THE AQUATIC ENVIRONMENT:

Microbial Transformations and Water Management Implications

Symposium Sponsored by Environmental Protection Agency Office of Water Program Operations

SYMPOSIUM SPEAKERS

Left to Right:

J. LeGall, R. K. Ballentine, F. Verhoff, R. H. Hardy, D. C. Hood, R. R. Colwell, F. Cordeiro, J. W. Payne, R. L. Sansom, K. M. Mackenthun, W. Fuhs, L. Keup, W. R. Finnerty, L. J. Guarraia.

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Symposium Sponsored by Environmental Protection Agency
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Edited by Leonard
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Richard K. Ballentine

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Mrs. K. Shorr

KEYNOTE SPEAKER

Robert L. Sansom
Assistant Administrator
for Air and Water Programs
Environmental Protection Agency

CHAIRMEN OF THE MEETINGS

Richard K. Ballentine*
Water Quality Protection Branch, Water Quality and
Non-Point Source Control Division, Office of
Water Program Operations

Leonard J. Guarraia
Water Quality Protection Branch, Water Quality and
Non-Point Source Control Division, Office of
Water Program Operations

Kenneth M. Mackenthun
Director, Technical Support Staff, Office of the
Assistant Administrator for Air and Water Programs

Lowell E. Keup
Deputy Director, Water Quality and Non-Point Source
Control Division, Office of Water Programs Operations

Donald Lear
Annapolis Field Office, Region III

---

* Currently with the Non-Point Source Control Branch, Water Quality and Non-Point Source Control Division

THE AUTHORS

F. H. Verhoff	*University of Notre Dame*
C. F. Cordeiro	*University of Notre Dame*
W. F. Echelberger, Jr.	*University of Notre Dame*
M. W. Tenney	*University of Notre Dame*
W. J. Payne	*University of Georgia*
J. LeGall	*University of Georgia*
W. R. Finnerty	*University of Georgia*
R. Hardy	*E. I. DuPont de Nemours and Co.*
R. D. Holsten	*E. I. DuPont de Nemours and Co.*
M. Alexander	Cornell University
C. W. Fuhs	*New York State Department of Health Laboratories*
R. R. Colwell	*University of Maryland*
D. Hood	*University of Alaska*
T. C. Loder	*University of Alaska*
R. K. Ballentine	*Environmental Protection Agency*
L. J. Guarraia	*Environmental Protection Agency*
R. S. Kennedy	*Department of Health, Education and Welfare*
D. J. Van Donsel	*Aquatic Health Center, Alaska, EPA*
E. E. Geldreich	*Water Supply Laboratory, National Environmental Research Center, Cincinnati, Office of Research and Monitoring, EPA*

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KEYNOTE ADDRESS

ROBERT L. SANSOM

Assistant Administrator for
Air and Water Programs
Environmental Protection Agency

It is a pleasure to welcome you to this symposium on aquatic microbial transformations and their implications in water management decisions. The projected results of this symposium will be a state-of-the-art assessment of the scientific knowledge on the actions of bacteria on the cycling of carbon, nitrogen, phosphorus and sulfur in the aquatic ecosystem and the relative importance of bacterial cycling of these major elements to water quality. The proposed dialogue on the application of mathematical models to natural processes is an area that will promote beneficial discussions between the economist and the microbiologist.

We of the Environmental Protection Agency are charged with cleaning up the environment, and it's our opportunity and our fate to make far-reaching decisions, often with imperfect knowledge. Any conclusion which can be offered by this type of symposium will help us to make better water management decisions.

Subjects that you will discuss affect decisions made by EPA in at least two areas. I know that recent developments in aeration, which affect the rate of bacterial action on organic matter, have influenced the design of sewage treatment plants. These developments have reduced holding time from 6 to 8-hours to 4 hours. Obviously this has a key impact on the capacity of major components of treatment plants and thereby an impact on their cost. Cleaning up our environment will require the

building of many additional municipal treatment plants. No matter how much money we have to construct such plants, it will never be enough. The more we learn about the role of bacteria in this process, the more we're going to be able to accomplish with the money allocated to us.

The second major area is eutrophication. Understanding how quickly nutrients are recycled is one of the key determinants of the rate of eutrophication. Discussions such as you are having here today will help us to understand and possibly exert some control over the process.

I would like also to reflect briefly on a broader theme. Attendance here today is composed of members of the academic community, EPA, and other Federal agencies. We of EPA want to remain aware of developments outside the Agency in order that we may make our decisions in the light of such awareness. Keep us informed. With assistance from you of the scientific community, we will be able to make our decisions enlightened by the latest scientific knowledge and expertise available.

Interrelationship of the Major Nutrients and Micro-organism

L. J. GUARRAIA

Development of successful water management programs and program planning depends upon as complete a knowledge as possible of both the physical and biological processes working within a particular system. The turnover rates and exchange of nutrients with the sediments are in part governed by biological communities. However, before proceeding, the term "nutrients" must be defined. Definition of "nutrient" depends upon the individual involved. "Nutrients" refer to not only organic material, simple and complex, but to trace elements, vitamins, and also the major inorganic elements: carbon, nitrogen, phosphorus and sulfur.

Although widely recognized as basic agents in the transformation of all classes of nutrients, the exact role of the microbial populations in terms of their contribution to the cycling of nutrients is in part obscure. The reasons for this are many. Techniques for the definition of microbial populations, size of these populations, metabolic rates or activities of these populations *in situ*, relationship of specific genera with each other and with higher life forms are for the most part unknown. These questions have been, and are the subject of many investigations.

The principle of microorganisms in nutrient cycling and biological transformations is the subject of this symposium. Whether one recognizes the fact or not man depends basically on two independent, but not mutually exclusive, processes to cleanse his immediate environment: oxidation, both chemical and photo, and biological oxidation or degradation. The first category with respect to the environment is beyond the im-

mediate control of society. The second category, that is, biological treatment or biological processes, has been taken advantage of and is apparent in the form of oxidation ponds. Microbial processes in the aquatic environment involve the cycling, transformation and synthesis of innumerable simple and complex compounds. The attention of this symposium is focused on the four basic nutrients—carbon, nitrogen, phosphorus and sulphur.

One nutrient which has received widespread attention is phosphorus. It is known that phosphorus can be limiting to phytoplankton and other organisms. However, most of the phosphorus in the aquatic environment is bound in the sediments as an insoluble phosphate salt with availability of insoluble salts being influenced by both the physical-chemical factors and bacterial metabolism. As seen in Figure 1 loss or precipitation of phosphates to the sediments and solubilization of insoluble phosphates from the sediments and exchange among the various biologic communities, is mediated in part by the bacterial community. Three general processes involved in phosphate solubility are: the direct metabolic proc-

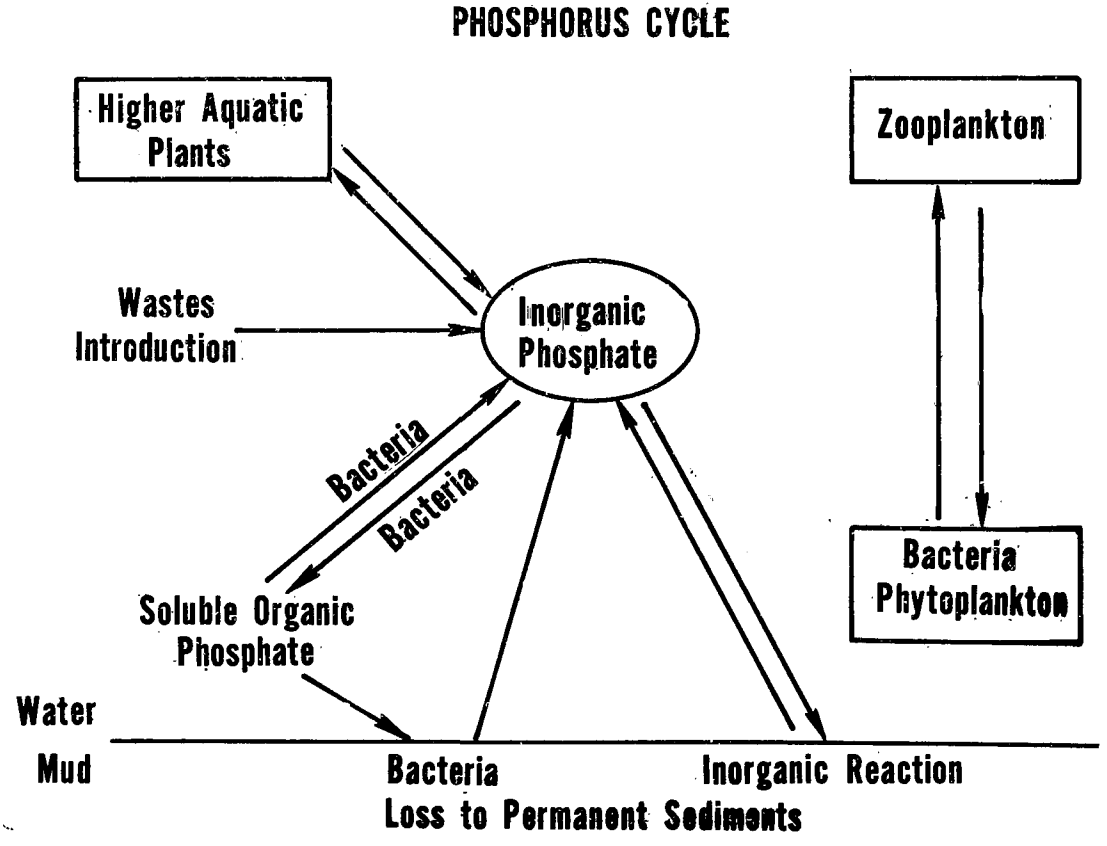

FIGURE 1.

esses involving enzymes, carbon dioxide production leading to a lower pH, and organic acid production. Inorganic phosphate is, in turn, used by higher aquatic plants—by zooplankton and phytoplankton. The microbial involvement in the phosphorus cycle will be discussed in detail later in the symposium.

As with the cycling of phosphorus, sulfur is cycled by the microbial populations in the aquatic environment. Although not widely recognized, the availability of sulfur can limit the productivity of the aquatic environment and has been linked to decreased productivity of fish.

Sulfate can be stoichiometrically reduced to hydrogen sulfide which, in turn, can be oxidized chemically, in the presence of oxygen, to elemental sulfur. Elemental sulfur, in turn, can be oxidized to sulfate.

Also the production of hydrogen sulfide by a specific class of bacteria, the anaerobic dissimilatory sulfate reducers, leads to the stoichiometric production of hydrogen sulfide and consequently an anaerobic environment. On the other side the oxidation of elemental sulfur by *Thiobacilli* leads to the production of sulfuric acid and their metabolic activity is evident in the acid mine drainage in certain areas of the country.

