).

Probably color and turbidity is of lesser importance in the production biology of shallow lakes than in the deeper ones. It is doubtful that color of most humic waters could have a significant effect on the growth of algae in cultures with proper illumination.

b. Toxins and antibiotics in humus.

Numerous observations have revealed that soil humus may contain substances injurious to plant growth. Among the compounds isolated and identified have been vanillin, benzoic acid, various aldehydes and dihydroxystearic acid (122, p. 355-358). None of these are very abundant, and they are probably constantly decomposed by fungi (54; 55; 83). Residues from the early stages of decomposition of various crop plants contain substances capable of reducing the respiration, germination and growth of tobacco seedlings. The water-soluble, heat-stable substances cause darkening and necrosis of root tip cells of the affected plants (83).

Certain bog waters in Ohio were found to inhibit growth of *Marchantia gemmae* and seedlings of corn, beans, and wheat (31, p. 133-137). Treatment of the water with lamp black carbon or calcium carbonate not only removed the toxic effect but the treated waters stimulated growth of the experimental plants. Certain bog waters around the Puget Sound area of Washington inhibited the formation of root hairs even in plants native to the bogs (94). These studies should be pursued further, because the reasons for the toxic effects are practically unknown. It is possible that the humic colloids of bog waters are poor in mineral nutrients due to the great adsorptive capacity of surrounding peat, or the colloids of the waters may be saturated by "swarms" of hydrogen ions (50, p. 151).

Substances antibiotic in nature are well known from soils as well as marine and freshwater habitats (121, p. 62). No doubt these are of ecological significance, but at present it is not possible to say to what extent they influence the growth of other organisms. The subject is still under study. Since these compounds and their ecological importance are outside the area of interest of this paper, no further discussion will be presented. Interested readers are referred to the reviews by Krauss (64, p. 227-230), Saunders (99, p. 396-399) and Vallentyne (121, p. 62-64).

c. Organic nutrition of algae by water humus.

Some algae, although otherwise autotrophic, require small amounts of preformed organic nutrients such as vitamins. Such algae are called "auxotrophic" (91, p. 48). The requirement for vitamins or other organic metabolites, although quite small, is absolute. For this reason, vitamins are considered to be of great ecological importance in natural waters (57, p. 825-827; 64, p. 227-230; 90, p. 295-302; 91, p. 48-56; 99, p. 394-396; 121, p. 58-61). Bacteria of various kinds are known to produce vitamins in soils, and it is possible that a high percentage of vitamins in lake waters enter with run-off or diffuse out of the sediments of lake bottoms. The well known stimulatory effect of soil extracts on the growth of algae (89, p. 40) may be due at least in part to vitamins in certain cases.

Certain algae, while capable of completely autotrophic nutrition, can utilize organic substances such as sugars or amino acids as energy and carbon sources in heterotrophic fashion. At low light intensities the heterotrophic and autotrophic mechanisms may work together. Most of the information on this type of nutrition has been obtained from cultural studies. Evidence is accumulating that free sugars and amino acids may be common in natural waters. Whether they exist in ecologically significant amounts is

questionable. Even where they are not detected in natural waters, they may be important because they may be utilized by the native flora as quickly as they are formed (90, p. 320). Heterotrophy in algae has been reviewed by Saunders (99, p. 391-394), Provasoli (91, p. 52-54), Fogg (43, p. 50-67) and Krauss (64, p. 218-227).

d. The buffer effect of humus.

The organic colloids of soils exhibit buffering properties, as might be expected (12, p. 69). All of the fractions of resistant humus, and especially the humic acid fraction, have weakly acidic phenolic hydroxyl groups as well as carboxyl groups (118, p. 132). Oden (74, p. 126-128) considered the acidic humus that he studied to be a true acid because it would go into solution in ammonium hydroxide and he considered this to be a neutralization reaction. Later he defined humic acids as substances which can split off hydrogen ions and form salts with strong bases (75, p. 105). Although our concept of humic acids has changed, there is no doubt of their buffer capacity. It was demonstrated by Gillam (47, p. 440-445) who used potentiometric titrations. Methylation of the humic acids greatly decreased their buffer capacity. This was assumed to indicate that non-carboxylic hydroxyl groups were converted to methoxyl groups, and that the weak acid (or buffering)

character of the humic acids was due to phenolic hydroxyl groups, although it was thought possible that part of the buffer effect could have been due to metallic ions held strongly on the reactive side groups of the humic acid nucleus. Strong buffering action in preparations of both synthetic and natural humic acids was shown by van Dijk (33, p. 152). Shapiro (107, p. 167) found that the yellow organic acids of certain lakes in Connecticut were good buffers.

Most algae grow in neutral or slightly alkaline waters, but it is characteristic of algae to show tolerance for changes in pH (16, p. 268). This might be expected of a wild species if it is to survive, but the possibility cannot be dismissed that algae may have a certain sensitivity to pH. As Tiffany (119, p. 295) has stated:

It is quite likely that many, if not most of the filamentous algae, are able to grow within a fairly extensive pH range, but best conditions for each species may reside in much narrower limits.

If one may safely generalize on the basis of data obtained from studies of laboratory strains, one may conclude that most blue-green algae grow best in an alkaline medium. All 30 cultures of Cyanophyceae isolated by Allen did well in the pH range 8.0 to 8.5, and none grew well below pH 7.0 (2, p. 47). Gerloff, Fitzgerald and Skoog found that their 22 isolates grew best in the pH range 8.0 to 9.5. They found that omission of sodium carbonate and

sodium silicate reduced the pH of the medium they were using to 7.1, and in this medium the growth of Nostoc muscorum was reduced to only 7.4 percent of its growth in the alkaline control medium (45, p. 216).

It is not possible at present to state whether pH effects are exerted directly upon the algal cells, but it can be stated with certainty that pH can exert important indirect effects upon the algae. For one thing, the form of mineral carbon available to the algae will be determined by pH. At pH values below 5.0, only free carbon dioxide is of quantitative importance in a dilute solution of salts. In the pH range 7.0 to 9.0, bicarbonate ion is of greatest quantitative significance. Above pH 9.0 carbonate ion appears in significant concentrations (57, p. 657). It would seem that blue-green algae use bicarbonate ion best in photosynthesis, and that algae that use bicarbonate preferentially would grow better at a higher pH than would algae that use only carbon dioxide. No algae are known to use carbonate ion in photosynthesis; in fact, it may even become toxic (43, p. 35). As algae remove the carbon dioxide and bicarbonate ion from the medium, the pH of the medium rises sharply. For this reason, water humus, if it is a good buffer, might be able to make the medium capable of supporting photosynthesis for a longer time than would a less well-buffered medium.

Another important effect of pH in a medium is its influence on solubility of mineral elements such as calcium, iron or phosphorus. Under circumneutral to alkaline conditions, calcium may exist as calcium phosphate or calcium carbonate, both of which are insoluble. Below neutrality, iron and phosphorus may precipitate as ferric phosphate (92, p. 188). The divalent (ferrous) state of iron is soluble only under anaerobic or strongly reducing conditions at pH ranges found in most natural waters. With the introduction of oxygen, the iron changes to colloidal ferric hydroxide which may or may not be available to algae. Thus, in water containing oxygen and at a pH near neutrality or above, there should be little or no iron or phosphorus in solution (35).

e. Effects of humus on the availability of mineral nutrients.

The most direct and obvious importance of humus in the mineral nutrition of plants is its function as a "storehouse" of minerals which are released by the activity of microorganisms. In lakes this occurs both in the water and the bottom sediments (123).

Humus has other effects on the mineral nutrition of plants. Humic colloids are negatively charged because of ionization of carboxyl groups, acidic hydroxyl groups or the adsorption of negative ions. Most humic material is amphoteric in nature, acting as an

acid at higher alkalinities and as a base in acidic surroundings (12, p. 65). The smaller colloids may act as electrolytes and move in an electric field (78, p. 253). It has often been demonstrated that the ion-exchange capacity (also called "base-exchange capacity") of organic colloids is high, even higher than that of clays (24, p. 120). The great adsorptive and ion-exchange capacity of colloidal humus is of utmost importance in the mineral nutrition of plants. These capacities are due to the reactive groups on the nuclei of the building units and, as mentioned earlier, humic acids can be considered analogous to ion-exchange resins. In addition, the smaller, less complex molecules of both nutrient and resistant humus are capable of forming stable complexes (salts), perhaps also chelates, with metallic ions.

The expression "chelate formation" is used to designate the formation of a stable ring structure when a metal ion combines with two or more electron donor groups of a molecule or ion (124, p. 4). Therefore, van Dijk (33, p. 154) is correct in criticizing the use of the term "chelation" in connection with humic acids, and Wallace (124, p. 6) is incorrect in naming humic acids as one of the naturally occurring chelating agents. Chelates generally are water soluble and, being smaller and less complex than humic acids, are readily absorbed by plants (126). Metals bound in

chelate rings lose their ionic characteristics and become less reactive, a fact of significance in plant physiology. The metals may be prevented from reaction with other ions to form insoluble compounds; e. g. , iron might not react with phosphorus, nor is chelated iron readily subject to oxidation. Undoubtedly there are many natural chelating agents in the total humus. In water humus, Shapiro's colored acids probably are chelating agents because they are dicarboxylic, water soluble, and able to keep iron in solution at a high pH for long periods of time (107, p. 173).

One of the consequences of the combined effects of chelation, complex formation, and ion-exchange activities of humus is that plants may be protected from potentially harmful environments. As an excellent example, the old question of "auximones" may be reviewed. During the years 1912 to 1924, a series of investigations were reported by W. B. Bottomley and his co-worker, Miss F. A. Mockeridge, dealing with the effects of a special preparation of peat (called "bacterized" peat) on the growth of various plants including species of Azotobacter and Lemna (17; 18; 19; 20; 72). Peat was mixed with various sources of protein, inoculated with aerobic soil bacteria and incubated for two weeks at 26^o C. The "ammonium humate" supposedly formed was extracted with water or alcohol. Dried extract was added to the culture media in which

the experimental plants were grown. A few milligrams of extracted dry matter greatly increased growth and nitrogen fixation in the species of Azotobacter used. In Detmer's or Knop's solutions without peat extract, the Lemna species grew badly and eventually died. When extracts of bacterized peat were added to these media, the plants grew large, green, and multiplied rapidly, producing as much as 60 to 70 times the dry weight of the controls. Bottomley concluded that green plants, in addition to carbon dioxide, water and mineral salts, require small amounts of some indefinite group of organic nutrients which he compared to vitamins and called "auximones." Mockeridge felt that auximones were also beneficial to non-green plants.

Bottomley and Mockeridge were severely criticized by many authors, among them Saeger (98), who duplicated Bottomley's work and in addition found that the two species of Lemna used could be grown in Knop's and Detmer's solutions diluted to one-tenth of their original concentration. Wolfe (129) also tested Bottomley's work and found undiluted Knop's and Detmer's media unfavorable for the growth of Lemna spp. Wolfe found another, less concentrated, medium (Shive's) to be more suitable. In an especially caustic attack on Bottomley's work, Wolfe suggested that the term "auximone" be dropped from the literature.

The criticisms leveled at Bottomley and Mockeridge were undoubtedly valid, but the critics failed to recognize the significance of the effect of peat extracts -- they had protected the experimental plants from the injurious effects of excessive concentrations of mineral elements in the unsuitable media.

As is well-known, the metals commonly known as "trace" elements can be toxic if present in excess of a plant's needs. These metals are also subject to precipitation at certain pH values. Some authors have suggested that in synthetic media, metals should be supplied as stable complexes or chelates, the idea being to create a "competition" between the enzymes of the plant cells and the chelating agents (58, p. 160-161). Living cells of plants contain chelating substances such as chlorophyll, cytochromes, catalase, peroxidase, polyphenoloxidase, cytochrome oxidase, etc. (126). A "competition" between these enzymes and the chelating agents could, in effect, produce a regulatory effect on the absorption of mineral elements by the plants. The presence of the chelating agent ethylenediaminetetraacetic acid (EDTA), allowed the green alga, Micrasterias, to grow and reproduce for a much longer period of time than would a medium containing the complexing agent, citric acid, or the control medium without chelating or complexing agents. Both precipitation and toxic effects

of heavy metals were prevented simultaneously by EDTA. Calcium and magnesium were kept in solution even at alkaline pH levels.

The range of pH that the alga would tolerate was greatly broadened by the presence of the chelating agent (125).

The green alga, Hydrodictyon, was killed in five days by 40 micrograms of copper per liter in a medium without soil extract. With soil extract in the medium, the alga showed only a slight decrease in growth even at a concentration of 170 micrograms of copper per liter. In the absence of soil extract, ten micrograms of copper per liter reduced the growth of the alga to only 30 percent of the controls (73, p. 50-55).

Fogg and Westlake (44) found that the polypeptides excreted by the blue-green alga, Anabaena cylindrica, could complex about 0.325 milligram of copper per milligram of polypeptide. The excretions could also form soluble complexes with zinc, iron, and the phosphorus from tricalcium phosphate. When the alga was washed free of polypeptides, it was killed by four milligrams of copper per liter; with polypeptides left in the medium, 32 milligrams of copper per liter were required to kill the alga.

The interaction of these polypeptides with phosphate is of great ecological importance because phosphate precipitates easily with iron and calcium. In addition, certain blue-green algae, e. g. ,

Gloeotrichia natans, are quite sensitive to the presence of free phosphate ions, and will grow only in media from which all detectable traces of soluble phosphate are removed or adsorbed (44, p. 229).

Lhotský recently reviewed several years of work on the influence of suspensions and extracts of soft lignite coal on the growth of algae in culture (67). The emphasis in the review was on the protective action of the humic colloids. The unfavorable influence of metabolic by-products in media seemed to be counteracted by the colloids. The toxicity of heavy metals was also counteracted by the colloids, and the toxicity of trace elements at higher concentrations was eliminated. The soft lignites helped the cells utilize phosphate ions, especially when the phosphates were present at suboptimal concentrations. Changes in pH exerted little influence on the action of the lignites. Higher concentrations of lignites were harmful to algae, possibly indicating competition between the cells and the colloids for the mineral nutrients.

Addition of humic acids at the rate of 2.5 milligrams per liter enabled rye seedlings in nutrient solutions to withstand greater amounts of nitrogen than in cultures without humic acids. In a medium containing humic acids, the plants were able to thrive at nitrate nitrogen concentrations up to 1200 milligrams per liter,

whereas nitrogen at 700 milligrams per liter became toxic and reduced growth of the plants in a medium without humic acids (28).

The most frequently stated reason for the stimulatory action of humus on growth of plants is that humus keeps iron in a more readily available form, i. e. , it prevents precipitation of iron. This stems from the works of Olsen (82) and Burk, Lineweaver and Horner (26; 27) who "proved," at least to their own satisfaction, that humus stimulates growth of plants by keeping iron in a readily available form. Olsen checked the work of Bottomley and Mock-eridge and found that extracts of "bacterized" peat stimulated growth and reproduction of the Lemna. However, iron added as a ferric citrate complex provided equal stimulation. In the control medium, iron was added as ferric chloride which began to precipitate at pH 3.5.

Burk, Lineweaver and Horner, after an extensive series of experiments, concluded that humic acids isolated from soil extracts could stimulate growth of Azotobacter vinelandii by virtue of their ability to make iron more rapidly and readily available to the organisms than in a completely mineral medium. The natural humic acids could be replaced by complexed organic iron, inorganic iron such as ferric sulfate, or metallic iron. The stimulation of the bacterium appeared as a more rapid beginning of the

logarithmic phase of growth. Over longer periods of time, addition of extra iron in any form produced as much growth as humic acid iron.

The conclusions of Olsen and of Burk, Lineweaver and Horner were not completely justified. Their interpretations were superficial because they did not consider the possibility that the components of the humic extracts would react with and influence the behavior of other mineral elements in the media. It might be argued that their experiments proved that iron was a limiting factor in their media. Nonetheless it is still widely believed that the beneficial or stimulatory effect of humus extracts on growth of plants is due to their complexing with iron to keep it more readily available to plants (57, p. 712; 62, p. 473-474; 95, p. 32). No doubt there are cases where this is true, but there is no hypothetical justification for limiting the reactions of humic substances to iron alone.

In at least one case, the stimulatory effect of soil extracts on the growth of microorganisms was due simply to the fact that they supplied a mineral nutrient that was limiting in the original medium. Taylor (117) found that a medium containing only agar and soil extract gave much higher plate counts of soil bacteria than any other medium in common usage. Even when a wide selection of organic compounds (including vitamins) were added to his

medium, many soil bacteria would not grow in the absence of soil extract. Treatment of the soil extracts with ion-exchange resins removed the stimulatory effect, but ashing did not. Therefore, Taylor tried likely constituents of the ash and found that calcium chloride would replace the soil extract.

If components of humus exert a regulatory influence upon ion absorption by plants, i. e. , if there is a competition between plant cells and the complex formers, as implied by Wallace (124, p. 70-72) and Hutner (58, p. 160), then the humus compounds should make ions less readily available rather than more available as so often claimed. Miller and Ohlrogge (71) found that water extracts of soil or barnyard manure, when added to nutrient solutions, greatly decreased the uptake of iron and zinc by corn and soybeans. The workers concluded that the complexing agents from soil extracts or manure could not readily enter the plant, since the complex formers or chelating agents (if any) in the extracts would have to enter the plants along with the zinc and iron and accumulate in the tops of the plants. This was an unwarranted assumption not supported by their data.

On the other hand, Chaminade and Blanchet (29) found that humic acids added to nutrient solutions at the rate of five to ten milligrams per liter increased the absorption of all essential

elements by rye plants. The authors offered a hypothesis that humic acids somehow increase the permeability of the cell membranes. Later, it was found that addition of small amounts of humic colloids accelerated the adsorption of potassium and phosphorus on the surface of the root hairs of the experimental plants (15). This was thought to explain the more rapid absorption of these elements in the presence of humic acids.

Saalbach (97, p. 108) grew rye plants 14 days in Knop's solution with and without added humic acids. In the presence of humic acids, the plants absorbed more nitrogen, potassium, phosphorus and sodium than the control plants without humic acids.

These types of evidence indicate that the beneficial effects of humus extracts can be due to something more than the ability of humus to hold iron in solution and keep it readily available.

There is a question, as yet unresolved, as to whether humic acids can enter a cell. There is evidence that they do not. Prát and Pospíšil (88) prepared radioactive humus by allowing sugar beets to assimilate radioactive carbon dioxide, then subjecting the leaves to three to five months of humification in soil. Radioactive humic acids were then extracted and added to Knop's solution in which corn plants were grown. Radioactivity appeared so slowly in the corn plants that entry of humic acids into the cells

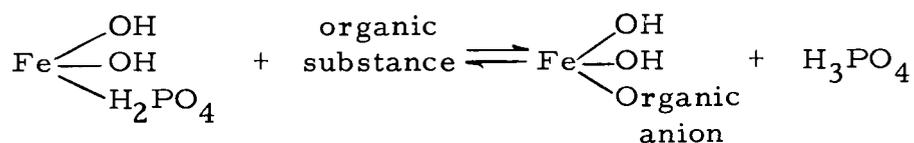
could not be claimed. The radioactivity that appeared in the plants could have been due to the entry of smaller compounds formed by decomposition of the humic acids because the cultures were not sterile.

The adsorptive capacity of humus has been of major interest to students of soil science. It appears that one important effect of this adsorptive capacity is to cause a "storage" of minerals in humic soils. Fiskel (38) found that the peat soils of Florida held relatively great concentrations of the trace elements copper, manganese and zinc. These could not be washed out of the soil with water, but could be extracted with sodium-EDTA. Fiskel claimed that the elements were held in the soil by some sort of "chelation" mechanism - a doubtful assumption. Chelation has a different meaning, as explained previously. If the elements were chelated, some of them should have been washed out by water. The amount of adsorbed metallic cations in peat may be 100 times the concentration of these elements in solution in the free water of bogs (50, p. 151, 162).

Another important function of humus in plant ecology appears to be the "mobilization" of elements "fixed" in soils in a precipitated or adsorbed state. Aqueous extracts of forest litter can not only keep iron in solution, but can bring the iron of ferric

hydroxide back into solution (68). An aqueous extract, when filtered, would not contain humic acids because they are insoluble in water. The fulvic acid fraction of peat extracts has the ability to reduce ferric iron to the ferrous state (10). The reducing power of this group of compounds may also be shown with various indicator dyes.

In the results of a series of experiments published by Sieling and his co-workers (21; 32; 116) it was demonstrated that organic acid anions and sugars commonly found in soils can increase the availability of phosphorus to plants, not only by preventing its precipitation with iron and aluminum, but also by dissolving that phosphate already precipitated or "fixed" in the soil. In one series of experiments, the absorption of phosphate by corn was five times greater in the presence of decomposing organic matter than in purely mineral media. The authors postulated that the organic anions pulled iron away from the phosphate as follows:



The direction in which the reaction will proceed depends on the stability of the "fixed" phosphate in relation to the stability of the organometallic molecule of iron. Different acids were effective at different pH levels. Considering all the anions and sugars tested,

precipitation of iron and phosphorus could be prevented throughout the pH range 3.0 to 9.0. The total microflora of the soil can generate organic substances of almost unlimited variety, so they may be important in preventing the precipitation of phosphorus in the soil, or in freeing that phosphorus already precipitated.

The ion-exchange, ion adsorption, and chelating activities of humus are important in the aquatic habitat also. Positive quantitative relationships have been found between the humus content of waters in southern Sweden and their content of ammonia and phosphorus (46, p. 141-143). The presence of humus in the water can cause Eloдея canadensis to absorb less phosphorus than from non-humic lake waters (46, p. 151-152). While humic waters may bring considerable amounts of mineral nutrients into a lake, they may "hinder" the use of these by the aquatic plants, and it is probable that certain ions are held fast by the humic colloids (46, p. 153).

Investigating brown-water lakes in northern Germany, Ohle (77, p. 592-596) found that after addition of ammonium hydroxide to humic waters, the resultant solution showed less electrical conductivity than the same amount of ammonium hydroxide in distilled water. The lowering of conductivity by the humic water indicated that adsorption complexes or salts with low dissociation constants

were formed between ammonia and the humus. This was true only in neutral or alkaline solutions. In strongly acid solutions, the organic colloids precipitated, their behavior resembling that of humic acids. When humic water was made slightly acid with hydrochloric acid, the conductivity of the resultant solution was greater than that of a solution containing an equal amount of hydrochloric acid in distilled water. This seemed to indicate that the mildly acid conditions caused a release of earth alkali carbonates and bicarbonates from the colloids. Ohle concluded that in humic lakes rich in calcium and other electrolytes, the electrolytes exist in an undissociated state and that in acid humic waters, poor in electrolytes, there is either dissociation of the minerals or cataphoretic activity of the colloids or both. Ohle felt that the adsorbed condition of the minerals was beneficial to aquatic plants growing in humic waters.

In another experiment on the adsorptive capacity of humus, Ohle (77, p. 658) found that the highly decomposed leaf "mull" from forest floors absorbed almost all the calcium and magnesium from bicarbonate solutions of these elements as they passed through the mull. Further study (79) definitely established that iron, phosphorus and ammonia were held by adsorption on the colloidal particles of humus in lake waters and soil extracts, especially under slightly alkaline conditions. Known quantities of ammonia, iron, and

phosphorus were added to humic lake waters or extracts of forest soils, and the solutions were passed through a "Cellafilter" with pores of 0.75 micron diameter. The filtrates contained lower concentrations of the elements than the original solutions. In the case of iron, this was true even at pH 4.5. Ohle criticized the expression "iron humate," still commonly used today, because it implies salt formation. It might be more correct to use "humic acid iron" to indicate iron held fast to the colloids by adsorption, but in the light of present-day knowledge of the reactive side groups on the humic acid nucleus, it may be that some iron is held in a complex in the manner of ion-exchange reactions.

In another study with the "Cellafilter" Ohle demonstrated that phosphorus can be held in an adsorbed condition by colloidal ferric hydroxide (80). Ohle placed a layer of ferric hydroxide gel over the filter, then overlaid this with solutions of phosphate at different pH values, drew the solutions through the ferric hydroxide, and analyzed the filtrate. It is interesting to note in this connection that ferric hydroxide has a positive charge at alkaline pH values (53, p. 224). It is evident that in alkaline humic waters, the relationships between iron and phosphorus may become quite complex.

f. Effects of humus on the enzyme systems of plants.

In recent years, many students of humus have directed their attention to the hypothesis that humus may exert a direct influence upon the enzyme systems of plants. Studies have been made with various preparations of natural humus as well as model compounds representing precursors of natural humic acids.

Sladky⁹ and Tichý⁹ (110) applied a mixture of fulvic, humatome-
lanic and humic acids to leaves of Begonia semperflorens. The
treated plants produced greater length of stems and roots com-
pared to untreated control plants. Fresh and dry weights of the
treated plants increased over the controls, as did chlorophyll con-
tent and rate of respiration. Overdosing caused growth of treated
plants to decrease; the leaves became wrinkled and the stems
showed abnormal branching.

In a later study, Sladky⁹ (109) determined the relative activity
of each of the three commonly separated fractions of resistant hu-
mus in producing the previously described effects. The fulvic acid
components were more active than the other fractions of humus
when applied to the leaves of Begonia, producing the greatest in-
crease in height and fresh and dry weights of stems and roots. The
activity of the humus components seemed to be inversely

proportional to the size and complexity of the molecules involved. Because these fractions were applied directly to the leaves, the indirect influence of humus on the environment around the roots was eliminated.

Humic substances may act as acceptors of hydrogen from terminal oxidase systems, particularly under conditions of poor aeration, or in the absence of oxygen (52). This hypothesis is supported by the work of Šmídová (112) who showed that humic acids added to water cultures of wheat, corn, or the gourd, Cucurbita maxima, increased respiration in the roots of all these species. The increase was reflected both in consumption of oxygen and evolution of carbon dioxide as measured by manometric techniques. The increase in respiration was accompanied by increases in length and dry weight of roots and stems of the treated plants.

These effects are possibly due to the presence of quinoid structures in humic acids (118, p. 156). A thymoquinone-thymohydroquinone system in the medium can increase the germination and early growth of cereals. The stimulation is especially obvious in poorly lighted cultures supplied with glucose. Stimulation of germination is accompanied by increased respiration in plants supplied with thymoquinone (40). Thymohydroquinone can influence the activity of several enzyme systems in rye plants grown in

nutrient solutions. The activities of aldolase and phosphatase are inhibited by 1.5×10^{-3} moles of thymohydroquinone, but the activities of amylase and sucrase are increased. The activities of the enzyme systems decrease with increasing concentrations of thymohydroquinone (41). Not many conclusions can be drawn from this work at present, but there is no doubt that enzymes are influenced by quinoid compounds.

In relation to this recent work on humus and enzymes it is interesting to review the discovery that humus extracts contain a supposed coenzyme, "Coenzyme R," which greatly increases the growth and respiration of several species of Rhizobium (5). Allison and Hoover (6) felt that "Coenzyme R" was the important factor in the stimulation of growth caused by humus extracts and not the supposed ability of these extracts to keep iron available. The iron requirements of rhizobia are quite small, and addition of iron in various forms did not stimulate growth of the organisms.

g. Summary of possible roles of humus in aquatic plant ecology.

The review of literature just presented was not intended to be a complete description of all the functions of humus in affecting the growth of plants. Only some of the more obvious effects of humus were presented. It seems desirable at this point to attempt a

synthesis of the functions of humus that might be of importance in the production biology of lakes.

Drainage lakes with a steady source of influent waters may receive great quantities of dissolved and colloidal humus which may be carrying considerable amounts of mineral and organic nutrients. On the other hand, seepage lakes, if they are surrounded by peat or humic soil, may become acid in reaction and poor in mineral nutrients because the great adsorptive power of the surrounding peat or humus may remove much of the mineral nutrition from the influent water.

Once in the lake, the colloidal and colored humus will limit the penetration of light into the water and thereby limit the zone of photosynthesis to the shallower layers. This effect should be more important in deep lakes than in shallow ones.

If the humus brought into a lake carries significant amounts of mineral elements, either dissolved or adsorbed on colloids, the humus may hold these elements in solution or suspension in the lake under conditions where they would precipitate in the absence of humus. This might be accomplished by chelation and complex-formation in the soluble humus, and by adsorption and complex-formation in the colloidal humus. In addition, the buffer capacity of the humus might minimize the pH effects that tend to

cause precipitation. It might also lessen the alterations of pH in the lake caused by photosynthesis of the native plants and respiration of native plants and animals.

The availability of the complexed or chelated nutrient elements may be regulated so that the organisms, while not subjected to toxic concentrations of any element, may absorb elements as needed. The ion-exchange capacity of the colloidal humus should also protect the aquatic plants from toxic concentrations of minerals, and regulate the availability of these elements to the plants.

Nutrient humus washed into the lake or produced there by native organisms may supply supplementary organic nutrition to certain algae, or it may contain toxic growth inhibitors. At the same time, part of the humus may be able to combine with the toxins and protect the plants.

As the total humus slowly accumulates on the bottom of the lake, its decomposition will release mineral and organic nutrients, and possibly toxins, into the water. If the concentration of oxygen near the bottom becomes limiting for aerobic organisms, the quinoid structures of humic acids might allow the organisms in and near the bottom to continue respiration.

It is evident that the effects of humus on the growth of plants are numerous and complex. The explanations of these effects must

be sought in the wide array of substances comprising the total humus and in the environmental conditions under which they are acting. The origin and age of the humus must be considered as well as the sensitivity or "needs" of the organism under study. Different fractions of the total humus will have different effects on the growth of plants and these effects may be exerted simultaneously or at different times. Stimulation or inhibition of growth of plants by humic substances may be due to any number of causes and, for this reason, all interpretations of these effects must be made with caution.

MATERIALS AND METHODS

1. Collection and handling of samples.

Samples of humic water were taken from the Williamson River at the bridge on the road to Kirk, Oregon, approximately one-half mile east of Highway 97, a few miles south of the southern margin of Klamath Marsh. Collections were made in the months of January and August, 1959, so that comparisons could be made of the characteristics of the water in two different seasons of the year. The samples were collected in polyethylene carboys to which chloroform was added immediately to prevent changes in the samples by activities of native organisms. The samples were stored at approximately 4° C. during the time of this study.

2. Examination of some physical and chemical characteristics of water from Klamath Marsh.

Physical and chemical studies of the water of Klamath Marsh had two objectives. The first was to obtain information concerning the commonly investigated characteristics of humic waters, such as pH, color, residue, mineral content, organic matter content, etc., which served as items for comparison with other humic waters. The second objective was to determine whether the water

contained any organic or mineral nutrients in sufficient quantity to be of ecological significance in the growth of a freshwater alga.

Color and turbidity in the humic water rendered chemical analyses difficult. The difficulties were increased by the low concentrations of most of the elements studied. These difficulties were overcome by concentration of samples, microfiltration, destruction of color and turbidity by wet or dry ashing, or avoidance of color and turbidity by distillation. Detailed descriptions of these procedures follow.

a. Determination of color.

The color of the water was determined by the method originated by Ohle (77, p. 393) and modified by Bennike (9, p. 9-10). The method consisted of visual comparison of samples of marsh water with the color of a standard prepared by adding known quantities of methyl orange to distilled water. Color determined in this manner is expressed in "Ohle degrees" or "Ohle units". One Ohle unit is the color imparted to a liter of distilled water by 0.01 milligram of anhydrous methyl orange, and is approximately equal to 2.8 milligrams per liter on the platinum-cobalt scale (57, p.413). Before the comparisons were made, turbidity of the samples was removed by micropore filtration, using a pore size of 0.45 micron.

Thus, the color determined was the "true color", i. e., the color of the water after removal of suspended material.

A standard solution was prepared containing ten milligrams of anhydrous methyl orange (1000 Ohle units) per liter. As one milliliter of this standard would impart one Ohle unit of color to one liter of distilled water, it was assumed that one drop of standard added to 50 milliliters of distilled water would impart one Ohle unit of color to the 50 milliliter aliquot. It was necessary to add two drops of standard to 50 milliliters of distilled water before any change of color could be discerned - a potential error of \pm one Ohle unit. It was found that differences in weakly colored solutions were easier to detect than changes in strongly colored solutions, so the marsh water was diluted by a factor of ten before color was determined. The total potential error in the determinations then became \pm one Ohle unit multiplied by the dilution of the marsh water, or \pm ten Ohle units

b. Determination of residue.