Biological nitrogen cycling involves, as does the cycling of sulfur and phosphorus, the transition of an elemental nutrient through various chemical states. Figure 2 is a schematic representation of the cycling of nitrogen. It is convenient to initiate the consideration of the nitrogen cycle at a point where fixation of gaseous nitrogen occurs. Relatively few species of microorganisms populating the earth are capable of metabolizing nitrogen from the air. Once fixed from the atmosphere nitrogen is converted by a relatively few species of bacteria and blue green algae to organic nitrogenous compounds.

The biochemical mechanisms involved in denitrification have only recently been elucidated in significant detail. These reactions result in the conversion of nitrate ultimately, to nitrogen gas and are apparently unique to a limited group of microorganisms.

The carbon cycle is composed of an integrated network of physically and biologically mediated pathways encompassing the synthesis, degradation, and transformation of innumerable

MICROBIAL CYCLING of INORGANIC NUTRIENTS

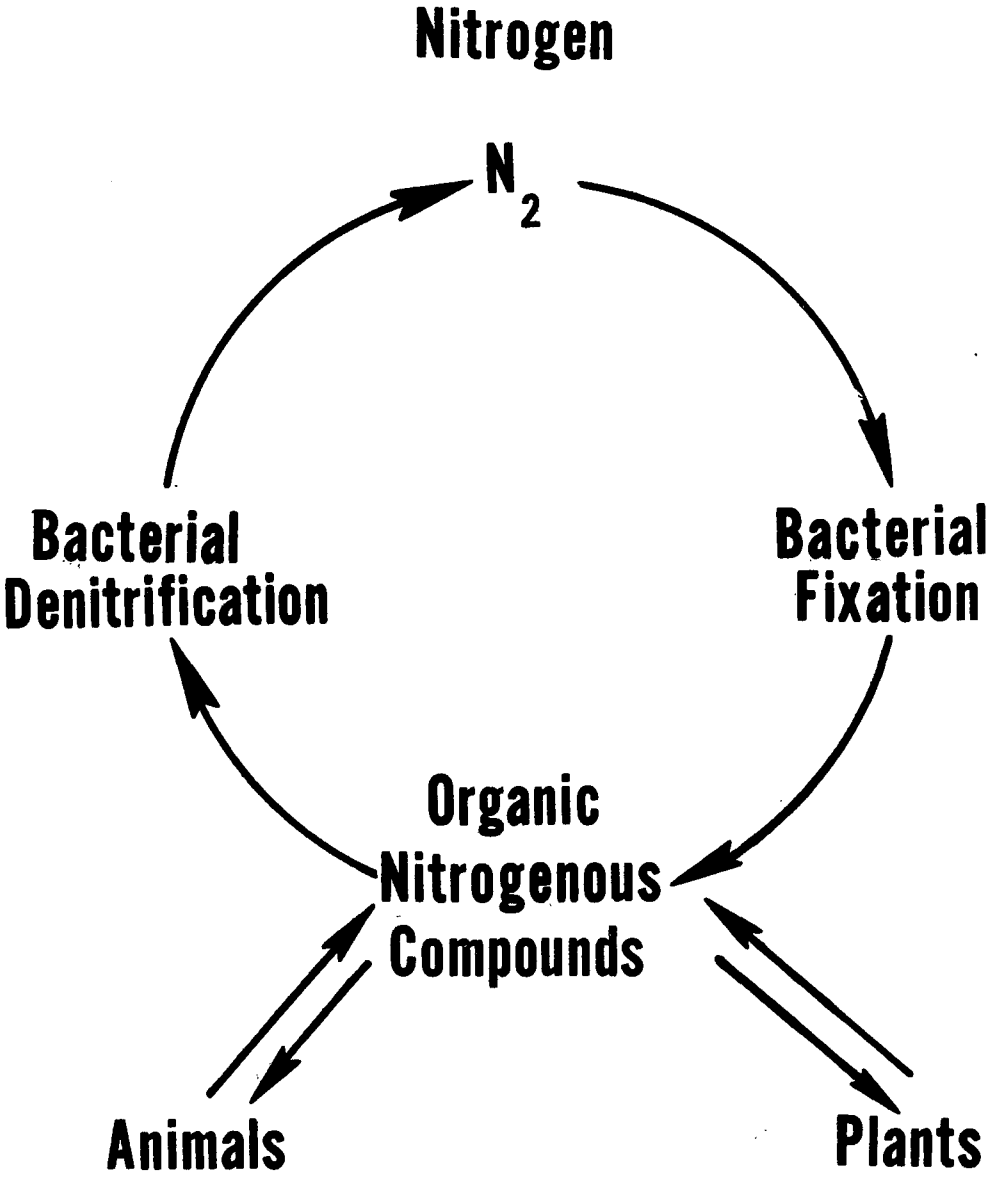

FIGURE 2.

simple and complex organic molecules. Superimposed on the carbon cycle are the controls exerted by nutrient availability, and the fixation and evolution of carbon dioxide. Various aspects of the organic carbon cycle in the aquatic environment have been examined with the emergent principle that an overall balance between the production, or synthesis, and decomposition of naturally occurring substances exists in nature. A simplified carbon cycle is seen in Figure 3. Photosynthetic carbon dioxide fixation by green plants is a major route by which carbon enters the organic carbon cycle. However, fixation

by autotrophic bacteria adds to the total carbon budget in the ecosystem. Once organic material has been introduced into the aquatic environment the endogenous flora and fauna can either utilize or contribute to, depending upon conditions, an existing reservoir of organic material. Some of the ecological questions relating to carbon that arise when considering the microbe's direct relationship to carbon cycling are: what effect does microbial synthesis of complex molecules such as vitamins, amino acids, carbohydrates, and lipids have on the aquatic biota; what is the contribution of the bacterial biomass as a food source for zooplankton; and, what is the significance of microbial degradation of suspended soluble or sedimented organic compounds?

Direct and complex relationships between diverse organisms have evolved based on the needs for various growth factors. Examples of these relationships are seen in the association of

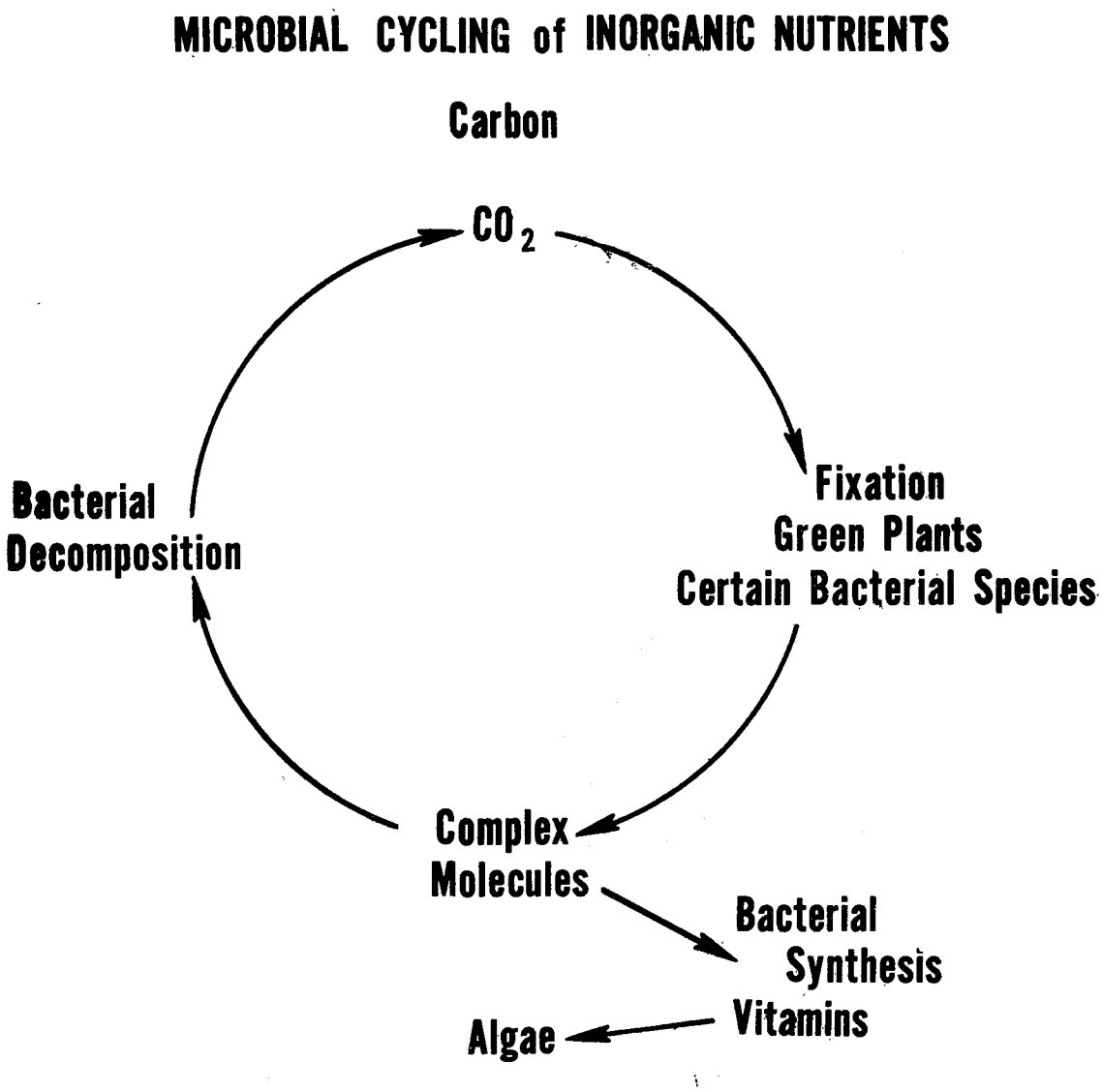

FIGURE 3.

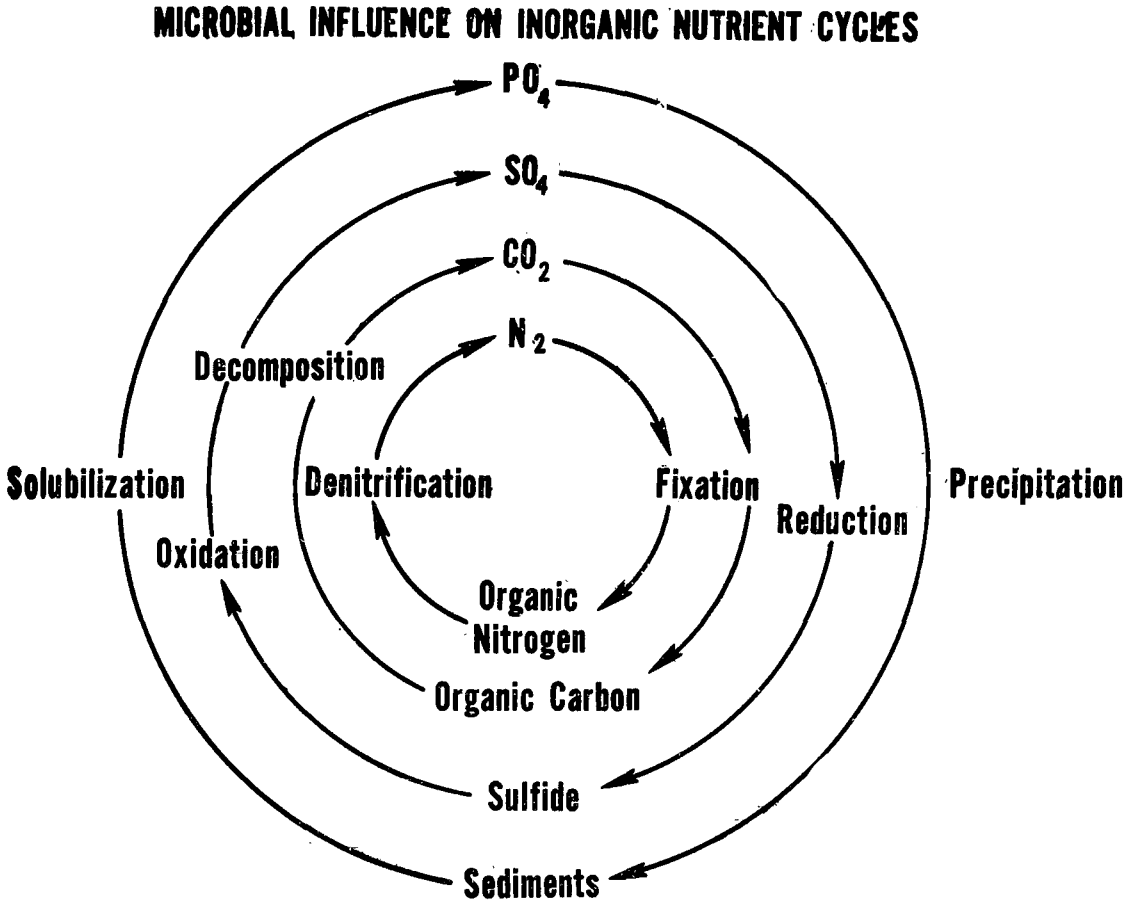

FIGURE 4.

various algae and bacteria in the marine and fresh water environments. Also the degradation of complex naturally occurring organic compounds such as chitin are affected by the microbial species.

As seen in Figure 4 microbial metabolic activity affects the cycling of the four major inorganic nutrients under consideration. The cycling of each of these nutrients—phosphorus, sulfur, carbon, and nitrogen—are interrelated in that any perturbation in one cycle has far reaching effects in the other cycles. For example, it has been shown that the sulfate reducing bacteria are capable, not only of nitrogen fixation, but degradation of carbon compounds to carbon dioxide and also to effect a solubilization of phosphate as a consequence of precipitation of insoluble iron sulfides. This is but one example. There are many examples of these interrelationships in microbial communities and it is these relationships, these rates, and these transformations which require elucidation for proper water management.

Engineering and Modeling the Microbial Influences on Nutrient Cycles

R. K. BALLENTINE

The engineering aspects section was included as part of this symposium for several reasons. The principal reason, however, was to demonstrate one type of analytical method used by engineers and others for the analysis of biological systems, that being the mathematical model, and how information developed by microbiologists is fundamental for the use of this analytical tool. Additionally, it is hoped that by becoming more aware of the scientific requirements of these analytical methods, microbiologists can produce many of the theoretical and laboratory developed coefficients necessary to allow the formulation of more realistic and complex mathematical models. Such developments ultimately will lead to a better understanding of ecological relationships in the aquatic environment.

As a brief introduction to the engineering section a few remarks about mathematical models are in order. I realize that to many in the audience mathematical models are used and developed routinely. Others, however, are not intimately familiar with these analytical tools.

Figure 1 is a schematic of a lake with various sources of nutrients which can be considered either organic or inorganic for purposes of the example. These sources include a waste treatment plant, direct discharges from residences close to the lake, drainage from agricultural lands and tributary inflow. Additionally, the outlet stream is shown which actually removes some nutrients.

Because of the input of these nutrients, a biological response will occur in the lake. Such response will generally include

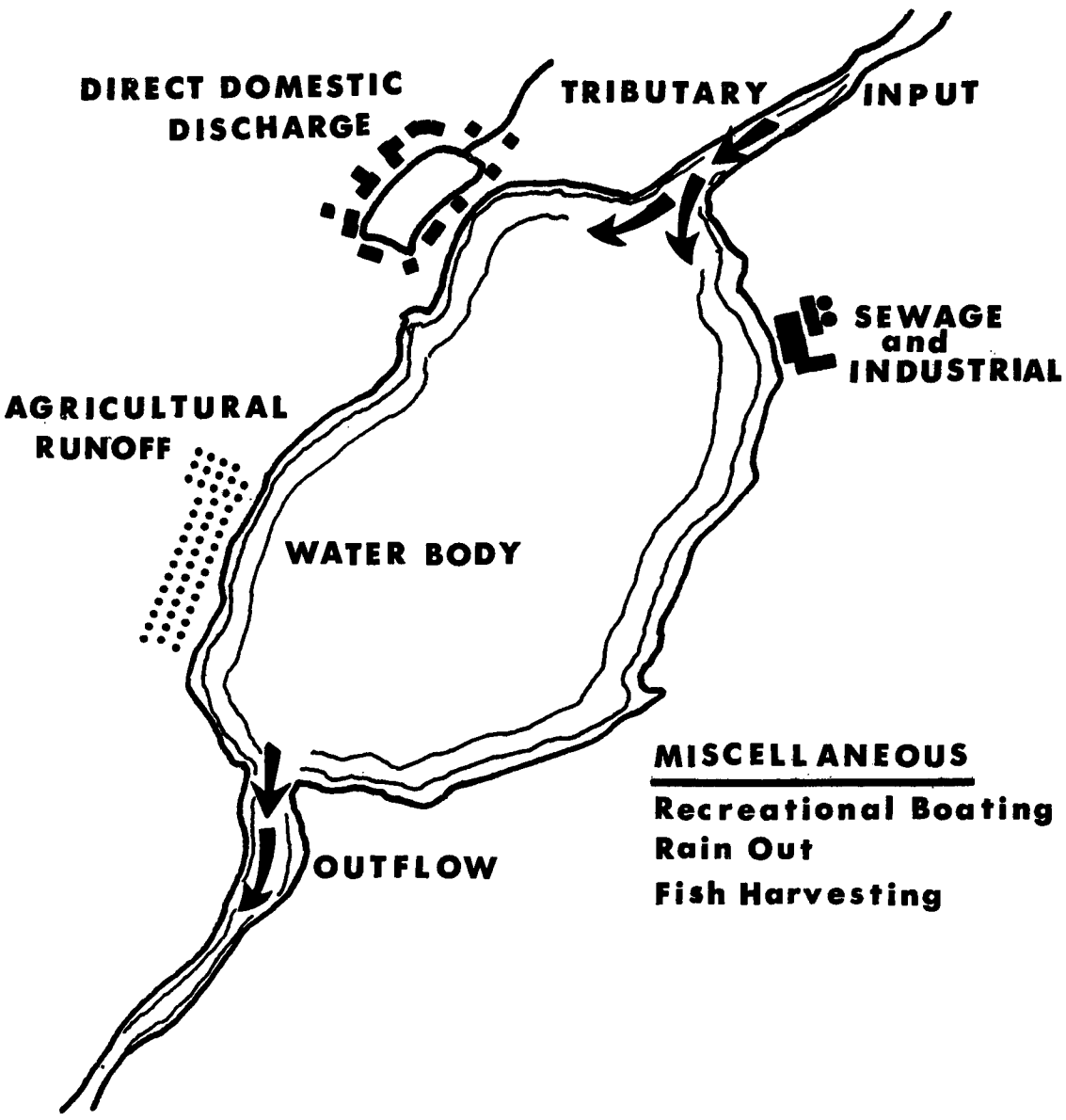

FIGURE 1.

increased microbial activity on organic nutrients and phytoplankton growth or primary production because of increased inorganic nutrients. In less than nuisance proportions these processes can result in desirable increases in productivity such as increased fish production and perhaps increases in recreational opportunity. When overloaded by either organic or inorganic nutrients the system becomes unbalanced and nuisance conditions develop (e.g. zero dissolved oxygen or algal blooms).

In an effort to avoid the development of nuisance conditions, the capacity of the lake to absorb nutrients must be described. Qualitative descriptions are insufficient for control—only a quantative description is sufficient and the concept of a mathematical description of the processes must be developed. This mathematical description is called the mathematical model.

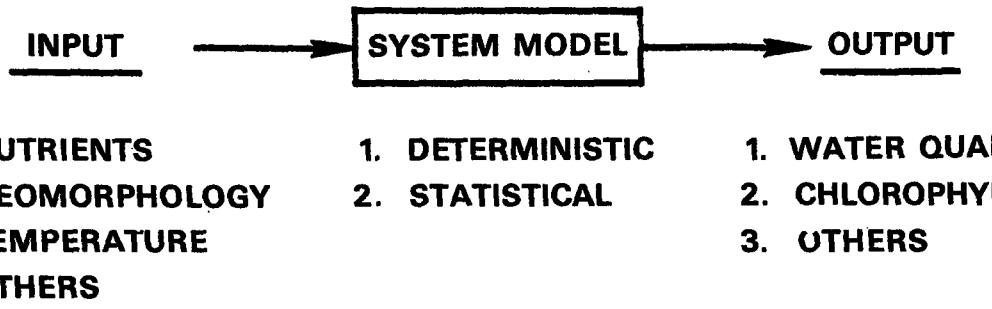

FIGURE 2.

The various biological processes themselves or the results of these processes are represented by mathematical expressions. These expressions are linked together to represent, or model, the system. These expressions and coefficients are then evaluated by field surveys and laboratory studies. This process is called calibration. The model is then tested against the prototype to see how well it describes known changes in the prototype. This process is called "verification". Once "verified" the model is used to gauge the lake or prototype response to various changes in the input variables. Figure 2 schematically represents the input–output model described.

This figure shows as inputs the nutrients and the geomorphological characteristic of a lake. The lake reaction mechanism is shown as a box in which the mathematical expressions are contained. The inputs are mathematically manipulated and the predicted output is generated. This output is water quality described by phenomena such as biological populations or chlorophyll concentrations.

The verified model can now be used to test abatement schemes including waste treatment or waste diversion in an attempt to control the adverse effects of excess nutrient addition.