Total residue was determined in duplicate aliquots of 250 milliliters from each collection that were evaporated on water baths to approximately 50 milliliters, then washed into tared cups made of aluminum foil and dried to constant weight in an oven at

105° C.

Suspended residue was determined in duplicate aliquots of marsh water from each collection that were passed through tared micropore filters with a pore size of 0.45 micron. Suspended residue was considered to be the increase in dry weight of the filters due to passage of 200 milliliter aliquots of water from the January collection or 100 milliliter aliquots from the August collection.

c. Determination of relative acidity.

The pH of the marsh water at the times of collection was determined potentiometrically with a portable pH meter. The effect of carbon dioxide and bicarbonates on the pH of the samples was determined in the laboratory. Samples of marsh water were boiled for five minutes, and the pH of the samples was determined with a pH meter while the samples were still boiling.

d. Chemical analyses.

Total carbon was determined in an apparatus designed for the dry combustion of small amounts of organic materials. Aliquots of two liters each from the January and August collections were evaporated to 50 milliliters on a warming plate under a stream of warm air from an electric heater. Duplicate ten

milliliter aliquots from each concentrate were mixed with 60-mesh granular aluminum oxide in platinum combustion vessels and dried. The dried samples were burned at 1200^o in a stream of purified oxygen. The carbon dioxide evolved in the combustion apparatus was passed first through a dessicant and then trapped in cartridges containing a CO₂ absorbent. The increase in weight of the cartridges was assumed to be evolved carbon dioxide. No correction was made for the presence of carbonates because of the relatively low pH (approx. 7.0) of the samples. Elemental carbon was calculated as follows: $C = CO_2 \times 0.2729$. Organic matter was assumed to be $C \times 1.92$, a conversion suggested by Waksman (122, p. 297).

Determinations of organic nitrogen were made on duplicate 250 milliliter aliquots of each collection. These were combined with ten milliliters of phosphate buffer and evaporated to less than 100 milliliters in erlenmeyer flasks, then transferred to 100 milliliter microkjeldahl digestion flasks and evaporated to dryness in an oven at 105^o C. The residue was analyzed for organic nitrogen by a standard "micro" method (56, p. 805). Controls were duplicate samples of distilled water given the same treatment.

Albuminoid nitrogen was determined on the same samples used for the determination of ammonia to be described later. The

standard method used (36, p. 141) consisted of reacting alkaline potassium permanganate with the organic matter to release ammonia which was distilled off and determined by nesslerization (36, p. 143).

Tests for amino acids and reducing sugars were performed on samples that had been concentrated ten times by boiling under reduced pressure at 45° C. in a flash evaporator. These were "spot" tests as outlined by Feigl (37, p. 282, 389), using ninhydrin to indicate amino acids and tetrazolium chloride to detect reducing sugars.

Analyses for sodium, potassium, calcium, and magnesium were performed by flame spectrophotometry using a Beckmann DU spectrophotometer. Recovery experiments were made at the same time with the above-named elements added to samples of marsh water at the rate of ten milligrams per liter.

Total iron was determined in duplicate 250 milliliter samples of water from each collection that were concentrated on water baths to approximately 50 milliliters, then washed into 100 milliliter microkjeldahl digestion flasks and evaporated to dryness at 105° C. The residue was ashed with hot concentrated sulfuric acid and the addition of drops of 30% hydrogen peroxide. The clear, wet ash was washed into graduated cylinders and neutralized with

12N potassium hydroxide. The wet ash of the residue from 250 milliliters of marsh water was diluted to 50 milliliters, making the concentration of iron in the developed samples five times greater than in the original samples. The iron was determined with ortho-phenanthroline by a standard method (36, p. 125). The optical density of the color in the developed samples was read in a spectrophotometer at 510 millimicrons, and corrected by comparison with a reagent blank in distilled water. The results were converted by comparison with a calibration curve prepared using dilutions of a standard solution of iron. In the recovery experiments, elemental iron was added at the rate of one milligram per liter to marsh water samples which were given the same treatment.

To determine phosphate phosphorus, duplicate samples of marsh water from each collection were given the same preliminary treatment as in the analyses of iron. The wet ash was analyzed for phosphate by a standard method with stannous chloride reagent (36, p. 169). The optical density of the developed samples was read in a spectrophotometer at 690 millimicrons, and corrected by comparison with a reagent blank in distilled water. Results were obtained by comparison of the optical densities with a calibration curve prepared using dilutions of a standard solution of phosphate. Phosphate was added to marsh water at the rate of 0.8 milligram

per liter for the recovery experiments.

Nitrogen as ammonia was determined in 500 milliliter aliquots of each collection by a standard distillation method (36, p. 143). The distillates were treated with Nessler's reagent and the optical densities of developed samples were read in a spectrophotometer at a wave length of 410 millimicrons and corrected by comparison with a reagent blank in distilled water. Absorbances of the developed samples were compared with a calibration curve prepared using dilutions of a solution of ammonium chloride. For the recovery experiment, ammonium chloride was added to an aliquot of marsh water from the August collection at the rate of one milligram of nitrogen per liter.

Nitrogen as nitrate was determined by an unmodified reduction method (36, p. 151). The ammonia produced by the reduction was distilled out and determined as previously described. For the recovery experiment, potassium nitrate was added at the rate of 0.8 milligram of elemental nitrogen per liter to an aliquot of water from the August collection.

For the determination of sulfate sulfur, duplicate 250 milliliter aliquots of water from each collection were concentrated on water baths to less than 25 milliliters. The concentrates were washed into porcelain crucibles and evaporated to dryness. The

residues were ashed in a muffle furnace at 700° C. The ash was dissolved in 0.5 percent hydrochloric acid. Each solution was brought back to the original volume of 250 milliliters, and the sulfate was precipitated by the addition of hot barium chloride (36, p. 196). No recovery experiment was performed because normally no other ion in natural waters will precipitate with barium from an acid solution.

3. Studies of the water of Klamath Marsh as an ecological factor in growth of a blue-green alga.

Two types of studies were used to investigate marsh water as an ecological factor in the growth of algae: (1) physical-chemical studies and (2) biological studies with cultures of Anabaena cylindrica. Four characteristics of the water were examined for their possible ecological importance: (1) the buffer capacity of the water; (2) the influence of the mineral elements in the water on the growth of the alga; (3) the effect of the humus in the water on the absorption of iron by the alga; and (4) the effect of the water humus on the solubility of iron and phosphorus in a culture medium.

a. The organism used.

The organism used in this study was a filamentous,

planktonic, blue-green alga, Anabaena cylindrica Lemm. (culture number 629) purchased from the algal culture collection of the University of Indiana (114, p. 80). This species was chosen for a number of reasons. It is a member of the Cyanophyceae, which group is of particular interest to the investigator. There is some knowledge of the physiology of the organism. It had been used previously in studies of nitrogen fixation (3). It is an obligate photosynthetic autotroph (43, p. 59), and grows well in a variety of media.

b. The culture medium.

The composition of the synthetic medium used in this study was derived empirically after consideration of the work of various authors, notably Rodhe (95, p. 57), McLachlan (70, p. 46), and Gerloff, Fitzgerald and Skoog (45). It is designated hereafter as M2 medium, and supplies cations as chlorides and anions as sodium salts. Thus it satisfies the sodium requirement of A. cylindrica (4). M2 medium is described in Table 1.

Stock solutions of nutrient elements were prepared by dissolving reagent grade compounds in double-distilled water. Media were prepared by appropriate dilutions of these stock solutions with distilled water. The concentration of a single element could

Table 1

The composition of M2 medium

Element	Mg/l	Supplied as	Stock g/l	Mg of element per ml stock	Ml of stock per l of medium
Ca	5	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	3.665	1	5
N	5	NH_4Cl	3.825	1	5
N	10	NaNO_3	6.100	1	10
Mg	2	$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	8.775	1	2
S	2	Na_2SO_4	4.425	1	2
K	2	KCl	1.912	1	2
P	2	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	4.450	1	2
Si	2	$\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	8.362	1	2
C	4	NaHCO_3	2.817	0.4	10
Fe	0.2	Ferric citrate Citric acid	0.556 0.556	0.1	2
Trace elements		Arnon's micro- nutrient solution	Supplied at 1/25 the concentration used for "higher" plants.		1

be changed while others were left unchanged. Trace elements were supplied in the mixture described by Arnon (7), but at 1/25 the concentration used by Arnon for vascular plants.

In preliminary experiments it was found that the experimental alga grew more rapidly if the medium was adjusted to pH 8.0 before inoculation. This was accomplished by addition of N/100 sodium hydroxide in all later experiments.

c. Conditions of culture.

Cultures were grown in a culture room at a temperature of $18 \pm 2^{\circ}$ C. The cultures were illuminated by "warm white" (red-dish) fluorescent tubes. The intensity of illumination at the level of the cultures was 100 foot candles. Except as otherwise noted, the cultures were grown in 38 X 200 millimeter Pyrex test tubes with cotton plugs. The tubes rested in a rack in a slanted position so that the cotton plugs did not shade the cultures.

d. Maintenance of cultures.

Both "stock" and "working" cultures of A. cylindrica were maintained throughout the study. Stock cultures were opened only to make transfers to new stock cultures or to inoculate working cultures. All cultures used in a particular experiment were

inoculated by transfer of uniform aliquots from a single working culture. After all cultures in an experiment were inoculated, the remainder of the working culture was discarded.

e. Inoculation of cultures.

The growth of A. cylindrica was clotted because the culture vessels were not agitated. When experimental cultures were started, the working culture was agitated vigorously and one milliliter portions of inoculum were withdrawn by pipette from the homogeneously dispersed areas of the culture suspension. Obvious clots of filaments were avoided. It was considered impractical to start each series of experiments with inoculum of the same age and physiological condition as the inoculum used in other experiments.

f. Harvesting the algae.

In all experiments comparing rates of growth of the experimental plant, growth was determined as the increase in dry weight of algal material over time. The cultures were harvested by passage through a plankton centrifuge. Periodic microscopic observations of the supernatant during the study indicated that if the algal suspensions were passed through the centrifuge at a sufficiently slow rate (about one milliliter per second) all algal cells were

removed.

The cells were washed from the centrifuge cup into tared aluminum foil cups and dried to constant weight in an oven at 105°C. Preliminary experiments showed that 24 hours in the drying oven was adequate drying time, and in all subsequent experiments the constant weight procedure was discontinued.

RESULTS

1. Results of the physical-chemical studies.

The physical features of the humic water of Klamath Marsh (Table 2) showed obvious differences in the color and residue in the two collections, but relatively little difference in pH.

Table 2

Some physical features of water from Klamath Marsh.

	January collection	August collection
pH at collection	6.8	7.2
pH after boiling	8.2	8.4
Total residue	103 mg/l	247 mg/l
Suspended residue*	5 "	85 "
True color	120 Ohle units	90 Ohle units

* Retained by a micropore filter with 0.45 micron pore size.

Studies of the inorganic chemistry of the marsh water (Table 3) revealed notable differences in the water from the two collections although the concentrations of elements studied were not especially high in either collection. Studies of the organic chemistry of the two collections (Table 4) also showed considerable differences between them.

Table 3

Concentrations of some inorganic constituents
of water from Klamath Marsh.

Element	As	January collection		August collection	
		mg/l	recovery	mg/l	recovery
Sodium	Na	6.5	95%	10.5	95%
Calcium	Ca	1.5	70	2.5	65
Potassium	K	1.7	113	4.2	108
Magnesium	Mg	5.0	110	8.5	95
Iron	Fe	0.2	91	0.9	86
Phosphorus	PO ₄	0.01	105	0.40	94
Nitrogen	NH ₃	0.04	--	0.15	93
Nitrogen	NO ₃	0.35	--	0.44	48
Sulfur	SO ₄	6.8	--	6.8	--

There was no correlation between the color of the water and the amount of organic matter it contained (Tables 2 and 4). Organic matter content was better correlated with total residue than with color.

Table 4

Concentrations of some organic constituents
of water from Klamath Marsh.

	Collection	
	January	August
Total Carbon	22.4 mg/l	34.3 mg/l
Organic matter (C X 1.92)	43.0 "	65.9 "
Kjeldahl nitrogen	0.7 "	1.8 "
Crude protein (N X 6.25)	4.4 "	11.3 "
Albuminoid nitrogen	0.2 "	0.5 "
Reducing sugars	None*	None*
Amino acids	None*	None*
Carbon:nitrogen ratio	32:1	19:1

* None found in a 10X concentration of the samples.

2. Marsh water as an ecological factor in the growth of an alga.

a. Preliminary studies.

Preliminary studies, using cultures of Anabaena cylindrica, were designed to determine the rate and amount of growth of the alga in M2 medium, and to determine the amount of stimulation of growth that the humic water would produce when substituted for distilled water in M2 medium. Modified versions of M2 medium were prepared in which one-fourth, one-half, and all of the distilled water was replaced with marsh water. All media were sterilized in an autoclave and cooled for 24 hours before inoculation. In the first trial, using water from the January collection, three cultures grown in each medium were harvested every five days. In the second trial, using water from the August collection, three cultures grown in each medium were harvested every ten days.

The first experiment (Table 5; Figure 1) showed that the strain of A. cylindrica used in this study grew relatively well in M2 medium, and that amendment of the medium with marsh water produced marked stimulation of the growth rate and total growth of the alga.

Table 5

Comparison of growth of *A. cylindrica* in M2
and M2 amended with marsh water.*

Amount of marsh water per liter of medium	Growth - dry weight - mg/50 ml					
	5 da.	10 da.	15 da.	20 da.	25 da.	30 da.
None	3.6	5.6	9.6	17.6	19.2	22.4
250 ml	5.5	8.5	16.5	22.4	24.7	27.3
500 "	5.1	7.5	16.6	23.8	25.7	32.9
970 "	6.1	8.1	16.4	25.0	28.2	37.8
LSD .05	0.9	1.0	2.1	1.5	1.1	2.7

* Marsh water from the January collection.

The results of the second experiment (Table 6; Figure 2), using marsh water from the August collection, matched quite closely the results of the first. In both experiments, the effects of marsh water were prolonged in direct proportion to the amount of marsh water added to the medium. The greater mineral content of the marsh water from the August collection did not appear to affect the results of these experiments.

Figure 1
COMPARISON OF GROWTH OF A. CYLINDRICA
IN M2 AND M2 AMENDED WITH MARSH WATER
FROM THE JANUARY COLLECTION

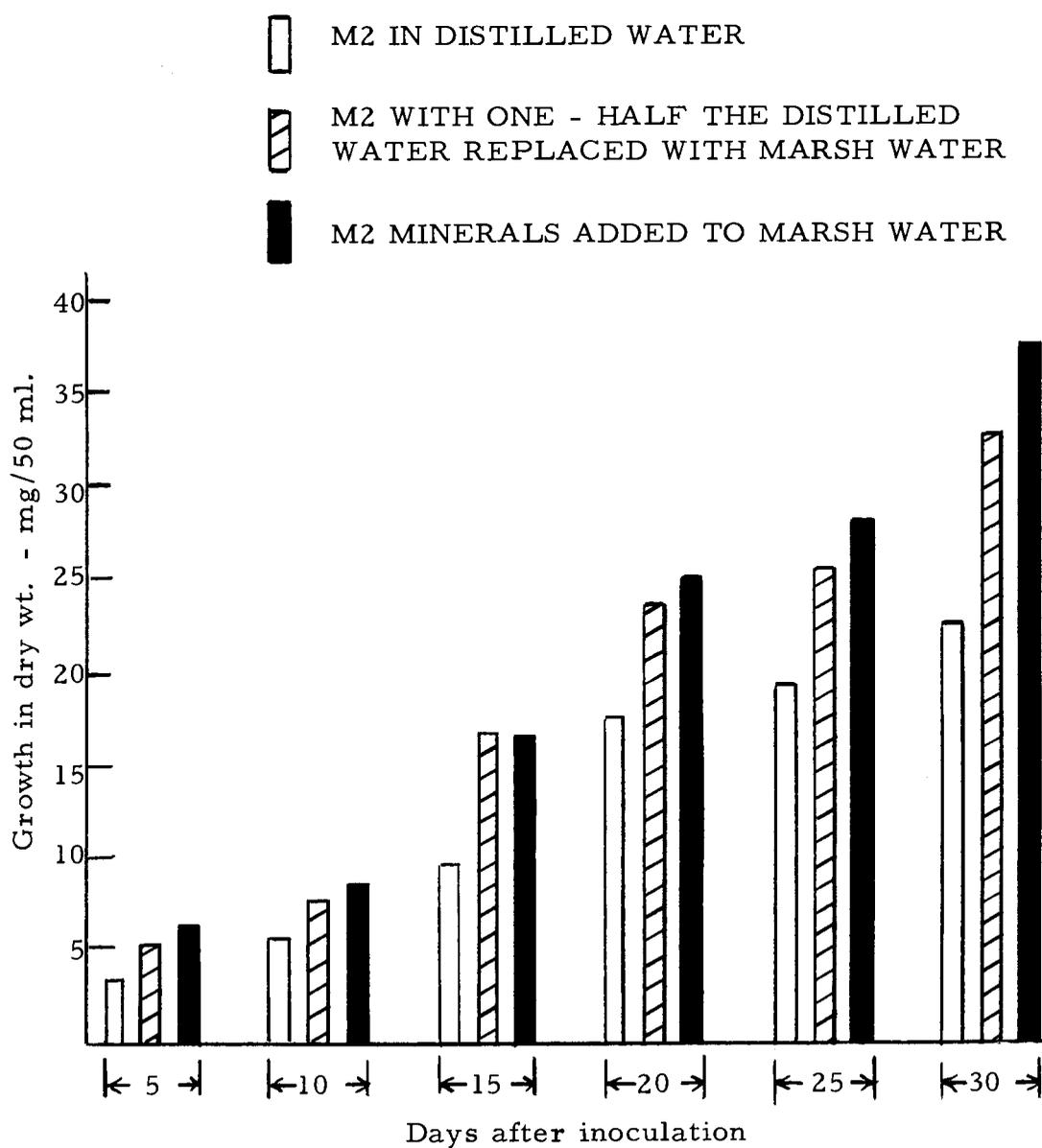


Figure 2

COMPARISON OF GROWTH OF A. CYLINDRICA
IN M2 AND M2 AMENDED WITH MARSH WATER

FROM THE AUGUST COLLECTION

-  M2 IN DISTILLED WATER
-  M2 WITH ONE-HALF THE DISTILLED
WATER REPLACED WITH MARSH WATER
-  M2 MINERALS ADDED TO MARSH WATER

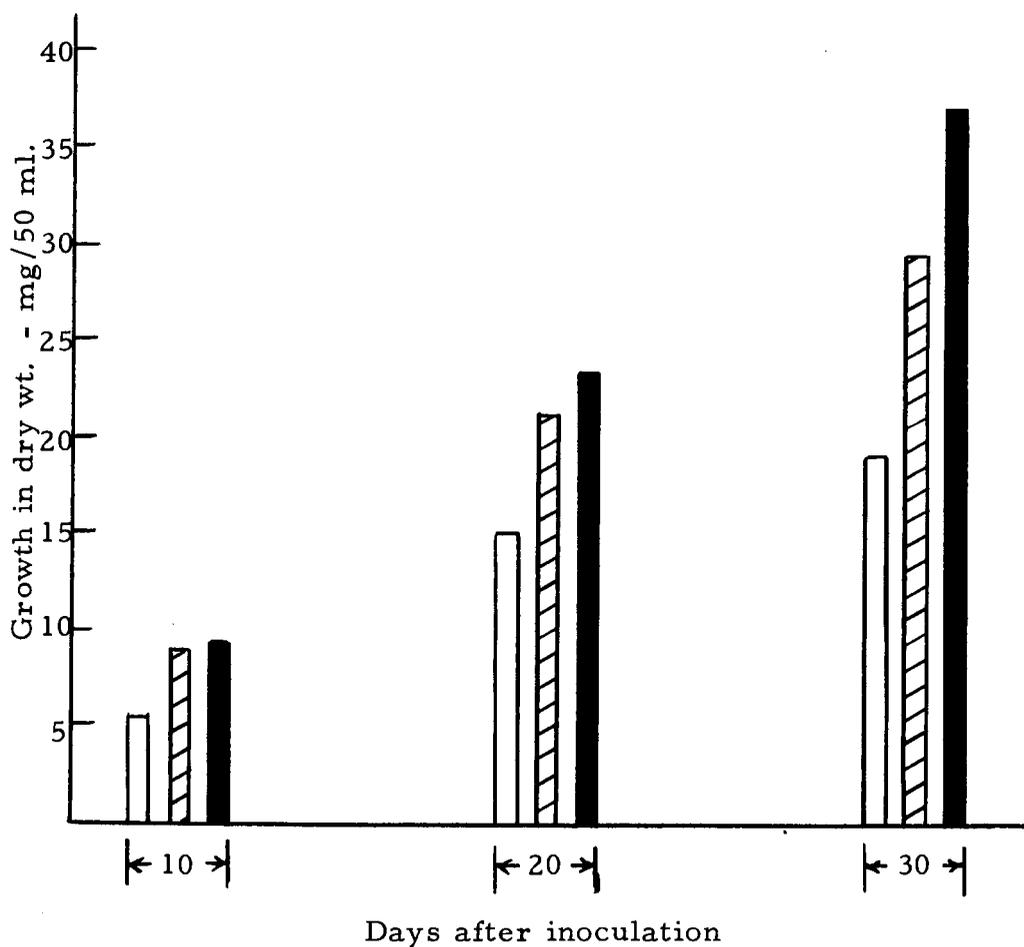


Table 6

Comparison of growth of A. cylindrica in M2
and M2 amended with marsh water. *

Amount of marsh water per liter of medium	Growth - dry weight - mg/50 ml		
	10 days	20 days	30 days
None	5.5	15.1	19.0
250 ml	6.5	20.4	25.9
500 ml	8.9	21.3	29.8
970 ml	9.2	23.7	37.2
LSD .05	0.9	1.9	3.2

* Marsh water from the August collection.

b. Buffer capacity of humic marsh water.

As mentioned earlier, the supposed buffering capacity of humic waters has been considered of possible ecological significance in the growth and nutrition of algae. By removal of carbon dioxide and bicarbonate ion during photosynthesis, algae may raise the pH of a medium so much that it becomes toxic (43, p. 35). Furthermore, the elevated alkalinity may cause precipitation of such minerals as calcium, iron, and phosphorus, possibly to the detriment of the algae. For these reasons the possibility that marsh water acts as a buffer was explored.

During the preliminary growth studies previously described, the reactions of the different media were determined at the time of

harvesting. The information thus obtained gave no indication that the marsh water had exceptional buffering qualities. In general, the more algal material a medium produced, the higher its pH at time of harvest.

To test more precisely the buffer characteristics of the marsh water, 50 milliliter aliquots of unfiltered water from each collection were titrated with N/100 sodium hydroxide. Separate aliquots were titrated with N/100 hydrochloric acid. Similar titrations were made on M2 in distilled water and a medium consisting of M2 minerals added to marsh water (designated "MWM2" medium). The resultant curves are compared in Figures 3 and 4. Neither collection of water had exceptional buffer capacity. Addition of M2 minerals to the marsh water increased the buffer capacity slightly. MWM2 medium with water from the August collection was the best buffer, but was more effective in the acid range.

The buffer capacity of marsh water was then tested biologically with young, physiologically active cells of A. cylindrica. A mass of cells was grown in two liters of M2 medium in an erlenmeyer flask. The medium was aerated in order to grow a large amount of algae in a relatively short time (ten days). A plankton centrifuge was used to concentrate the cells into 100 milliliters of M2 medium. This also served to break up the filaments, resulting

Figure 3

COMPARISON OF TITRATION CURVES
OF MARSH WATER (JANUARY COLLECTION), M2, AND MWM2

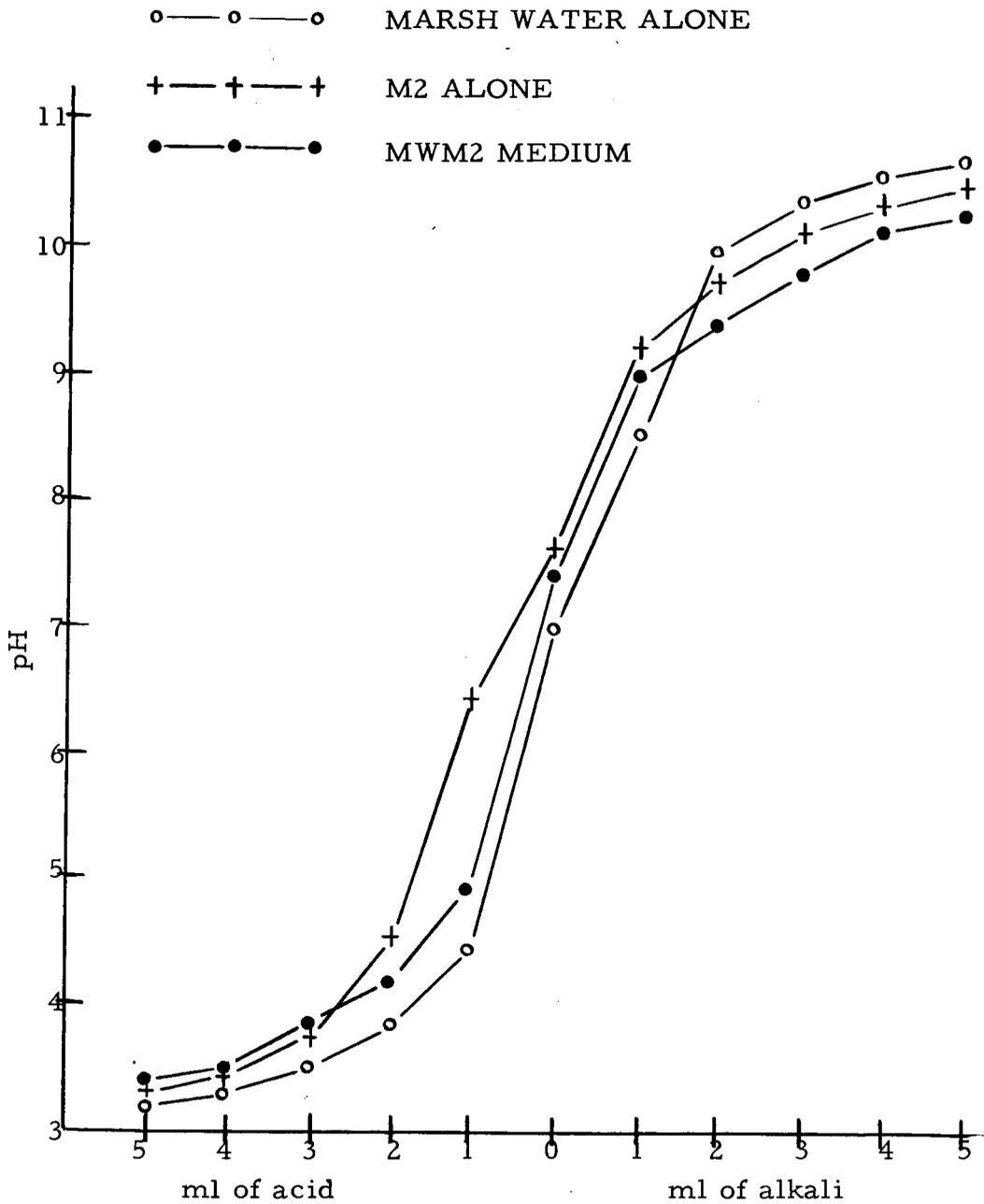
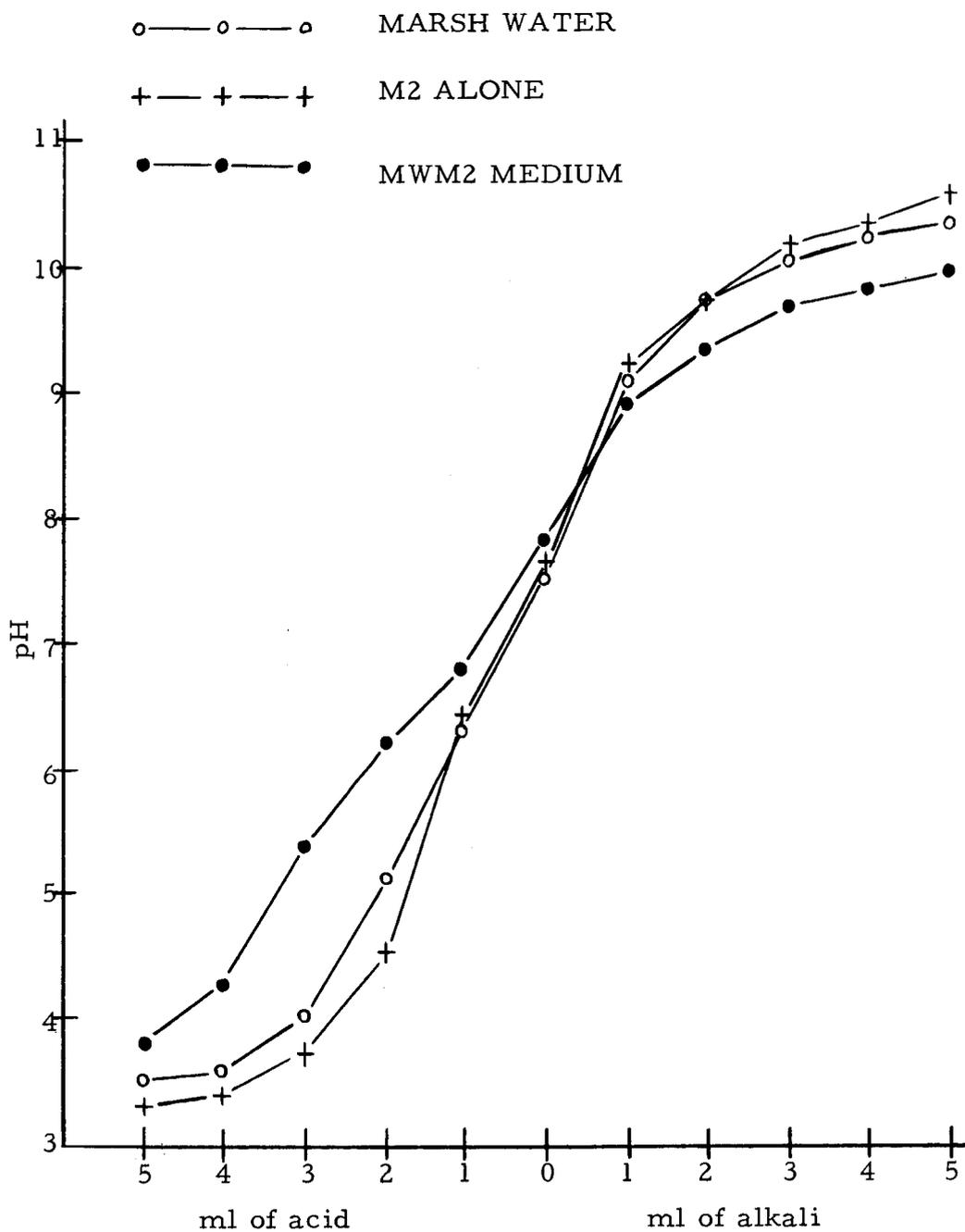


Figure 4

COMPARISON OF TITRATION CURVES
OF MARSH WATER (AUGUST COLLECTION), M2, AND MWM2



in a thick, homogeneous mass of cells which could be measured accurately with a pipette. Ten milliliter aliquots of this algal suspension (15 milligrams dry weight) were added to 50 milliliters each of the following media: M2, MWM2 with water from the January collection and MWM2 with water from the August collection. The cultures were illuminated continuously for six hours and the reactions of the media were measured at hourly intervals during this period. The cultures were then covered with a wood box painted matte black inside and out, and the lights in the culture room were turned off. As the cells respired, the reactions of the media were checked at hourly intervals for six hours.