Following the construction and operation of engineering structures, it is informative to follow the biological changes that actually occur and compare these to the predicted changes. In this way mathematical models can be truly verified and internal improvements incorporated. Such monitoring and validation requires as much microbiological input as does the initial formulation.

Modeling of Nutrient Cycling in Microbial Aquatic Ecosystems

Part I. Theoretical Considerations

F. H. VERHOFF, C. F. CORDEIRO, W. F. ECHELBERGER, JR.* and M. W. TENNEY*

University of Notre Dame
Department of Chemical Engineering
Notre Dame, Ind. 46556

ABSTRACT

This paper summarizes work being done on the construction of ecosystem models of nutrient cycling through the microbial populations in an aquatic environment. The principle factors to be considered are the rate of microbial reactions, i.e., the microbial growth rate, the stoichiometry of the microbial reactions, and the conservation of mass for the major microbial nutrients.

A new mechanistic model of microbial cell growth is reviewed. This model assumes that microbial cell growth occurs in two separate processes, the transport of nutrients into the cell and the conversion of these nutrients into protoplasm. The rate of transport is dependent upon the external nutrient concentrations and the conversion rate is dependent upon light and temperature. This model is compared with known experimental facts of cell growth.

A model of an aerobic aquatic environment including the cycling of carbon, oxygen, and phosphorus has been con-

* Department of Civil Engineering

structed. This model has been simulated on a yearly and a diurnal basis; qualitative comparisons between the predictions of this model and known experimental facts are made. The effect of the chemical element composition of the bacteria and the algae upon the response of the ecosystem is investigated.

A discussion of the model alterations necessary to include the nitrogen cycling and the bottom sediments is given. The stoichiometry of the bacterial and algal reactions is altered significantly with the addition of the nitrogen in its different forms. The stoichiometry and the rate of the reactions occurring in the bottom muds of deep water bodies appears to be nebulous and hence these reactions are difficult to include in the model.

INTRODUCTION

The term modeling usually implies an attempt to quantify relationships between components of a system; in this particular paper it means the quantification of the dynamic relationships among the microbial populations existing in a given aquatic environment and the nutrients that are associated with their growth. To prevent the model from becoming unwieldy, the microbial populations are grouped into large categories based upon the type of nutrient transformations accomplished by the microbe. A realistic dynamic description of the growth of these categories is a necessity, and the stoichiometry of the microbial reaction, as well as the chemical composition of the microbe, must be known. To complete the model of nutrient cycling in aquatic microbial ecosystem, the purely chemical reactions of the nutrients and the mass transfer of nutrients from the atmosphere to the water and among different aquatic environments should be included. This following discourse presents the approaches of the authors to this modeling task.

This paper summarizes a more realistic quantitative dynamic description of microbial cell growth. The cell growth process is assumed to occur via two distinct processes; (1) the transport of the nutrient from the exterior of the cell to the interior and (2) the conversion of nutrients to cell protoplasm and the subsequent cell division. The transport process is separated from other cell processes because the rate of this transport process is dependent upon external nutrient concentrations.

An aerobic water model which includes the cycling of carbon, oxygen, and phosphorus through the algal and bacterial populations is included. That the composition of the algae and bacteria (both assumed to be the same) effects the dynamics of the ecosystem is shown. The ecosystem model was used to simulate seasonal and diurnal variations of population and nutrient population.

Nitrogen in its various chemical forms is included in the last microbial ecosystem model to be presented. The division of the microbes into population categories for particular chemical transformation of nitrogen and the corresponding stoichiometry of the transformation is presented.

General ecological models have been studied since the original work of Volterra and Lotka (*3*) on the interaction of predator and prey. Since then, various types of investigations into ecological systems have been made; some of these investigations would have to be considered theoretical and others practical. The theoretical investigators (see for example Garfinkel (*9*), Smith (*25*), or Verhoff (*33*) were usually interested in mathematical properties of the system of equations which describe the ecological system. Stability, effects of density dependent factors, and number of trophic levels are among the factors studied by these authors. The practical studies were usually motivated by some ecological phenomena observed in many ecological systems (see for example Bloom (*1*) and Chen (*4*)) or by the specific ecology of a particular location (see for example, Parker (*20*)). The commonality of these papers is the attempt to simulate and predict actual concentrations of nutrients and biological organisms as a function of time and possibly position for the ecological system studied.

Chen (*4*), Parker (*20*), DiToro, O'Connor and Thoman (*7*), O'Brien and Wroblewski (*19*), and Patten (*21*) all have developed ecological models which consider the movement of nutrients through the microbial populations. However, none of these models complete the nutrient cycles, e.g., they do not consider the exchange of CO_2 on N_2 with the atmosphere, nor do any consider the nitrogen fixation capability of certain aquatic microorganisms. Parker's model follows the passage of a nutrient through the trophic levels up to the fish and O'Brien

and Wroblewski follow the cycling of a single conserved nutrient through the lower trophic levels. The O'Brien analysis is similar to that of Verhoff (*33*) except that the hydrology of the continental shelf is included.

DiToro, et al. consider the effects of phosphate and nitrate nutrients upon the dissolved oxygen of natural waters as caused by the growth of algae; the cycles of these nutrients are not completed. Patten considers the effect of these nutrients upon the growth of algae.

It is believed by the authors that the cycling of nutrients through the microbial populations plays a significant role in dynamics of water quality. Hence to determine the effects of the nutrient cycling, the complete cycle of the nutrients must be mimicked by the model. The first attempt at the construction of such a model, including the cycling of carbon, phosphorus, and oxygen, was reported recently and is summarized herein. Chen partially completed the cycling of nitrogen, phosphorus, and oxygen in a stream model.

Also, the quantitative description of the rate of microbial cell growth plays a key role in the dynamics of nutrient cycling (and hence water quality) in aquatic ecosystems. All of the above investigators use a Monod-type expression to relate the growth rate of microbial organisms with the nutrient concentrations in the natural water. To account for two limiting nutrients, all investigations use a multiplication of Monod factors. This Monod equation is used despite the fact that it does not represent the dynamics of microbial cell growth (*30*). Although DiToro et al. justify the multiplication of Monod factors by using data taken for one nutrient limiting at a time. It is known that this multiplication does not express growth rate when two nutrients are limiting growth at the same time (*6*). This paper then includes a synopsis of the work done by the authors on microbial cell growth (*35*).

Microbial Cell Growth

The principal mathematical expression used to relate rate of microbial cell growth to limiting substrate concentration is the Monod equation (*18*). It is recognized that there are many

cases in which observed values of cell concentration do not correspond to those predicted by this equation in batch cultivation. In particular, this expression does not predict the lag phase, nor does it predict the endogenous phase of batch cultivation. In continuous cultivation, this equation rarely predicts the experimental results partly because the yield of cell mass from limiting substrate is not constant (see Herbert (*12*, *13*)). Still other phenomena such as that involved in the biosorption process of sewage and waste treatment (*31*) are totally unexplained by this equation of microbial cell growth.

Other authors have developed different empirical expressions relating cell growth rate to limiting substrate concentration, e.g., Teissier's (*28*) exponential expression for growth rate, and the two phase proposal of Garret and Sawyer (*10*). Kono (*16*) developed a model for the case of batch cultivation taking into account four phases of growth. He bases his model on a similarity to chemical kinetics and introduces the new concepts of critical concentration and the coefficient of consumption activity. He applies the model to various available data and finds the fit to be good with proper choice of parameters. Other authors (e.g., Ierusalimskii (*15*) and Contois (*5*)) worked on cell concentration effect on growth rate. Still others (e.g., Powell (*23*)) modified Monod's equation. In particular, Powell included the effects of mass transfer in the Monod growth model. He compared his growth model with that of Monod and with that of Teissier and found that his model fitted the experimental data better than the others. Several of the above authors, as well as others, also worked on the problems of yield and endogenous respiration; some have corrected their models for this latter effect. An excellent review of the application of these models is given by Gaudy (*11*).

The important point to note about the above models is that they are primarily empirical, i.e., the experiment data were fit with an equational form. None of the authors propose a mechanism for growth because it is always assumed that the metabolic processes are quite complex, involving a variety of rate laws, and that these processes are among the rate determining steps for growth. Although, there is extensive knowledge about the mode of growth (the sequence of processes involved in cell

growth), none of the authors use this information in developing a model.

The fact that such diverse organisms as bacteria, yeasts, and even molds (see Pirt and Callow (*22*) for mold growth) exhibit the same growth dependency seems to indicate that the metabolic processes do not play a major role in the growth rate dependency on limiting substrate. In the authors' opinion, only a few facts about the metabolic activity of the cell are required along with a knowledge of the mode of growth in order to explain the growth rate—limiting substrate dependency.

In this paper, a mechanism based upon the mode of growth is proposed by the authors. We assume that every cell is either growing (however slowly) or it is lysed. The mode of growth assumed in this model supposes that the cell growth occurs in a two-step manner; the first step involves the accumulation and possible loss of the limiting substrate by the organism and the second step involves the ingestion of the limiting substrate by the organism and possibly a subsequent cell division. This mechanism suggests that the microbial population should be divided into two categories as well as it suggests certain mathematical forms for the rate of each step. These rates are then combined to predict the microbial growth rate as a function of limiting concentration. This mechanism along with a simple mechanism for microbial lysis are applied to the continuous stirred tank reactor (chemostat) and to the batch reactor. The relationship of organism and substrate concentration to such variables as flow rate and input substrate concentration for the continuous culture case are developed. The variation of yield with flow rate is also shown. In batch culture, this mechanism predicts the lag phase through to the endogenous phase.

Mechanism of Microbial Cell Growth

For a simplified version of the cell growth process, we might consider a viable cell in a growing culture just after a cell division. This cell selectively assimilates or adsorbs the nutrients from the culture medium. The cell reaction systems then convert the nutrients which have been assimulated into protoplasmic material characteristic of the particular organism. This process of assimilation followed by conversion may occur once

or several times between cell divisions; the theory to be developed will be the same for all cases with the change of one parameter. After some chemical rearrangement of the protoplasmic material and the production of an increased amount of nuclear substance, the cell ultimately divides producing two new viable cells which will repeat the process.

This process can be put into symbolic form to represent the stoichiometry (conservation of mass) as shown below.

$$A+\eta S_s \underset{r_2}{\overset{r_1}{\rightleftarrows}} B \xrightarrow{r_3} (1+\epsilon)A+\rho_2(\eta-\epsilon)S_s. \tag{1}$$

This symbolism in words says that a viable cell just after cell division, A, combines with η amount of limiting substrate, S_s, to form an ingesting cell, B. Since we will be primarily dealing with mass units, we will define the mechanism as one mass unit of A combines with a fraction η of mass units of S_s to form $1+\eta$ mass units of B. One mass unit of B then can undergo one of two processes; it could release the assimilated nutrients and revert back to its original form of A and S_s, or it could ingest the substrate and produce $1+\epsilon$ units mass of A. This ingestion could include cell division; if cell division always accompanies ingestion as is probably the case with some bacteria, then ϵ is approximately equal to one. During the ingestion and possible cell division, some of the substrate may be returned to the medium for use again by other cells; thus the weight ρ_2 $(\eta-\epsilon)$ represents the weight of substrate returned to the medium. Since ρ_2 is normally considered to be near zero without changing the mechanism, it could be assumed that part or most of the ingestion occurs during what has been described as the assimilation step.

From the symbolic form (equation 1) it can be seen that the rate of growth will depend upon the three rate processes r_1, r_2, and r_3 indicated. For the development of the rate functions, we will consider A, S_s and B to be mass concentration, i.e., mass per unit volume of solution. The rate of absorption of nutrient per unit volume of solution r_1 should be linearly proportional to A with all other quantities held constant, i.e., a larger mass of cells per unit volume of solution will give a proportionally larger rate of absorption per unit volume of solution. Also, we

would expect the absorption process to be mass transfer limited, either in the cell membrane or in a liquid film around the cell. With all other quantities held constant, the rate of absorption per unit volume is proportional to the difference between the substrate concentration in solution, S_s, and some low substrate concentration, S_c, maintained by the cell. Combining these two facts, the rate of absorption per unit volume can be written as the following equation.

$$r_1 = k_1 A S,$$

where

$$S = S_s - S_c.$$

Because S_c is very small, S is approximately equal to S_s. For all of the analysis to follow in this paper, we will assume that k_1 is independent of all substances whose concentrations change during growth.

The rate loss of limiting substrate per unit volume by ingesting cells B (i.e., rate r_2) will be proportional to the concentration of B in the solution assuming all other quantities are constant. Again we will assume in the subsequent analysis that k_2 in the following law expressing this relationship is independent of all other changing substances in the solution.

$$r_2 = k_2 B.$$

The rate at which B ingests substrate or ingests substrate and divides is assumed proportional to B with all the other quantities held constant. It is also assumed that all other quantities that change during an experiment do not affect the ingestion rate; in particular, it is assumed that the ingestion rate is independent of the concentration of limiting substrate in the solution. The rate of ingestion and cell division per unit volume is then

$$r_3 = k_3 B.$$

Growth Rate Predicted by Mechanism

To develop mathematical expressions for microbial cell growth rate dependencies, we will assume that a certain mass of cells are placed in a media with a constant concentration of limiting substrate. No cells are assumed to lyse. The equations that

express the rate of change of concentration of the absorbing cells, A, and of the ingesting cells, B, are given below:

$$\frac{dA}{dt} = -k_1 AS + \frac{1+\epsilon}{1+\eta} k_3 B + \frac{k_2}{1+\eta} B. \tag{2}$$

$$\frac{dB}{dt} = (1+\eta) k_1 AS - k_3 B - k_2 B. \tag{3}$$

Associated with these equations are the initial conditions:

$$A = A_1$$
$$@\ t = 0.$$
$$B = B_0$$

Since S is constant, equations (2) and (3) are a linear set of differential equations.

Specific growth rate, the quantity of interest, is given by the following formula.

$$= \frac{1}{A+B} \frac{d(A+B)}{dt}.$$

Solving for the largest eigenvalue gives for the specific growth rate

$$\mu = \frac{-(k_1 S + k_2 + k_3) + [(k_1 S + k_2 + k_3)^2 + 4\epsilon k_1 k_3 S]^{1/2}}{2}.$$

This equation can be reduced such that there are three groups of numbers to be plotted; two on the axes of the graph and one as a parameter. The form suitable for such plotting is

$$\frac{\mu}{k_3 \epsilon} = -\left(\frac{k_1 S}{2k_3 \epsilon} + \frac{k_2 + k_3}{2k_3 \epsilon}\right) + \left[\left(\frac{k_2 S}{2k_3 \epsilon} + \frac{k_2 + k_3}{2k_3 \epsilon}\right)^2 + \frac{k_1 S}{k_3 \epsilon}\right]^{1/2} \tag{4}$$

Figure 1 shows a plot of equation (4) with $(k_2 + k_3)/\epsilon k_3$ as the parameter. The shapes of these curves closely resemble those of the Monod equation.

For larger values of substrate concentration, the specific mass growth rate approaches the rate of ingestion and cell division times the weight fraction of substrate converted. From a biological point of view, this would be the expected limiting growth rate.

In order to see the effect of various parameters, we will

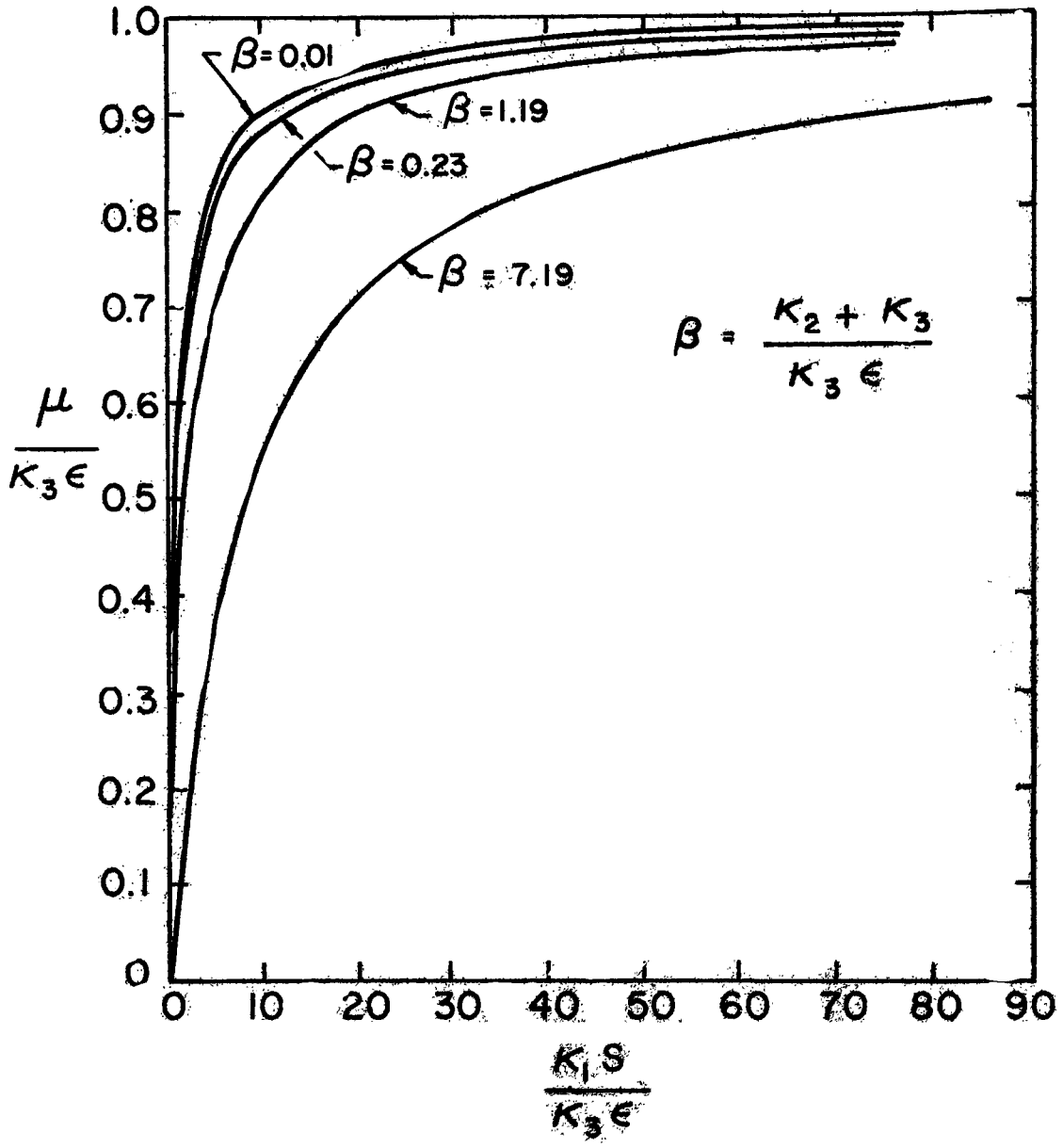

FIGURE 1.—Dimensionless growth rate vs dimensionless limiting nutrient concentration.

investigate various limiting cases of them. Taking the limit as k_1 gets extremely large, the specific growth rate is a constant value for all but the very lowest values of substrate concentration S. This would be expected because in this case, the rate limiting step is the ingestion and cell division.

For the case of k_3 approaching zero (equivalent to k_1 and k_2 getting large), it can be shown that

$$\frac{\mu}{\epsilon k_3} = \frac{S}{\frac{k_2 + k_3}{k_1} + S}$$

which is the Monod equation for limiting substrate. This equation would be expected for this case because it closely corresponds to the equilibrium assumptions involved in the development of the Michaelis-Menton equation for enzymatic reactions. In this case, the reaction rates r_1 and r_2 are approximately in equilibrium with r_3 being much slower. Figure 2 shows the relationship between steady state growth from this mechanism and that of Monod. It can be seen that there is very little difference in the steady state characteristics for one substrate.

To modify the above description of microbial cell growth for two limiting nutrients is very simple since the only modification is in the transport process. A multiplication of the concentration of the two limiting nutrients was used in the ecological models discussed in this paper, although this is not the true dependency as can be obtained from the microbiological literature. Work is progressing on an improvement of transport quantification along the lines of a recent publication (*34*).