MWM2 medium had slightly more buffer action than M2 under these conditions (Figure 5). The effect was more pronounced in the acid range than in the alkaline range, and water from the August collection had more buffer action than water from the January collection.

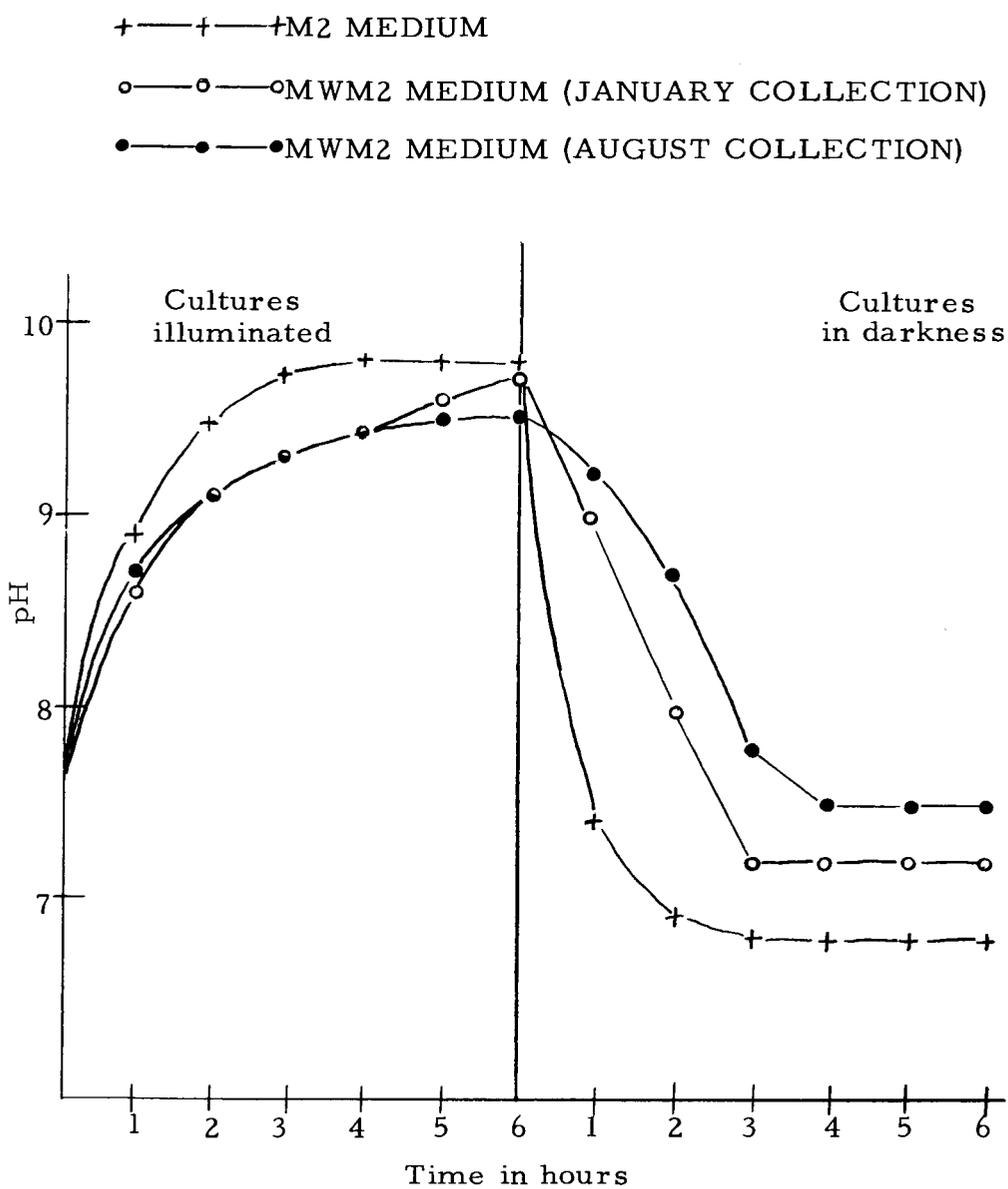
c. Marsh Water as a source of mineral nutrients.

(1) Test of M2 medium for limiting factors.

On the assumption that certain essential elements might be present at sub-optimal concentrations in M2 medium, experiments were designed to test the effect of increasing the concentration of

Figure 5

COMPARISON OF pH CHANGES
 ASSOCIATED WITH PHOTOSYNTHESIS AND RESPIRATION
 BY A. CYLINDRICA IN M2 AND MWM2 MEDIA



minerals in M2 on the growth.

In the first experiment, A. cylindrica was grown in M2, MWM2, M2 with double the normal amount of minerals (2XM2), double-strength M2 in marsh water (2XMWM2), M2 with one-half the normal amount of minerals (1/2M2), and one-half strength M2 in marsh water (1/2MWM2). The marsh water was from the January collection. Three cultures grown in each medium were harvested every ten days for 30 day.

The results (Table 7; Figure 6) showed that certain factors were limiting in M2 because doubling or halving the concentration of minerals in the medium produced definite changes in the amount of growth of the alga. The effect of adding marsh water to the media was obvious.

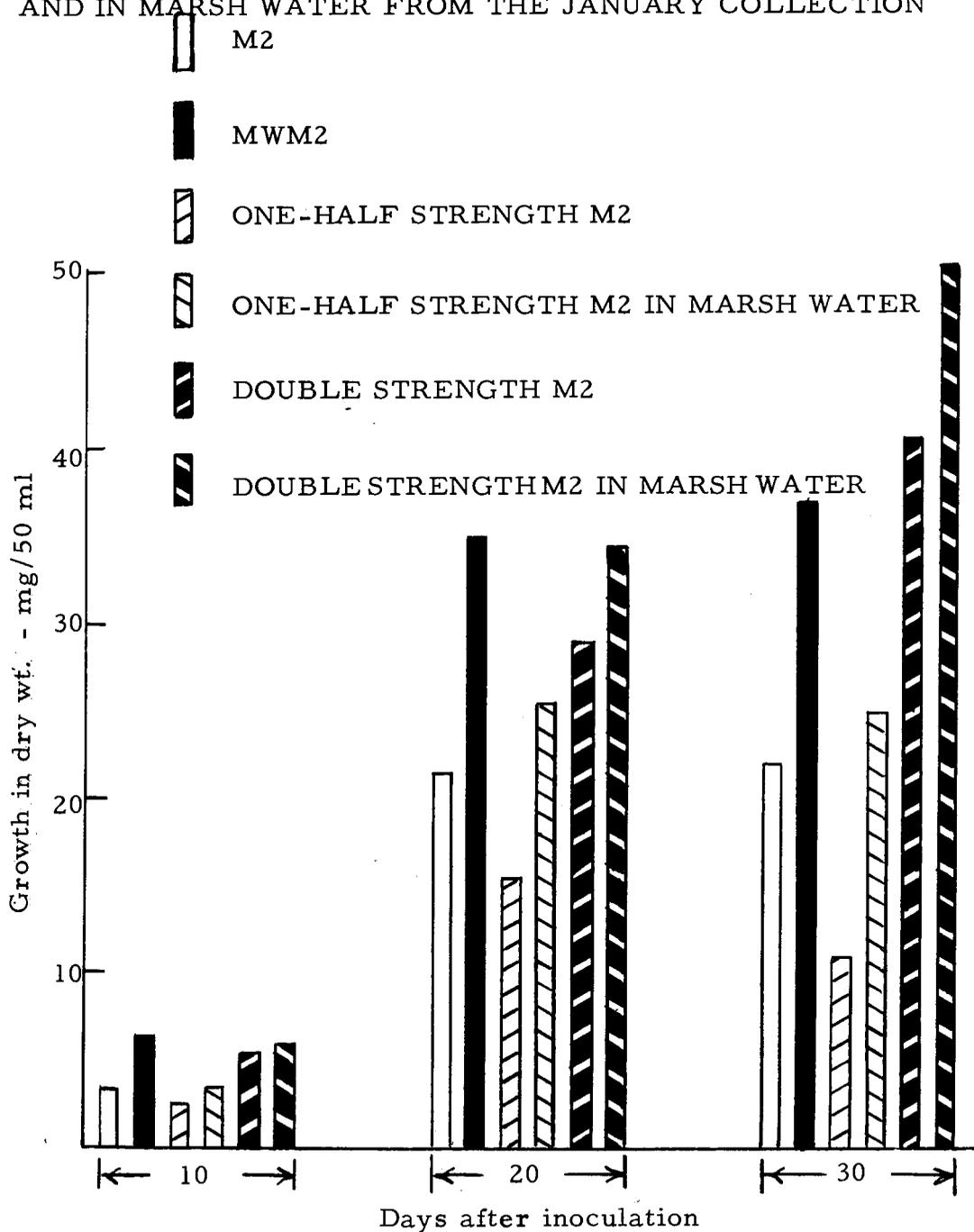
Table 7

Comparison of growth of A. cylindrica
in different concentrations of M2 and MWM2

Concentration of medium	Growth - dry weight - mg/50 ml		
	10 days	20 days	30 days
1/2 M2	2.5	15.5	11.0
1/2 MWM2	3.5	25.5	25.0
M2	3.5	21.5	22.0
MWM2	6.5	35.0	37.0
2XM2	5.5	29.0	41.5
2XMWM2	6.0	34.5	50.5
LSD .05	0.8	3.6	2.6

Figure 6

COMPARISON OF GROWTH OF A. CYLINDRICA IN DIFFERENT CONCENTRATIONS OF M2 MINERALS IN DISTILLED WATER AND IN MARSH WATER FROM THE JANUARY COLLECTION



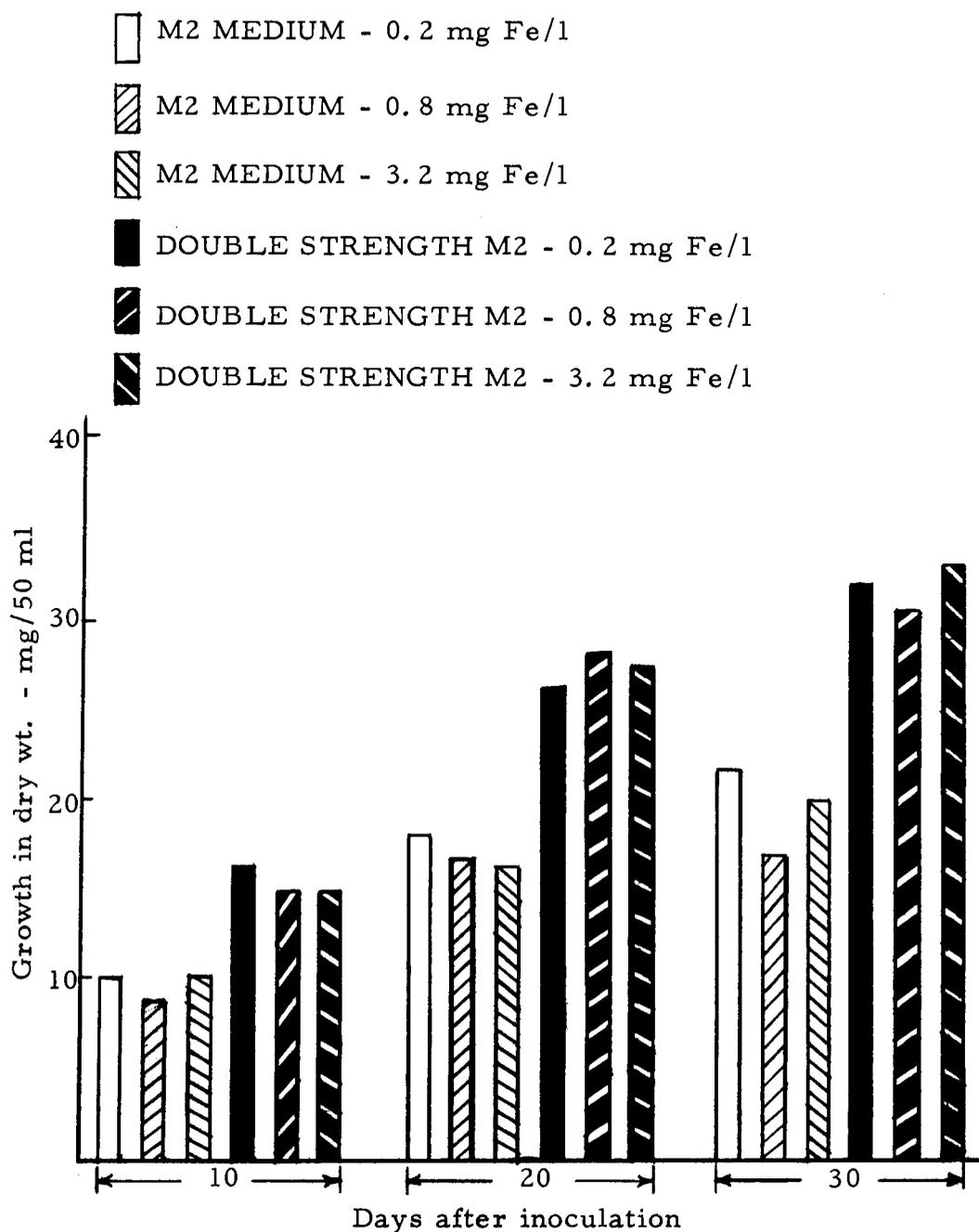
The second experiment in this series was designed to test the effect of adding iron to M2 upon growth. One of the most commonly proposed reasons for the observed stimulation of plant growth by humic substances or extracts is that humus may supply iron in a more readily available form than completely mineral media (26, p. 447; 82, p. 15-16). To test the hypothesis that iron could be the limiting factor in M2 medium, different media were prepared with the following amounts of iron: 0.2 milligram per liter (control), 0.8 milligram per liter and 3.2 milligrams per liter. One trial was prepared in normal strength M2 medium, and another in M2 with double the normal amount of minerals added to preclude the possibility that some other element might also be or become limiting. It is evident (Table 8; Figure 7) that under the conditions of this experiment, the addition of extra iron to the medium had no effect on the growth of A. cylindrica. The effect of doubling the concentration of elements in the medium was again pronounced.

Table 8

Comparison of growth of <u>A. cylindrica</u> in M2 and double strength M2 with different amounts of iron				
Medium	Amount of iron	Growth - dry weight - mg/50 ml		
		10 days	20 days	30 days
M2	0.2 mg/l	10	18	22
2XM2	0.2 mg/l	16	26	32
M2	0.8 mg/l	9	17	18
2XM2	0.8 mg/l	15	28	31
M2	3.2 mg/l	10	16	20
2XM2	3.2 mg/l	15	27	33
LSD		1.7	1.7	1.7
		.05		

Figure 7

COMPARISON OF GROWTH OF A. CYLINDRICA IN DIFFERENT
AMOUNTS OF IRON IN M2 AND DOUBLE STRENGTH M2



The third experiment in this series was designed to determine specifically which element or elements were limiting the growth of A. cylindrica in M2 medium, and to determine whether the concentration of one of the elements found in marsh water could cause the observed stimulatory action of marsh water.

Modifications of M2 medium were prepared with extra amounts of elements added individually to different trials at the rate they were found to occur in the marsh water of the August collection, the only exceptions being that phosphorus was added at the rate of one milligram per liter rather than 0.02 milligram per liter as found in the marsh water and calcium was added at the rate of five milligrams per liter. The effect of adding nitrogen was not tested because little nitrogen occurred in the marsh water and also because A. cylindrica is capable of fixing atmospheric nitrogen (3). The experiment was set up in acid-washed glassware. Three cultures grown in each medium were harvested every ten days for 30 days.

It is evident (Table 9) that the addition of extra calcium or sulfur to M2 medium produced stimulation of growth similar to that produced by marsh water. Addition of extra potassium had a slight stimulatory effect, but not so great as that of marsh water.

Table 9

Comparison of growth of A. cylindrica
in M2 enriched with certain elements

Medium	Growth - dry weight - mg/50 ml		
	10 days	20 days	30 days
M2 - Control	11	19	21
M2 plus 5 mg/l extra Ca	12	25	31
M2 plus 4 mg/l extra K	12	21	25
M2 plus 7 mg/l extra S	11	23	33
M2 plus 1 mg/l extra P	12	17	22
M2 plus 8 mg/l extra Mg	11	20	23
M2 plus extra Ca, K, S, P and Mg	10	21	34
LSD .05	1.4	2.0	3.4

(2) Bioassay of marsh water.