Also, the dynamics of this model as operating in a chemostat were investigated (*36*). This model suggested that under most circumstances the response of this system should be non-

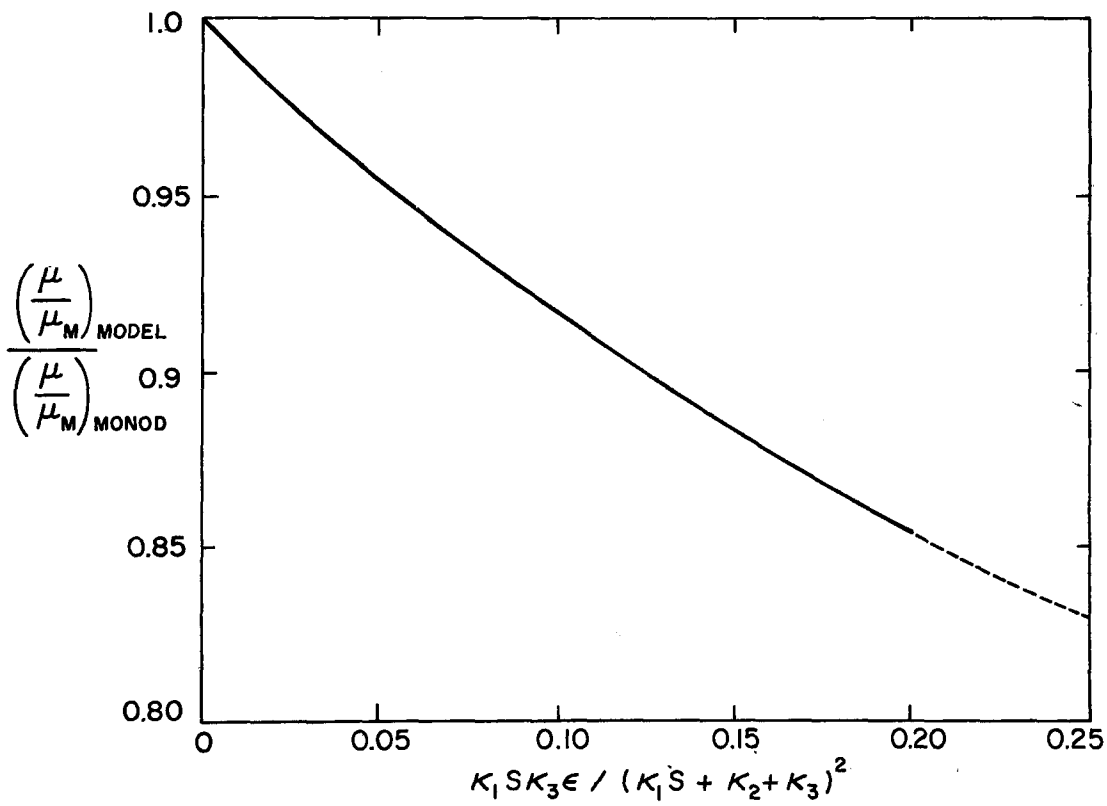

FIGURE 2.—Comparison of model growth rate with rate from Monod's equation as a function of the dimensionless parameter.

oscillatory. However, under certain conditions, oscillation was predicted by the model; under similar experimental conditions, these oscillations also were observed.

Application of Model to Aerobic Waters

SEASONAL VARIATIONS

Since the use of system modeling for prediction of water quality is a recent development, much is unknown about the effects of various system parameters on the dynamics of such systems and hence many areas are available for research. A cursory search of the literature indicates that the empirical composition of algae and bacteria is not well known and thus the stoichiometric constants which describe the composition of these organisms are among the system parameters whose effect upon the lake dynamics needs to be investigated. This study presents the formulation of an aquatic microbial model and the results of a sensitivity analysis of these stoichiometric constants by the simulation of a simplified model of the surface waters of a lake.

The simplified model including only the surface waters (i.e., the algae and bacteria in the aerobic zone) rather than the model which also includes the anaerobic bottom muds was chosen for this study for two reasons: (1) the stoichiometry of the reactions in the bottom muds is almost totally unknown, and (2) the inclusion of the bottom muds only complicates the investigation of the composition of algae and bacteria in the upper waters. The model to be simulated in this paper also applies directly to any lake which never develops an anaerobic lower water layer.

Stumm (*27*), Redfield (*24*), and Eckenfelder (*8*) suggest that most mixed algal cultures common in surface waters and mixed bacterial cultures as found in waste treatment processes, have an average stoichiometric relation between carbon, nitrogen, and phosphorus of about 106 to 16 to 1 atoms. They propose the chemical formula for such algae and bacteria to be $C_{106}H_{180}O_{45}N_{16}P$. This is one of the chemical formulas we will use for the composition of bacteria and algae. McCarty (*17*),

Speece (*26*), Hoover (*14*), and Burkhead (*2*) conclude that a typical empirical formulation for bacterial cells is $C_5H_7O_2N$. Extrapolating this to the same nitrogen to phosphorus ratio (16 to 1) as that proposed by Stumm (*27*) and others cited above gives the formula $C_{80}H_{112}O_{32}N_{16}P$ for the bacterial and algal cells. Extrapolating this formulation to the carbon to phosphorus ratio (approximately 105 to 1) gives yet another empirical formula $C_{105}H_{145}O_{42}N_{21}P$ for the composition of these living cells. Various other formulations as suggested by these other authors were used in the model of this paper.

The purpose of this modeling venture is to simulate the interactions of microorganisms and nutrient cycling which affect water quality. Water quality can be defined in different ways, and in this instance, we are primarily focusing on the algae. Included in this model must be all quantities thought important in the growth of algae and all factors affecting these quantities. The final model will then be essentially closed with only external influences, such as weather and stream flows (set equal to zero) serving as inputs to the system.

Essentially a model of the water quality of aerobic waters involves the nutrient cycling between components in these waters and the influence of weather on these components. Each cell of bacteria and algae cannot be considered as a particular component (nor can each species of bacteria or algae for that matter) of the system because the size of the system to be simulated would be prohibitive. Hence the components of the system must be a lumped group of organisms. In the model used in this paper, all algae are included as one component (see Chen (*4*) for a division of the algae) and all bacteria as another. The other chemical components of the system include the HCO_3^-, $H_2PO_4^-$, O_2, carbonaceous material with phosphorus included and the carbonaceous material without phosphorus. These components and the flow between components are shown in Figure 3.

The bacteria are assumed to have the same composition as the carbonaceous material with phosphorus included. Thus when bacteria or algae lyse the products enter this component. If a different composition had been chosen for the algae and bacteria, two components of carbonaceous material with phos-

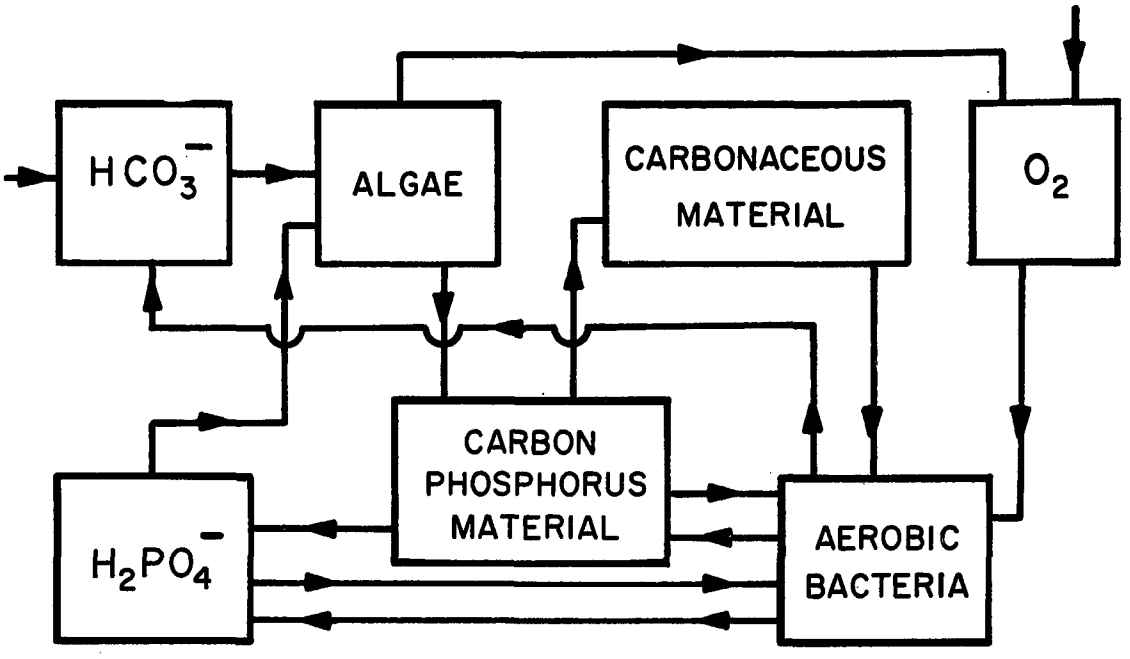

FIGURE 3.—Flow diagram of components in the surface water model.

phorus included would be necessary. The carbonaceous material without phosphorus has the same composition as the bacteria and algae with a $H_2PO_4^-$ removed. The requirements of algae for carbon and phosphorus were assumed to be supplied by HCO_3^- and $H_2PO_4^-$ respectively; these choices were then used in the stoichiometric equations. This division of components might appear rather simplistic, however, it is required to make the problem tractable.

Kinetics Used in Model

There are essentially four types of transfers of nutrients in a lake ecological system: (1) convective, i.e., hydrodynamic, (2) diffusive, i.e., simple diffusion of substances, (3) chemical reaction, i.e., spontaneous reactions occurring in the lake possibly mediated by radiation, and (4) complex biological reactions, i.e., those involved in bacteria and algae. The kinetics of the first three types of transfer are understood to some extent and reasonable transfer rate functions can be formulated. Since hydrology has been neglected, no transfer rates are required for this process (see Tenney (*29*) for hydrological considerations). There are two diffusive transfer mechanisms necessary for this model; the oxygen transfer between the water and air and the similar transfer of CO_2 or HCO_3^-. The rate of transfer per unit volume of shallow lake is then given by the following

formulae assuming that the main resistance to mass transfer is in the water.

$$r_8 = k_8(Y_{eq \cdot HCO_3^-} - Y_{HCO_3^-})$$

$$r_9 = k_9(Y_{eq \cdot O_2} - Y_{O_2})$$

where

r_8 = mass accumulation of HCO_3^- per unit volume of lake per unit time.

r_9 = mass accumulation of O_2 per unit volume of lake per unit time.

k_8 = mass transfer coefficient for HCO_3^-. This constant varies with time due to lake overturn.

k_9 = mass transfer coefficient for O_2. This constant varies with time due to lake overturn.

$Y_{eq HCO_3^-}$ = water mass concentration of HCO_3^- in equilibrium with the air.

$Y_{eq \cdot O_2}$ = water mass concentration of O_2 in equilibrium with the air.

$Y_{HCO_3^-}$ = water mass concentration of HCO_3^-.

Y_{O_2} = water mass concentration of O_2.

There is assumed to be one homogeneous chemical reaction occurring in the water; the carbon-phosphorus material decomposes into carbonaceous material and phosphate $H_2PO_4^-$. This reaction is assumed to be first order, i.e., the rate of the reaction is given by the following formula.

$$r_7 = k_7 Y_{CP}$$

where

r_7 = mass of carbon-phosphorus material reacted per unit time per unit volume of lake.

k_7 = first order reaction constant.

Y_{CP} = mass concentration of carbon-phosphorus material in the lake.

The algae and bacteria kinetics are somewhat more complicated since a dynamic description of microbial cell growth as discussed previously was used. This mechanism essentially divides the algal and bacterial masses into two parts, the absorbing part and the ingesting part.