To test the ability of marsh water to supply its nutrient elements to algae and to confirm biologically the results of the chemical analyses, a bioassay of the marsh water was performed with cultures grown in acid-washed culture tubes. Three types of control media were used: M2 in distilled water, MWM2 with all essential elements added, and marsh water medium to which no essential elements were added - only silicate and bicarbonate. To assay for

a particular element, all the essential elements except one were added to the marsh water. The cultures were grown for 30 days, after which time each culture was transferred to fresh medium using one milliliter of inoculum from the old culture. The remainder of each of the old cultures was then harvested and weighed. In cases where the medium would not support the growth of the alga for 30 days, new cultures of that particular trial were inoculated from the control cultures in M2 medium.

In this experiment, the ability of strain 629 of A. cylindrica to fix atmospheric nitrogen was tested also. All M2 elements except nitrogen were added to distilled water and to marsh water from each collection. These cultures were treated as the cultures used in the bioassay just described. All experiments were performed in duplicate.

The results (Table 10) show that neither collection of marsh water stimulated growth of A. cylindrica without addition of sulfur or phosphorus, and the January collection did not stimulate growth of the alga without addition of potassium. Algae in marsh water media lacking added phosphorus would not grow for the full 30 days. Marsh water media without sulfur produced algae that were chlorotic and "unhealthy" in appearance. Other than the controls, the cultures with the most "healthy" appearance from the standpoint of

color and vigor were in marsh water media lacking added iron, magnesium, calcium, nitrogen, and (in the case of the August collection) potassium.

Table 10

Bioassay of mineral elements
in the water of Klamath Marsh

Medium	Element omitted	Growth - dry weight - mg/50 ml			
		1st Mo.	2nd Mo.	3rd Mo.	Mean/Mo.
M2	None	14	16	23	18
Jan MWM2	None	35	35	38	36
AugMWM2	None	43	45	49	46
Jan MWM2	All	3	4	0	2
AugMWM2	All	10	10	5	8
M2	N	16	12	17	15
Jan MWM2	N	29	25	37	30
AugMWM2	N	46	36	43	42
Jan MWM2	P	4	4	6	5
AugMWM2	P	11	11	12	11
Jan MWM2	Fe	32	32	36	33
AugMWM2	Fe	47	43	45	45
Jan MWM2	Ca	32	33	35	33
AugMWM2	Ca	43	40	43	42
Jan MWM2	Mg	33	28	39	33
AugMWM2	Mg	44	43	47	45
Jan MWM2	K	19	19	20	19
AugMWM2	K	38	40	48	42
Jan MWM2	S	20	25	23	23
AugMWM2	S	21	23	20	21
LSD .05		4.4	4.0	3.4	5.0

d. Effect of marsh water on solubility of iron and phosphorus.

One of the most widely held opinions regarding the ecological importance of the organic substances of water humus is that they form chelates or complexes with certain ions and hold them in solution under conditions that would ordinarily cause precipitation. Iron is quite susceptible to precipitation as ferric hydroxide or ferric phosphate at all pH ranges found in natural waters (35, p. 386), and phosphorus precipitates readily as calcium phosphate at neutrality or above (92, p. 188).

To test the ability of the humic marsh water to stabilize iron and phosphorus in the presence of relatively large amounts of calcium and at a high pH, two different media were prepared - one in distilled water and one in marsh water from the August collection. The media were made with the same concentration of minerals as the M2 medium except that iron was added at the rate of five milligrams per liter as ferrous sulfate, phosphorus was added at the rate of five milligrams per liter as acid phosphate, and calcium was added at the rate of ten milligrams per liter. The media were adjusted to pH 8.5.

After allowing four hours for precipitates to form in the media, experiments were performed to determine the amounts of iron and phosphorus remaining in solution. The solutions were

stirred vigorously and 50 milliliter aliquots were withdrawn and passed through micropore filters with a pore size of 0.45 micron. The filtrates were dried by evaporation and these residues were ashed separately from the precipitates on the filters. All residues were ashed by the wet-ashing method previously described. The rate of recovery of iron or phosphorus was determined by adding the amount of each element found in the filtrate to the amount found on the filter as precipitate. The results (Tables 11 and 12) show a relatively good rate of recovery of both iron and phosphorus.

The rate of precipitation of iron and phosphorus from the solutions was determined by use of two 500 x 70 millimeter test tubes with outlets at the bottoms. These were filled with the media, M2 in one and MWM2 in the other. After various periods of time, duplicate samples were withdrawn from the bottom of each tube. Each sample was divided into equal parts and one part was analyzed for iron and the other part for phosphorus. After the samples were withdrawn, the original height of the column of medium in each tube was restored from stocks of extra medium and each column was agitated vigorously with air and the next period of precipitation was started. Samples were withdrawn after two, four, eight, 16, 32 and 64 hours.

Table 11

Comparison of precipitation
of iron from M2 and MWM2 media at pH 8.5

Medium	Begin	2 hrs.	<u>mg/l in samples</u>				32 hrs.	64 hrs.
			4 hrs.	8 hrs.	16 hrs.			
M2	4.5*	8.25	21.50	24.38	27.00	11.25	19.50	
MWM2	5.1**	5.25	6.00	7.00	6.25	5.75	6.50	

* 3.9 mg removed by micropore filter; 0.6 mg/l in filtrate.
This is a recovery rate of 90%.

** 4.1 mg removed by micropore filter; 1.0 mg/l in filtrate.
This is a recovery rate of 84%.

Iron precipitated readily from M2 medium, but quite slowly from MWM2 (Table 11; Figure 8). In both media, most of the iron was in suspension rather than solution as most of the iron was removed by the micropore filter at the beginning of the experiment.

Phosphorus also precipitated readily from M2 medium, but not at all from MWM2 medium (Table 12; Figure 9) even though most of the phosphorus was in suspension rather than solution.

Figure 8

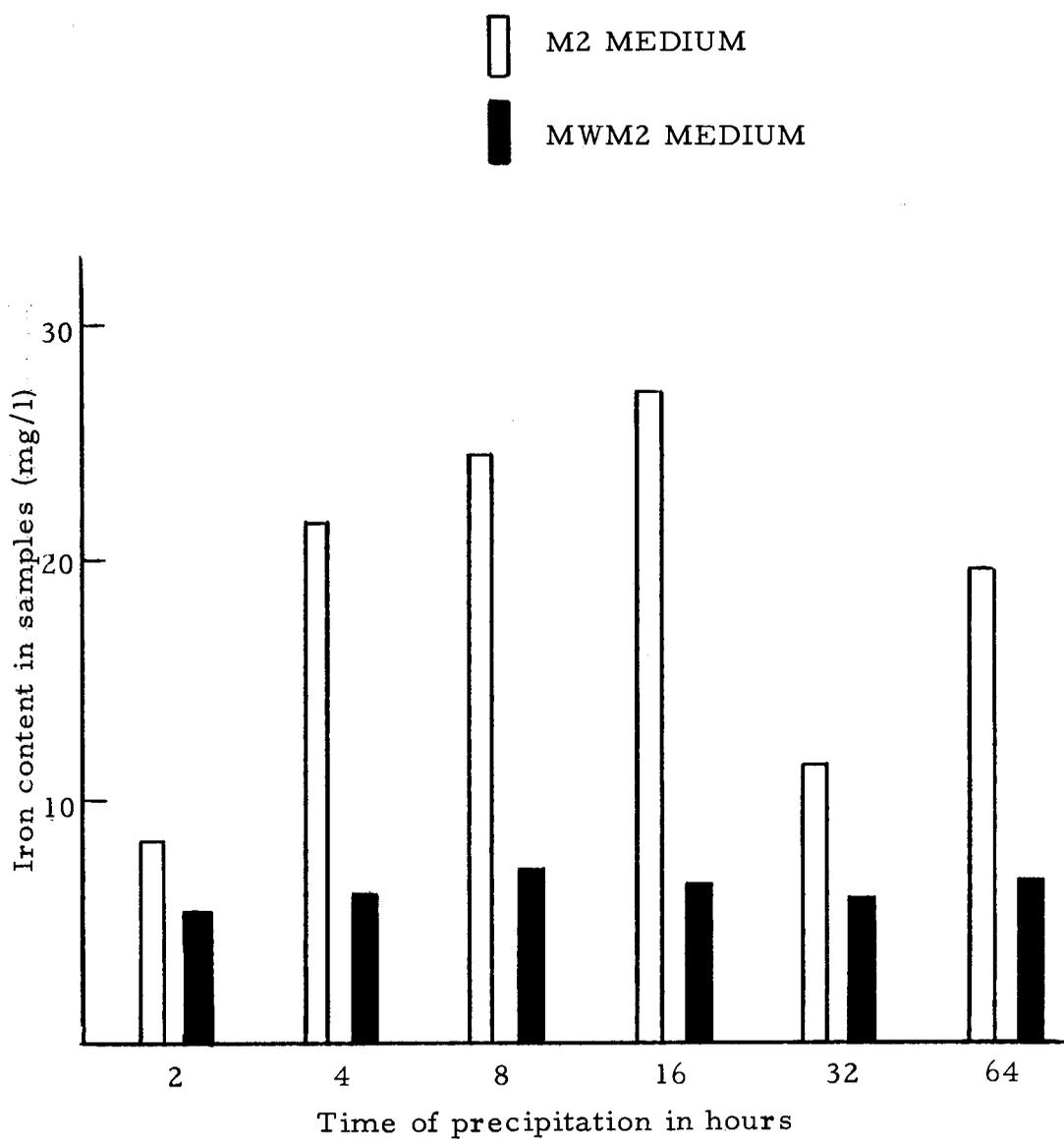
COMPARISON OF THE RATES OF PRECIPITATION
OF IRON FROM M2 AND MWM2 MEDIA

Figure 9

COMPARISON OF THE RATES OF PRECIPITATION
OF PHOSPHORUS FROM M2 AND MWM2 MEDIA

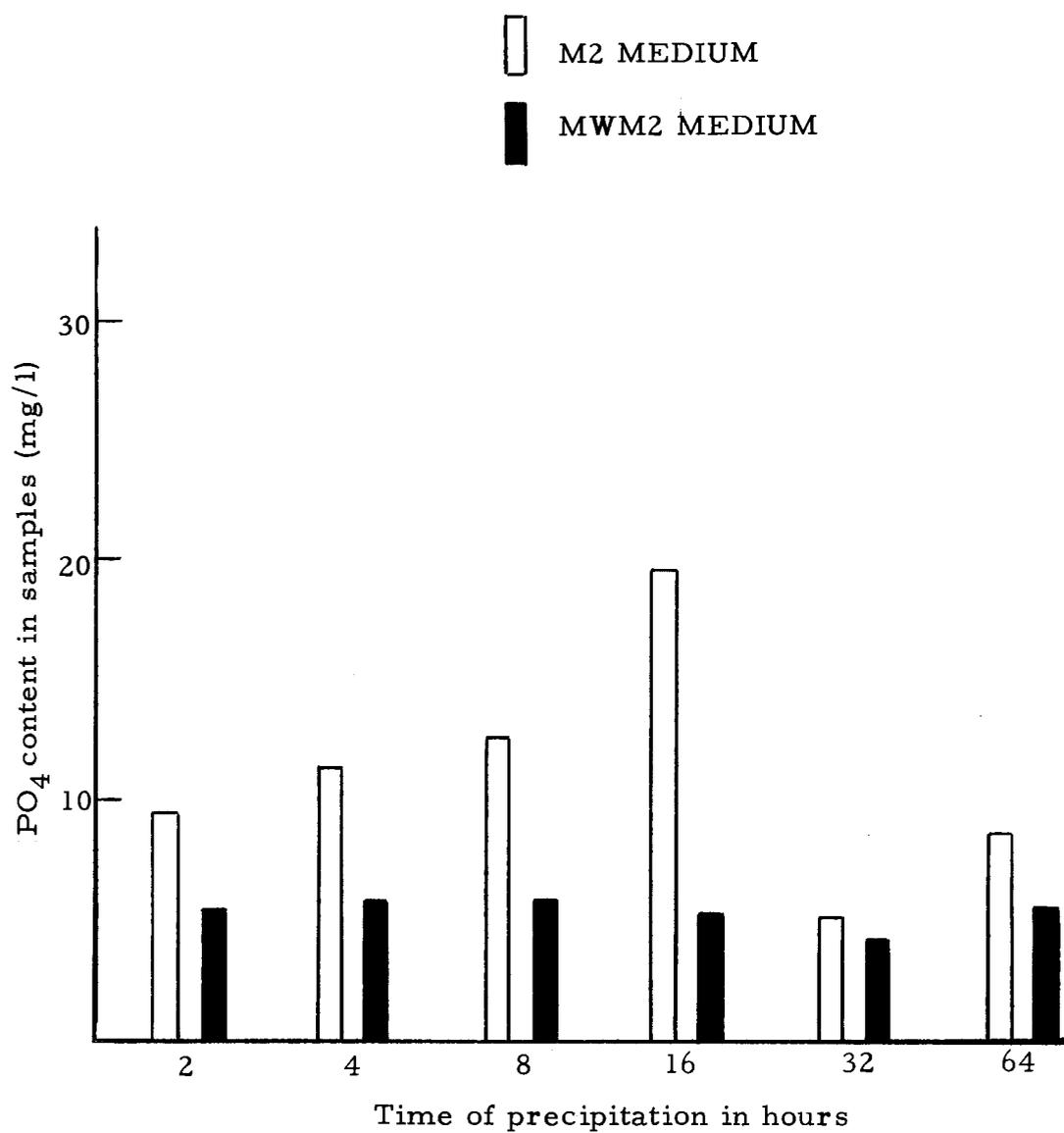


Table 12

Comparison of precipitation
of phosphorus from M2 and MWM2 media at pH 8.5

Medium	mg/l in samples						
	Begin	2 hrs.	4 hrs.	8 hrs.	16 hrs.	32 hrs.	64 hrs.
M2	4.7*	9.4	11.3	12.5	19.5	5.00	8.4
MWM2	5.3**	5.4	5.8	5.8	5.1	4.0	5.4

* 4.3 mg/l removed by microspore filter; 0.4 mg/l in the filtrate.
This is a recovery rate of 94%.

** 4.5 mg/l removed by micropore filter; 0.8 mg/l in the filtrate.
This is a recovery rate of 98%.

e. Effect of marsh water on the absorption of iron by A. cylindrica.

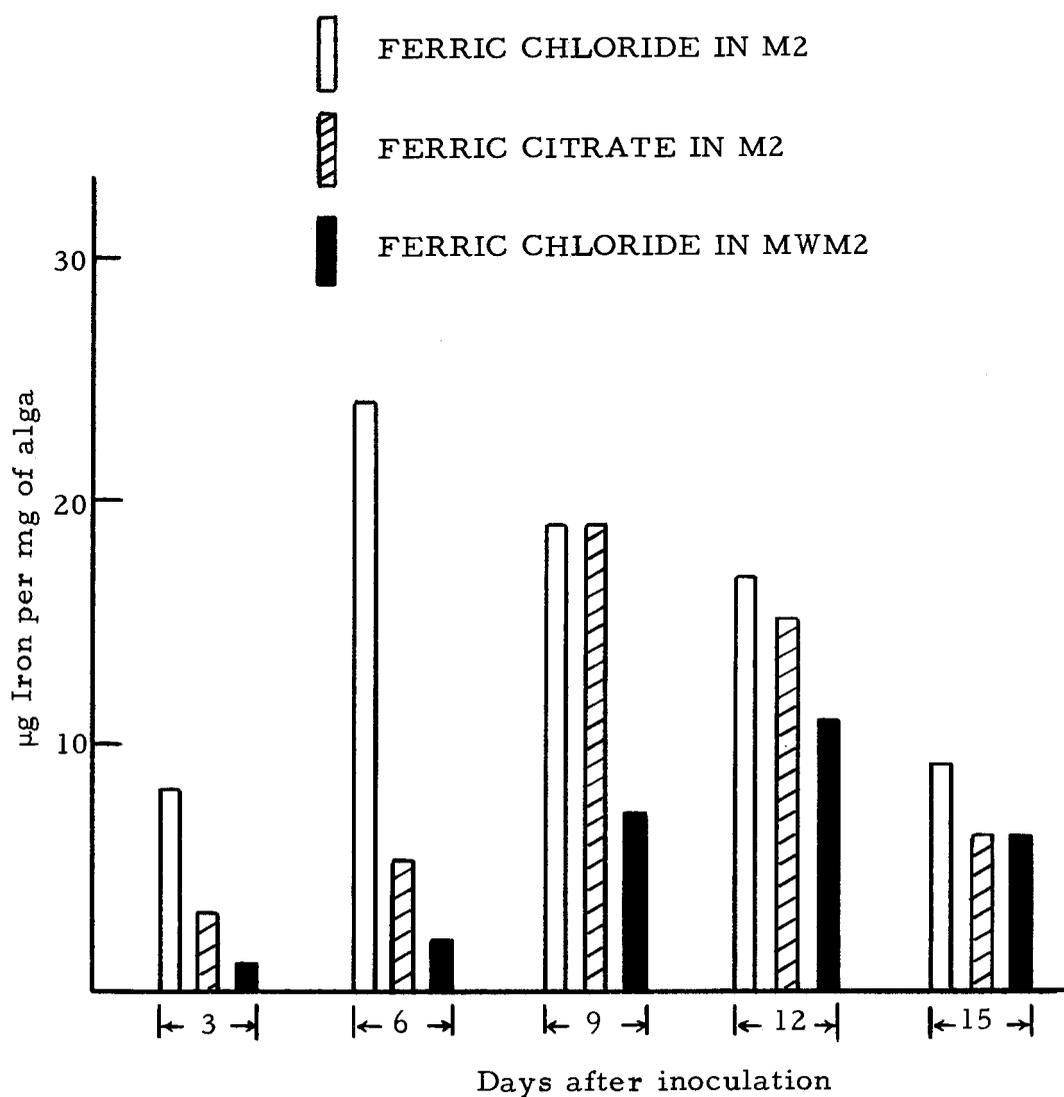
The opinion is widely-held that humus in a medium may be able to furnish nutrient elements, especially iron, to plants in a more readily available form than would water lacking humus (26, p. 447; 82). This hypothesis was tested in the following manner. Twenty tubes of each of the following media were prepared: (1) M2 with five milligrams of iron per liter as ferric chloride; (2) M2 with five milligrams of iron per liter as ferric citrate stabilized with citric acid; (3) MWM2 with five milligrams of iron per liter as ferric chloride. The marsh water used was from the August collection. The media were adjusted to pH 8.0, and each culture tube

was inoculated with ten milliliters of an algal suspension prepared as follows: a mass culture of A. cylindrica was grown in aerated M2 medium with a minimal amount of iron (0.05 milligrams per liter) for 90 days. The medium was changed after 30 and 60 days of growth. A plankton centrifuge was used to concentrate the cells in 250 milliliters of M2 medium, making a homogeneous mass which could be pipetted accurately. The ten milliliter inoculum added 9.8 milligrams of algae to each tube. Four cultures grown in each medium were harvested every three days. The harvest from the four cultures were mixed and then divided into four equal parts, two of which were dried to obtain the weights of algae in each harvest. The other two parts were placed in separate porcelain crucibles, dried, and ashed in a muffle furnace at 700° C. The ash was dissolved in 3N hydrochloric acid and transferred to small flasks. The acid was neutralized with potassium hydroxide and the resultant solutions were analyzed for iron using ortho-phenanthroline reagent (36, p. 125). To check the accuracy of analysis, duplicate samples of MWM2 medium were passed through the centrifuge and dried and ashed in the same manner as the algae. Recovery of iron by this method averaged 86 percent.

It is evident (Table 13; Figure 10) that A. cylindrica absorbed iron readily from the colloidal ferric form, as very little iron

Figure 10

COMPARISON OF ABSORPTION OF IRON BY
A. CYLINDRICA IN DIFFERENT MEDIA



would have been in solution in M2 medium with ferric chloride and at a pH of 8.0. Iron supplied as a ferric citrate-citric acid complex was absorbed less rapidly, and iron adsorbed or complexed on particles of water humus was absorbed least rapidly. The effect of the water humus was to make iron less readily available to the alga rather than more available as stated in the original hypothesis.

Table 13

Comparison of absorption of iron
by A. cylindrica in different media

Medium	$\mu\text{g Fe per mg of alga}$				
	3 days	6 days	9 days	12 days	15 days
M2 - iron supplied as ferric chloride	8	24	19	17	9
M2 - iron supplied as ferric citrate	2	5	19	15	6
MWM2 - iron supplied as ferric chloride	1	2	7	11	6
LSD .05	1.5	1.0	2.5	1.0	2.0

f. Adsorption of minerals on humic colloids.

Evidence from the last two mentioned experiments suggested that adsorption of mineral elements on the colloidal particles of the water humus might explain the stimulatory action of the marsh water on the growth of algae. Such an opinion was expressed by Ohle (77, p. 597). Gessner (46, p. 153-155) suggested that algae, by releasing oxygen during photosynthesis might obtain large quantities of nitrate nitrogen and phosphate phosphorus by oxidizing the humic colloids of brown waters and breaking down the mineral-humus complexes.

To test these hypotheses, an experiment was designed to adsorb mineral elements on the humic colloids of the MWM2 medium, remove any mineral-colloid complexes from the medium, and then determine the effect on growth of A. cylindrica in the treated medium.

M2 and MWM2 media were prepared and then treated with activated carbon. The carbon had been washed in acid, then leached 48 hours in two changes of distilled water, and finally rinsed in six rinses of distilled water (100 milliliters each rinse). Approximately two grams of carbon were stirred into a liter of medium and the resultant mixture was passed through coarse filter paper. This treatment removed all the color as the carbon and humus were

removed from MWM2 medium.

Two types of controls were used in this experiment: in one case, M2 and MWM2 were prepared in water that had been treated with activated carbon before minerals were added; the other controls were M2 and MWM2 with no treatment. Three cultures grown in each medium were harvested every ten days for 30 days.

Table 14

Comparison of growth of A. cylindrica
in media treated with activated carbon.

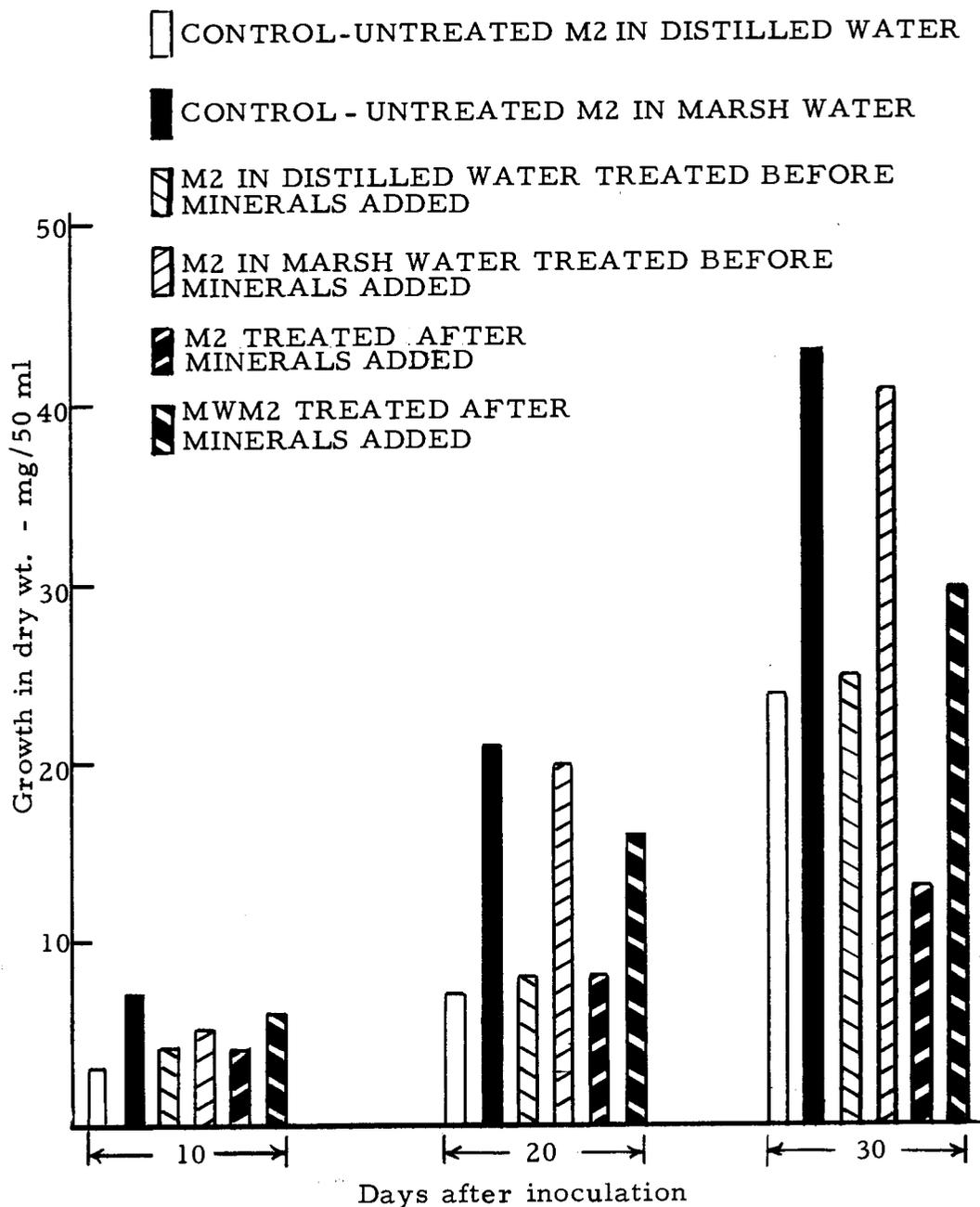
Treatment	Medium	Growth - dry weight - mg/50 ml		
		10 days	20 days	30 days
None	M2	3	7	24
None	MWM2	7	21	43
Water treated before minerals added	M2	4	8	25
	MWM2	5	20	41
Medium treated after minerals added	M2	4	8	13
	MWM2	6	16	30
LSD .05		0.3	3.0	3.0

The activated carbon did not appear to add anything capable of stimulating growth (Table 14; Figure 11), as M2 or MWM2 in water treated before addition of minerals produced the same amount of growth as the untreated controls. On the other hand,

treatment of the media after addition of minerals reduced the amount of growth in both M2 and MWM2 as compared to the controls. This suggests that removal of humic colloids by the activated carbon also removed some of the essential elements. The reduced growth in M2 was probably caused by removal of ferric citrate and citric acid by the carbon.

Figure 11

COMPARISON OF GROWTH OF A. CYLINDRICA
IN MEDIA TREATED WITH ACTIVATED CARBON



DISCUSSION

Interpretations of the physical and chemical data obtained in this study must be tempered by considerations of possible effects of long-term storage of the samples. It is generally recommended that samples be analyzed as soon as possible after collection (36, p. 4-5). The physical and chemical data reported here were obtained over a period of approximately one year. However, the water samples were treated with a generous quantity of chloroform and stored in darkness at low temperature, so it is unlikely that major changes occurred. Adsorption of cations on the walls of the storage containers was improbable in view of the chemical inertness of polyethylene and of the adsorptive and ion-exchange capacity of the humic colloids in the water. If significant changes had occurred in the water samples, they probably would have been accompanied by evident changes in color and/or pH, but no such changes occurred.

The color of water from Klamath Marsh does not increase as the pH of the water is raised. This is in contrast to the humic waters studied by Ohle (81, p. 147) and the colored acids isolated from lake waters by Shapiro (107, p. 170). The color of the marsh water is as dark as any reported in the literature and is quite stable.

No change occurred in a sample stored six months at room temperature in a normally lighted laboratory.

This study corroborates the findings of several other students of humic waters, namely, that color is not necessarily correlated with the total content of organic matter (Tables 2 and 4). The sample collected in August contained much more organic matter, but was visibly lighter in color, than the sample taken in January. In this respect the water of Klamath Marsh resembled that of Lake Gribso in Denmark (11, p. 105) which showed a minimum of color in August. Apparently this phenomenon is at least partly related to run-off because the flow of the Williamson River in late summer and fall is very much reduced from that during winter and spring. In late summer and fall, much of the marsh is dry, whereas a much greater area is under water during winter and spring. As the brown color mainly represents peat extracts, this would tend to increase the color of the water in winter and spring.

Although the total residue in the August sample was only 2.4 times greater, and the content of organic matter only 1.5 times greater than in the January sample, the suspended residue of the August sample was 17 times greater than in the January sample. It is possible that the great difference in suspended residue concomitant with the relatively slight increase in organic matter and

total residue in the August sample is explainable on the basis of increased amounts of decay of vegetation during the summer. This might increase the content of bicarbonates in the water. The bicarbonates thus formed would probably adsorb on the colloids of the water humus (77, p. 596). This could account for a relatively great increase in colloidal material without a correspondingly great increase in organic matter content.

Difficulties in analyzing the colored, turbid samples were successfully overcome in most cases, as shown by the rates of recovery of elements (Table 3). The analyses of metallic cations by flame spectrophotometry were satisfactory, although there was some interference with the determination of calcium. The analysis of iron by the wet-ashing technique was satisfactory. The analysis of ammonia was satisfactory, but the determination of nitrate nitrogen was considered unsuccessful. The standard method using phenoldisulfonic acid (36, p. 149) was unsatisfactory because the colored samples interfered with the developed color. In addition, the presence of organic matter caused a darkening of the developed samples for which it was impossible to compensate in the spectrophotometer. Attempts were made to destroy the organic matter with 30 percent hydrogen peroxide in one case and with five percent sodium hypochlorite in another. Both attempts were unsuccessful;

only 35 percent of added nitrate nitrogen could be recovered. The standard reduction method (36, p. 151) was the best method tried even though only 48 percent of added nitrate nitrogen was recovered. This could have been caused by incomplete reduction of the nitrate to ammonia or by losses of ammonia from the apparatus employed (36, p. 151). The analyses of sulfate ion are suspect because the amounts determined were the same in both collections, whereas in the case of all other elements, the August sample contained greater concentrations.

The water of Klamath Marsh is unusual in that the concentration of magnesium exceeds that of calcium. In most freshwaters of the world, the reverse is the case (92, p. 179). The concentrations of inorganic elements in both collections were rather low compared to most freshwaters of the world (49, p. 19).

Before the spot tests for amino acids and reducing sugars were performed, 100 milliliters of water were evaporated to ten milliliters; therefore, the tests were equivalent to analyzing a liter of water. Feigl (37, p. 284) claims a sensitivity of 0.4 microgram for glycine to ten micrograms for hydroxyproline, and a sensitivity of 0.2 microgram for reducing sugars (37, p. 390), so the concentrations of these compounds were quite low. It should be noted, however, that the absence or extremely low concentrations of these

compounds do not prove that they are unimportant in the water, as they may be utilized by native organisms as quickly as they are formed. Probably they would also disappear first in storage.

"Crude protein" comprised 17 percent of the organic matter in the August collection, but only ten percent of the organic matter in the January collection. The less colored, more proteinaceous nature of the water of the August collection could result from decomposition of the standing crop of vegetation in the marsh. This vegetation would be absent or mostly decomposed in January. Thus, the water in the January collection would contain relatively more extractives from peat and these would be less proteinaceous than the products of the early stages of decay of recent vegetation. These assumptions are based on the statements of Hutchinson (57, p. 886) and Reid (92, p. 197) that autochthonous organic matter (from native algae and vascular plants) tends to be less colored and more proteinaceous than highly colored extractives of peat. The work of Birge and Juday (13, p. 461-472) shows that humic waters may be quite colored and yet contain less decomposable organic matter than a less colored water. Indirect evidence for the more active decomposition of vegetation in the marsh in late summer is the fact that all elements studied (except sulfur) were present in greater concentrations in the August sample despite the smaller

area of marsh under water.

There is little to be gained in attempting a comparison of the physical features and chemistry of the water of Klamath Marsh to other humic waters of the world because of the great variability in such waters.