The rate functions describing the absorption of nutrients from

the waters are then described by the following formulae written in terms of mass changes in A_2 and H_2 respectively.

$$r_1 = K_1 Y_{A_1} Y_{H_2PO_4^-} Y_{HCO_3^-}$$

$$r_4 = k_4 Y_{H_1} Y_{O_2} (Y_C + N Y_{CP})$$

where

r_1 = mass of A_2 formed per unit time per unit volume of lake.

r_4 = mass of H_2 formed per unit time per unit volume of lake.

Y_{A_1} = mass concentration of algal cells A_1.

Y_{H_1} = mass concentration of bacterial cells H_1.

$Y_{H_2PO_4^-}$ = mass concentration of $H_2PO_4^-$.

Y_C = mass concentration of carbonaceous material.

N = preference factor for carbon-phosphorus material over carbonaceous material.

k_1 = rate constant.

k_4 = rate constant.

This formulation of the absorption step is very empirical; however, work is progressing on a more realistic approach using facilitated and active transport. The disappearance of A_2 and H_2 by ingestion is assumed to be first order in mass concentrations of A_2 and H_2. Note that this is equivalent to zero order reaction inside the algae and bacteria; such zero order reactions are known to occur, e.g., the replication of DNA.

$$r_2 = k_2 Y_{A_2}$$

$$r_5 = k_5 Y_{H_2}$$

where

r_2 = mass of A_2 disappearing per unit time per unit volume of lake.

r_5 = mass of H_2 disappearing per unit time per unit volume of lake.

Y_{A_2} = mass concentration of algae A_2.

Y_{H_2} = mass concentration of bacteria H_2.

k_5 = first order rate constant; this constant is a function of time due to light and temperature change.

Also the lysing rates of the algae and bacteria have to be

specified; for this the usual first order law is used as shown below.

$$r_3 = k_3 Y_{A_1}$$
$$r_3' = k_3 Y_{A_2}$$
$$r_6 = k_6 Y_{H_1}$$
$$r_6' = k_6 Y_{H_2}$$

In these equations r_3, r_3', r_6, and r_6' represent the disappearance of mass per unit time per unit volume of lake for A_1, A_2, H_1, and H_2 respectively. The first order rate constants for these processes are k_3, k_3, k_6, and k_6 respectively. The lysing constant for A_1 and A_2 (similarly H_1 and H_2) could have been made different, but for the purposes of this presentation, the assumption that they are the same will suffice.

The rates of transfer of nutrients throughout the lake ecological system is then described by nine rate constants. However, before the model is complete, the stoichiometry of these interactions must be specified.

Stoichiometry Used in Model

Stoichiometric relationships are required for the absorption, ingestion, and lysing processes of the bacteria and the decomposition of the carbon-phosphorus material. If the composition of the algae, the bacteria, and the carbon-phosphorus material is assumed to be given by $C_aH_bO_cN_dP$, then stoichiometry associated with the decomposition of carbon-phosphorus material is given by the following formula

$$C_aH_bO_cN_dP \rightarrow C_aH_{b-2}O_{c-4}N_d + H_2PO_4^-$$

The absorption stoichiometry for the algae and bacteria respectively is as follows.

$$(C_aH_bO_cN_dP)_{A_1} + \eta_1[aHCO_3^- + dNO_3^- + H_2PO_4^- + ((b-a-2)/2)H_2O] \rightarrow (C_aH_bO_cN_dP + \eta_1[\quad])_{A_2}(C_aH_bO_cN_d)_{H_1} + (\epsilon_2 + (\eta_2 - \epsilon_2)S_2)\begin{bmatrix}(C_aH_bO_cN_dP)_{CP} \\ (C_aH_{b-2}O_{c-4}N_d)_{CC} + H_2PO_4^-\end{bmatrix} + (\eta_2 - \epsilon_2)(1 - S_2)\left[\left(3a + 3d + 4 + \frac{b-a-2}{2} - c\right)/2O_2\right] \rightarrow (C_aH_bO_cN_dP +)_{H_2}$$

These stoichiometric relations are essentially mass balances showing the mass of nutrients which combine with a unit mass of A_1 and H_1 respectively to form A_2 and H_2. Note that the stoichiometry for the carbonaceous form of bacterial substrate is quite similar to that of the carbon-phosphorus form.

The ingestion stoichiometry for algae and bacteria respectively is then given by the following expressions.

$$(C_aH_bO_cN_dP + \eta_1[\quad])_{A_2} \rightarrow (1+\epsilon_1)(C_aH_bO_cN_dP)_{A_1} \tag{5}$$
$$+(1-S_1)\eta_1(3a+3d+4+(b-a-2)/2)/2O_2$$

$$(C_aH_bO_cN_dP+)_{H_2} \rightarrow (1+\epsilon_2)(C_aH_bO_cN_dP)_{H_1} \tag{6}$$
$$+(\eta_2-\epsilon_2)[aHCO_3^- + dNO_3^- + H_2PO_4^- + ((b-a-2)/2)H_2O]$$

The ingestion reactions given for the algae and bacteria are not reactions, i.e., the separate production and energy consuming reactions are not presented for the algae. For the bacteria part of the substrate is used for synthesis and part is oxidized.

The overall reaction for both the algae and bacteria is given in the next chemical equation.

$$aHCO_3^- + dNO_3^- + H_2PO_4^- + [(b-a-2)/2]H_2O \rightleftarrows C_aH_bO_cN_dP$$
$$+[3a+3d+4+(b-a-2)/2-c]O_2$$

The algae uses, of course, the forward reaction and the bacteria the reverse.

The lysing of A_1 and H_1 is assumed to produce pure carbon-phosphorus material and the lysing of A_2 and H_2 is assumed to follow the same stoichiometry as equations (5) and (6) except the produced $C_aH_bO_cN_dP$ enters the carbon-phosphorus pool instead of the A_1 and H_1 pools respectively.

Differential Equations of Model

Mass balances are performed for a volume of the surface waters resulting in nine differential equations to be simulated.

These equations were simulated with the following rate constants.

$$k_1 = 45.0$$

$$k_2 = 0.3(0.525 + 0.475 \sin\phi)$$

$$k_3 = 0.015$$

$$k_4 = 0.03$$

$$k_5 = 0.5(0.55 + 0.45 \sin\phi)$$

$$k_6 = 0.1$$

$$k_7 = 0.05$$

$$k_8 = 1.0(0.55 - 0.45 \sin\phi)$$

$$k_9 = 0.2(0.55 - 0.45 \sin\phi)$$

$$\eta_1 = 1.5$$

$$\eta_2 = 1.0$$

$$\epsilon_2 = 1.5$$

$$N = 2.0$$

$$Y_{eq \cdot HCO_3^-} = 0.44$$

$$Y_{eq \cdot O_2} = 9.1$$

where

$$\phi = 6.283/2t/360 \qquad t = \text{days}$$

From experimentation estimates can be obtained for constants k_2, k_3, k_5, k_6, k_8, and k_9. The physical meaning of k_2 and k_5 are explained by Verhoff (*35*) and the values given above are reasonable values. Values for the other four rate constants are in the same range as used by Chen (*4*). Constants k_1, k_4, and k_7 were obtained by intuitive guesses along with the four constants η_1, η_2, ϵ_2, and N. The stoichiometric coefficients in the composition of bacteria, algae, and carbon-phosphorus material were varied in the simulation.

Results

The differential equations were programmed for the computer using the Runge-Kutta subroutine in the IBM Scientific Subroutine Package. The simulation was performed until a steady curve was reached; the criteria used to determine if transients had damped out was that the integral over the year of the deviation of the oxygen and bicarbonate from the equilibrium value was zero. This says that at a steady state for the model assumed the net transfer of oxygen and carbonate over a year interval should be zero. Various integration step sizes were

tested and the longest possible step size that could be used without significant error was one day. The three values for the organic composition of algae, bacteria, and carbon-phosphorus material used in the simulation are shown on the plots of the concentrations as functions of time. All three substances have the same composition in a simulation.

Figure 4 is a plot of the algae concentration in mg/l throughout the year. The algae peak occurs around October 1 which might be somewhat late, but other variations of k_2 as a function of time probably would be more realistic and give a peak nearer the measured value. The choice of the algae composition causes up to 50% change during certain times of the year and also causes changes in the shapes of the curves.

The effect of organic composition on the bacterial curves is very small as can be seen in Figure 5. The bacterial concentration are much higher during the summer season as would be expected.

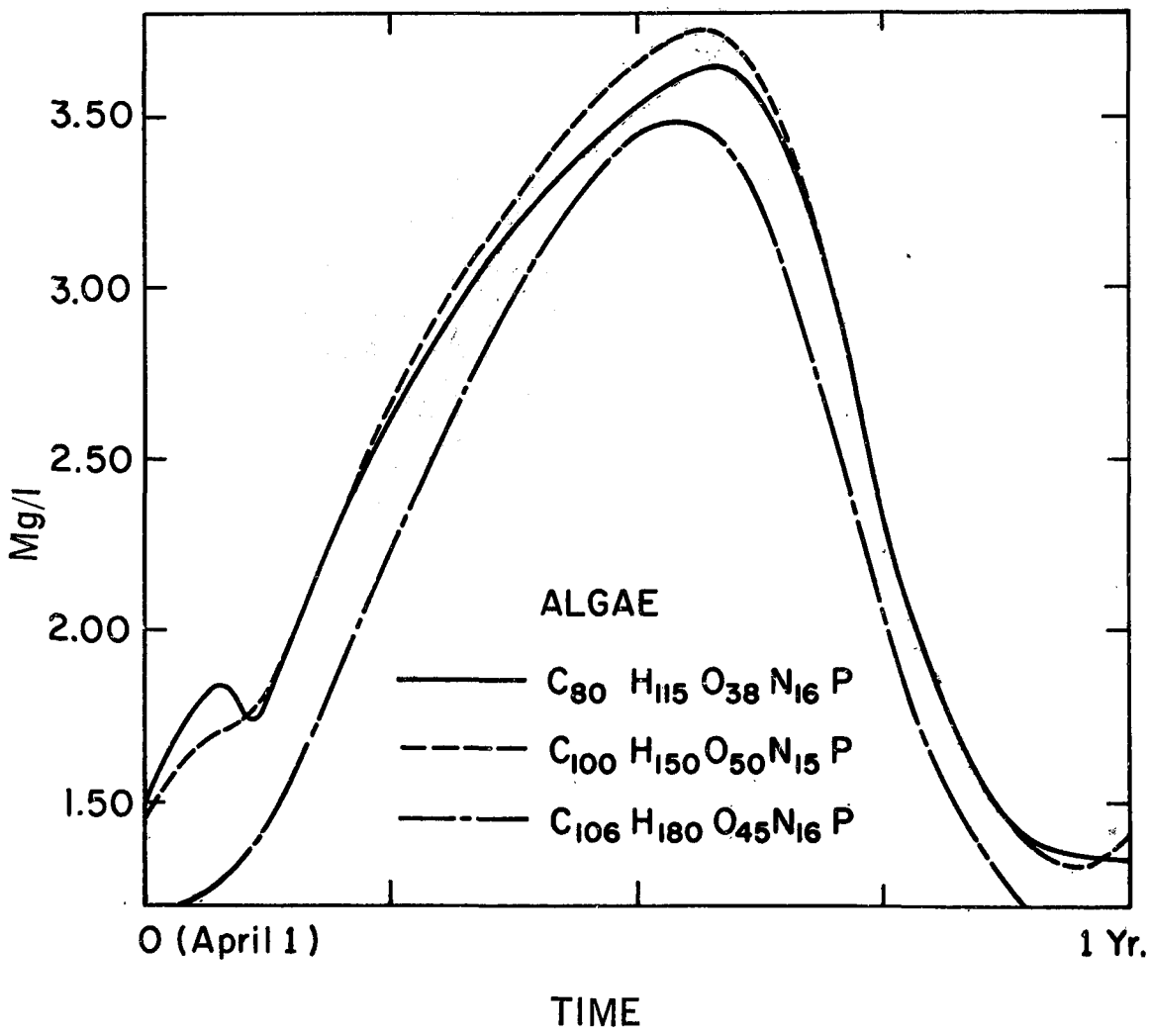

FIGURE 4.—Modeled seasonal variation of algal concentration.