This study has illustrated the inadequacies of the "standard" methods of water analyses used in sanitary engineering and public health work when such methods are applied to humic waters. Most of the analyses of mineral elements were performed by destructive techniques, and for this reason, do not indicate in what form the elements existed in the water. For example, the determination of iron made no distinction between soluble, complexed, adsorbed or colloidal iron. The same can be said of the flame spectrophotometric analyses of other metallic cations, and the analyses of sulfur and phosphorus which could have existed in the organic state.

Before a clear understanding of water humus and its ecological role can be reached, non-destructive methods of analyses must be developed. Shapiro (108) found the yellow organic acids common in colored lake waters show different R_f values on paper when complexed with different metallic cations. This fact may prove of value in determining the state in which certain cations exist in the water. Micropore filters can be obtained with pore sizes ranging

from one to 500 millimicrons (48, p. 195). These may be of use in separating colloidal and dissolved organic matter and in determining the sizes of colloids in humic waters. Ultracentrifugation might also be useful. Various fractions of total humus could be separated by means of paper and/or column chromatography, electrophoresis, and the use of newly developed reagents. Soil scientists are making important advances in the study of humus with these techniques (101; 105; 106). If these techniques were combined with use of radioisotopes under controlled conditions in the laboratory, much knowledge could be gained about the state of matter in water humus.

Suspended material filtered from the marsh water formed a grayish-brown mass reminiscent of the descriptions of preparations of humic acids from soils (47, p. 433). These colloids should be studied using the many techniques outlined by Scheffer and Schlüter (101) to determine whether they are similar to typical humic acids of soils.

Stimulation of growth of A. cylindrica by marsh water was quite obvious (Tables 5 and 6; Figures 1 and 2). It is difficult to determine whether the stimulation was due to addition of mineral elements to the medium or some effect of the water humus on the environment. The latter cause might be suspected from the fact

that marsh water from the August collection, although it contained more essential elements than the January collection, did not stimulate growth of the alga more than water from the January collection.

The possibility that marsh water stimulated growth by supplying organic nutrients can probably be ruled out, as A. cylindrica is known to be an obligate photosynthetic autotroph (43, p. 59), and to the writer's knowledge, the alga has never been shown to be stimulated by or to have a requirement for vitamins or any other preformed organic nutrient. The action of quinoid compounds, probably present in most humus, as hydrogen acceptors for terminal oxidase systems may have been operative in these experiments, but the possibility was not studied, nor were possible actions of water humus on other enzyme systems of the alga.

That the water of Klamath Marsh is not an exceptional buffer substance is evident from the results of titrations (Figures 3 and 4). Water from the August collection was a better buffer than water from the January collection, but the difference was slight. Changes in pH of media caused by photosynthesis and respiration of the alga (Figure 5) showed that, under the conditions of the experiment, the media with marsh water were buffered somewhat better than unamended M2 medium. This was especially true of the medium made with marsh water from the August collection. It is possible

that the media containing humic colloids absorbed bicarbonates formed during respiration or supplied bicarbonate ion during photosynthesis. Then the medium with the greatest amount of colloidal material would have shown the greatest buffer effect, i. e. , the marsh water from the August collection. In a natural situation, with alternating periods of light and darkness, the buffer effect of marsh water might be cumulative, but the possibility seems doubtful because of the magnitude of changes in pH caused by photosynthesis or respiration during such short periods of time (Figure 5). In all other experiments reported in this thesis, illumination was constant, and at time of harvest the media in marsh water frequently had pH levels higher than M2 medium. The pH in all media usually was well over 9.0 after 20 days of growth. At this pH, carbonate ions form in the media and the colloids of the water humus may have absorbed these in some manner. If carbonate ion is toxic to A. cylindrica as it is to certain other algae (43, p. 35), the humic colloids could have protected the alga.

It does not appear that the marsh water stimulated growth of A. cylindrica by supplying extra iron or by keeping iron in a "more readily available" form as postulated by Olsen (82, p. 12) and Burk, Lineweaver and Horner (26, p. 447). Iron was not a limiting factor in M2 medium (Table 8; Figure 7). Iron supplied as a ferric

citrate-citric acid complex is stable if it is sterilized separately and added after a culture medium has cooled (95, p. 35). Iron supplied in complexed form was less available than iron in the form of ferric hydroxide (Table 13; Figure 10). The same result might not be obtained with other species of algae, especially those which secrete less organic matter than A. cylindrica. The hindrance of absorption of iron caused by water humus is comparable to the results of Miller and Ohlrogge (71, p. 228) who found that corn and soybeans absorbed less iron and zinc from media containing extracts of soil or manure than from purely mineral media. The results of these experiments lend indirect support to the hypothesis that water humus regulates the supply of mineral nutrients to plants by acting as a stable "reserve" from which plants may obtain ions as needed, by ion exchange, yet may not absorb ions in excess of their needs.

There is little doubt that several nutrient elements in the MWM2 media could have been adsorbed or complexed on the colloids of the water humus. The greater part of iron and phosphorus quickly attached to the colloids (Tables 11 and 12), and were removed by micropore filters. These results are supported by data from the experiment growing A. cylindrica in media treated with activated carbon (Table 14; Figure 11). Some mineral elements

were removed together with the organic matter by the activated carbon. This experiment should have included analyses of the marsh water and media after treatment with carbon. The carbon may have caused a release of certain ions from the colloids of the water humus because treatment of the marsh water before addition of mineral elements did not reduce growth of the alga. An explanation of this result is not available at present. More knowledge is needed concerning the action of activated carbon on mineral-humus complexes. This experiment should be improved and repeated to allow definite conclusions, but it suggests the possibility that the stimulatory action of water humus in these experiments was not due to the effects of organic matter, a possibility that seems unlikely in view of present day knowledge of the composition and reactions of humic acids.

There is no doubt of the ability of humic colloids in marsh water to keep iron and phosphorus in suspension (Tables 11 and 12; Figures 8 and 9). Under laboratory conditions, i. e., in a culture without agitation, the presence of water humus would keep the algal cells in better contact with essential elements, but in the natural turbulence of a lake the ability of humic colloids to keep these elements in suspension might not be of significance. Perhaps of greater significance was the fact that after four hours, 67 percent

more iron and 100 percent more phosphorus were in solution in MWM2 medium than in M2. Thus, in spite of the fact that neither medium kept a high percentage of iron or phosphorus in solution, the presence of water humus maintained more of these elements in a soluble state.

The available data may be used to argue both for and against the possibility that water humus stimulated growth of A. cylindrica by supplying one or more elements that were present at sub-optimal concentrations in M2 medium (Tables 7, 8 and 9; Figures 6 and 7). From Table 9, it might be assumed that marsh water stimulated growth by supplying needed calcium, potassium and sulfur. The bioassay of essential elements in the marsh water (Table 10) seems to support this with regard to potassium and sulfur. It shows that most of the sulfur in the marsh water was available to the alga. In the bioassay of the January collection of marsh water, growth of the alga in the marsh water medium lacking added sulfur was equal to growth in the M2 control, but only 64 percent of the growth in the marsh water medium with all essential elements added. In the bioassay of the August collection, growth of the alga in the marsh water medium lacking added sulfur was also equal to the control in M2, but only 46 percent of the growth in the marsh water medium with all essential elements added. As M2 contains

two milligrams of elemental sulfur per liter, it can be concluded that both collections of marsh water contained approximately two milligrams of assimilable sulfur per liter. The chemical analyses (Table 2) showed 6.8 milligrams of sulfate per liter (equal to 2.24 milligrams of elemental sulfur), so the bioassay supported the accuracy of the chemical analyses and indicated that the extra sulfur added to M2 with the addition of marsh water might have stimulated growth of the alga.

Slight stimulation of growth of the alga was caused by increasing the concentration of potassium in M2 medium (Table 9), and apparently the concentration of potassium in the January collection of marsh water was limiting (Table 10). In the bioassay of the January collection of marsh water, the marsh water medium lacking added potassium contained approximately two milligrams of potassium per liter and supported growth equal to that in M2, but only 53 percent of the growth in the marsh water control medium containing four milligrams of potassium per liter. In the bioassay of the August collection, the marsh water medium lacking added potassium contained approximately four milligrams of potassium per liter, and supported growth equal to the marsh water control medium containing six milligrams of potassium per liter. As these results were consistent over a period of three months, the writer is of the opinion that the strain of A. cylindrica used in

this study had a definite requirement for potassium, as did the strain of this species studied by Allen and Arnon (4, p. 656). Earlier, Allen (2, p. 46) reported a strain of A. cylindrica that was able to substitute sodium for potassium.

Increasing the concentration of calcium in M2 medium had a stimulatory effect on growth of the alga (Table 9), and it might be assumed that the marsh water stimulated growth in part by supplying needed calcium. The bioassay (Table 10) did not support such a conclusion. The M2 medium with added calcium contained ten milligrams of calcium per liter. The January collection of marsh water contained only 1.5 milligrams of calcium per liter and the August collection only 2.5, yet the marsh water media lacking added calcium produced as much growth as marsh water media with added calcium. It appears that less calcium was required by the alga in the presence of water humus.

The validity of the data in Table 9 must be questioned. Because of a necessary change in culture rooms, the experimental cultures were grown for the first 12 days at a higher temperature than previously. In the new location, the temperature was uncontrolled, and varied from approximately 22 to 26° C., whereas the alga had been growing for several months at 18° C. Apparently the strain of alga used was adapted to the lower temperature,

because the experimental cultures did not appear to be doing well. They were moved back into a cooler location where differences in growth in the different media then began to appear. The experiment should be repeated.

In general, the writer is of the opinion that the stimulatory effect of marsh water on the growth of A. cylindrica resulted at least in part from the addition of mineral nutrients that were present at sub-optimal concentrations in unamended M2 medium. According to Allen (2, p. 36), growth of most blue-green algae is affected little, if any, by variations in the concentrations of salts in the range from 0.01 percent to 0.1 percent. The M2 medium contained 0.018 percent salts, so these studies do not support Allen's statement. Doubling the concentration of salts in M2 medium greatly stimulated growth of the alga (Table 7; Figure 6). Doubling the concentration of minerals in M2 medium prepared in marsh water produced even greater stimulation of growth. This increase may have been due either to a protective action by the humic colloids or to the additional concentration of mineral salts. Further experiments are necessary to determine the exact cause of these phenomena, and to determine the optimum concentrations of essential elements for the growth of the particular alga used and the conditions of culture used. It is likely that algae will tolerate higher

concentrations of salts in the presence of humic colloids than in purely mineral media.

In conclusion, it can be stated that humic marsh water probably stimulated the growth of A. cylindrica by a number of actions including: (1) supplying certain nutrient elements (especially potassium and sulfur) which were present at sub-optimal concentrations in M2 medium, (2) adsorbing and/or complexing mineral nutrients on the humic colloids of the marsh water and thereby preventing certain ions, such as calcium, iron and phosphorus, from precipitating from the medium in some cases and may have regulated the absorption of mineral elements by the alga in other cases. In other words, the water humus probably acted as a "stabilizer" of the mineral elements in the media, while simultaneously regulating the absorption of these elements.

The previously stated hypotheses were supported, not proved, by the data. The data indicated only the following: (1) certain essential elements were present at sub-optimal concentrations in M2 medium, (2) these elements were present in the marsh water in quantities sufficient to affect the total growth of the experimental alga used, (3) the experimental alga required less calcium in the presence of water humus than when water humus was absent from the medium, (4) iron and phosphorus formed complexes with the

humic colloids of marsh water and did not precipitate from a medium containing water humus, even at a high pH and in the presence of relatively high concentrations of calcium, (5) the experimental alga absorbed iron less readily from an iron-humus complex than from a ferric citrate complex or from the hydrated, oxide form, and (6) the humic water of Klamath Marsh is not an effective buffer.

Before much further progress can be made in studies of the action of water humus on the growth of algae, a medium must be developed containing the optimum concentration of each element essential for the alga being used and for the conditions under which the alga is to be grown.

SUMMARY

This thesis reports an exploratory study of some ecological aspects of the humic water flowing from Klamath Marsh in southern Oregon. The study was based on the hypothesis that the marsh water could be an important factor in the growth of freshwater algae by supplying physiologically significant amounts of essential elements or by producing beneficial physical effects in the environment, e. g. , buffering, or chelating and complexing mineral elements and thus regulating the absorption of these by algae, or by keeping certain mineral elements in a readily available form by preventing their precipitation under conditions that ordinarily cause precipitation from the medium in the absence of water humus.

The objectives were to obtain a brief physical-chemical description of the humic marsh water and to determine some of its effects on the culture of Anabaena cylindrica Lemm.

Large samples of marsh water were taken in January and August, 1959, preserved with chloroform, and stored in darkness at 4°C. in polyethylene carboys. Physical and chemical investigations of the marsh water revealed that the concentrations of mineral elements were low in comparison to the average freshwaters of the world. The marsh water was more highly colored in winter than in

summer, but the summer collection contained more total and suspended residue, more mineral elements, more organic matter and a narrower carbon:nitrogen ratio than the winter collection. The color and pH of the water were quite stable in storage. The study revealed the inadequacy of standard methods of water analyses used in hygiene and sanitary engineering when applied to strongly humic waters. Methods were suggested for improving methods of analysis and study of humic waters.

Two types of investigations were used to determine the ecological importance of the marsh water in the growth of the experimental alga: (1) physical-chemical studies and (2) biological studies performed with pure cultures of Anabaena cylindrica (strain number 629 from the culture collection of the University of Indiana) grown under controlled conditions of temperature and illumination. The following characteristics of the marsh water were studied for possible ecological significance: (1) the buffer capacity of the water, (2) the importance of the concentrations of essential elements in the water to the growth of the experimental alga, (3) the effect of the humus in the water upon the absorption of iron by the experimental plant, and (4) the effect of the water humus upon the stability of iron and phosphorus in an alkaline culture medium.

The mineral composition of the medium used was derived

empirically by averaging concentrations of mineral salts in media used by other workers successful in growing blue-green algae. The medium grew the alga well, but replacement of the distilled water in the medium with water from Klamath Marsh stimulated growth. Further studies revealed that under the conditions of culture used, the synthetic medium would have been more suitable for growing the alga if higher concentrations of mineral salts had been supplied, because addition of extra quantities of calcium, sulfur, or potassium increased the growth of the alga. The same effect was obtained by doubling the quantities of all minerals supplied in the synthetic medium.

More calcium was required by A. cylindrica in a purely mineral medium than in a medium containing water humus. Both collections of marsh water contained enough sulfur to increase growth of the alga, and the summer collection contained enough potassium to increase algal growth. The strain of A. cylindrica used in this study probably had a requirement for potassium. It had been reported in one case as capable of using sodium in place of potassium, and in another case as requiring both elements.

The presence of marsh water prevented precipitation of iron and phosphorus from an alkaline, aerated, culture medium containing a relatively high concentration of calcium. Most of the iron and

phosphorus in this solution were adsorbed or complexed on humic colloids and were removed by micropore filters with a pore size of 0.45 micron. Stimulation of growth was not due to the ability of the marsh water to keep iron in solution and suspension under conditions of high pH, as has been claimed by other authors, nor was the addition of extra iron contained in the marsh water the cause of stimulation, because addition of extra quantities of iron in a ferric citrate-citric acid complex had no effect on growth of the alga in M2 medium. The presence of marsh water greatly decreased absorption of iron by A. cylindrica as compared to the absorption of iron by the alga from a ferric citrate-citric acid complex or from colloidal ferric hydroxide. The possibility was suggested that absorption of other ions might also be inhibited by the presence of water humus in the culture medium.

Titration of culture media containing marsh water revealed that the marsh water was not an exceptional buffer substance. Changes in the pH of media due to photosynthesis and respiration were not greatly lessened by the presence of marsh water.

The results suggested, but did not prove, that the presence of water humus in a culture medium stimulated growth of A. cylindrica by stabilizing the mineral salts in the medium, by regulating absorption of minerals by the alga, and by supplying extra quantities of

essential elements, thereby providing beneficial conditions for growth.

The opinion was expressed that much further progress in the study of water humus depends upon development of non-destructive methods of analyses of both organic and inorganic compounds in humic waters. Studies of the ecological effects of water humus upon growth of algae must always be preceded by development of culture media containing optimum concentrations of each element essential for the particular alga used and for the particular environmental conditions in which the study is performed.

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