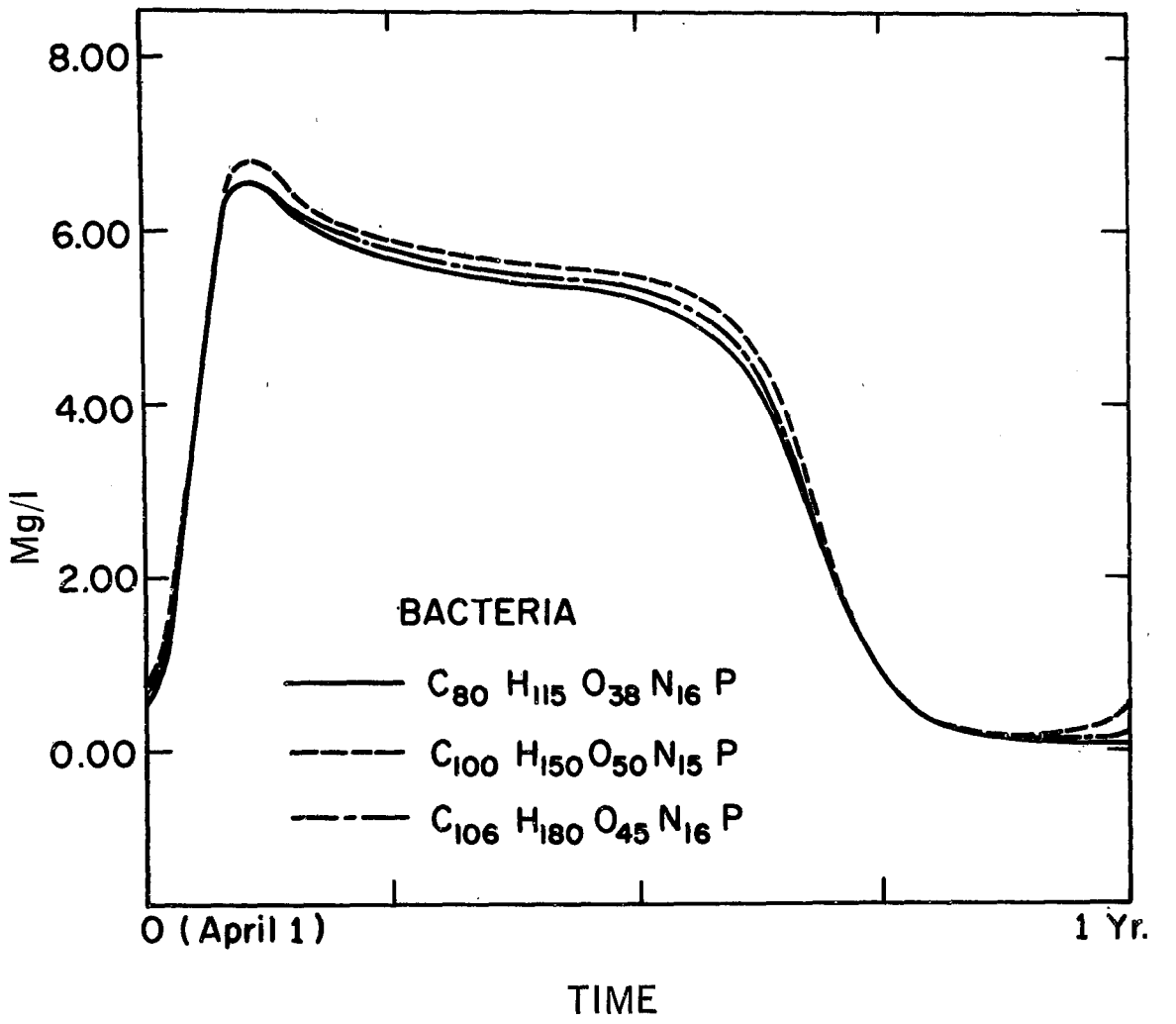

FIGURE 5.—Modeled seasonal variation of bacterial concentration.

The bicarbonate concentration varies significantly throughout the year. During the spring, CO_2 is transferred out of the water, and it is absorbed by the water during the rest of the year. The effect of organic composition on the time variation of bicarbonate is small except at the beginning of summer when the effect is about ten percent. Figure 6 shows the bicarbonate functionality.

The phosphate variation, shown in Figure 7, exhibits the low value during the summer months as characteristic of surface waters. The effect of organic composition on phosphate concentration is as large as 15% during winter.

The carbon-phosphorus material shows two peaks, one in early spring and the second much larger in late fall. Since it is rather difficult to estimate the spontaneous decomposition rate of this material, these peaks could be much smaller if the rate were higher. As seen in Figure 8, the organic composition in the ecological system has a small effect on the carbon-phosphorus material.

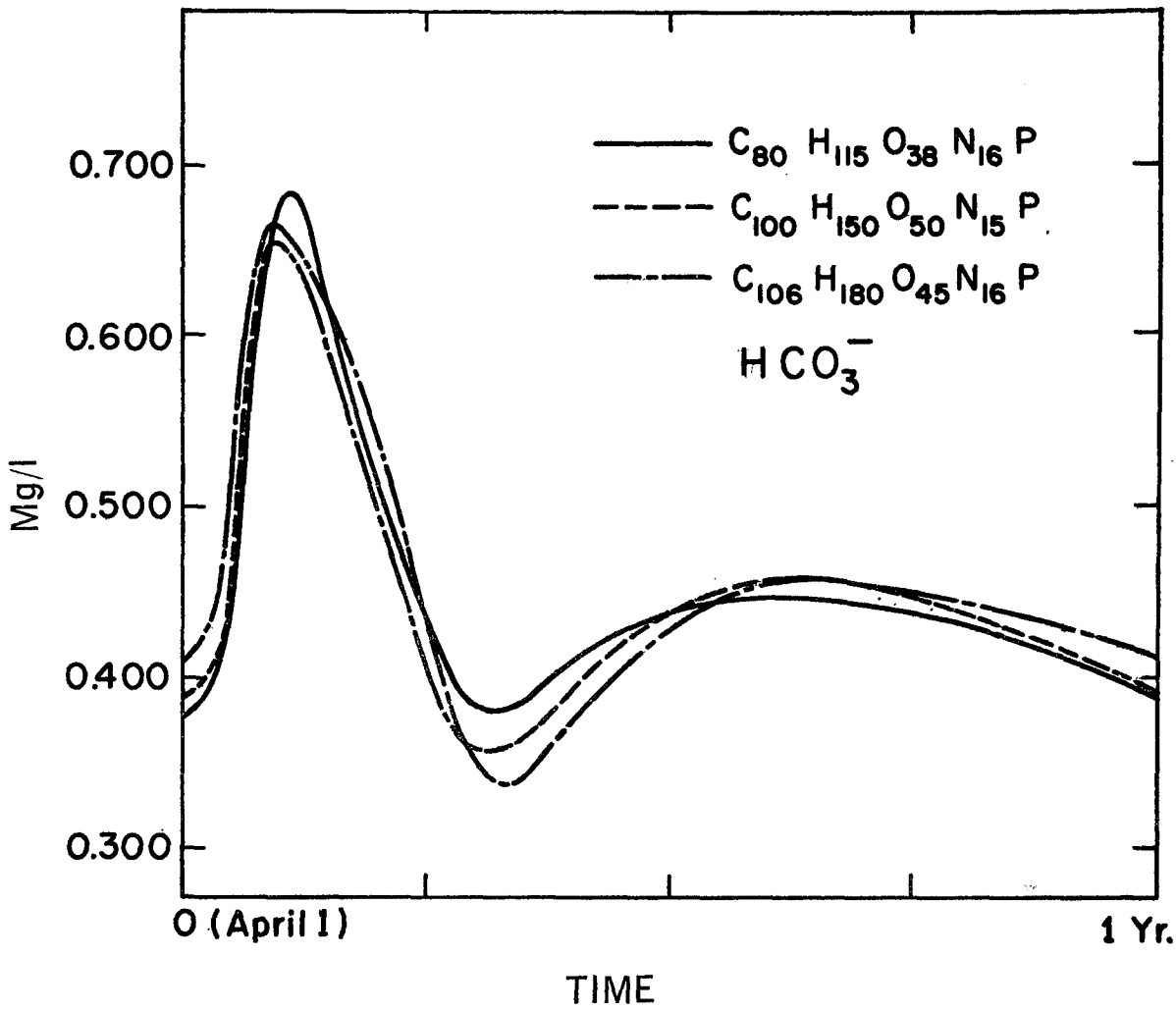

FIGURE 6.—Modeled seasonal variation of HCO_3^- concentration.

Figure 9 shows that the carbonaceous material is very low during the summer and relatively high during the winter. The effect of organic composition is negligible on the carbonaceous material.

The oxygen concentration in the surface waters drops down in spring indicating a transfer of oxygen into the water at this time of the year and transfer out during the rest of the year. The organic composition can cause as much as a five percent change in composition as can be seen in Figure 10.

The overall dynamics of the nutrient cycling are indicated by Figures 4 through 10. During the year, the algal growth rate gradually becomes more restricted by the HCO_3^- concentration. The phosphates drop rapidly to a low value and remain there. The HCO_3^- needed for rapid algal growth in early spring is supplied by the bacterial action on the carbonaceous material left from the winter. Other interactions of this type can be noted.

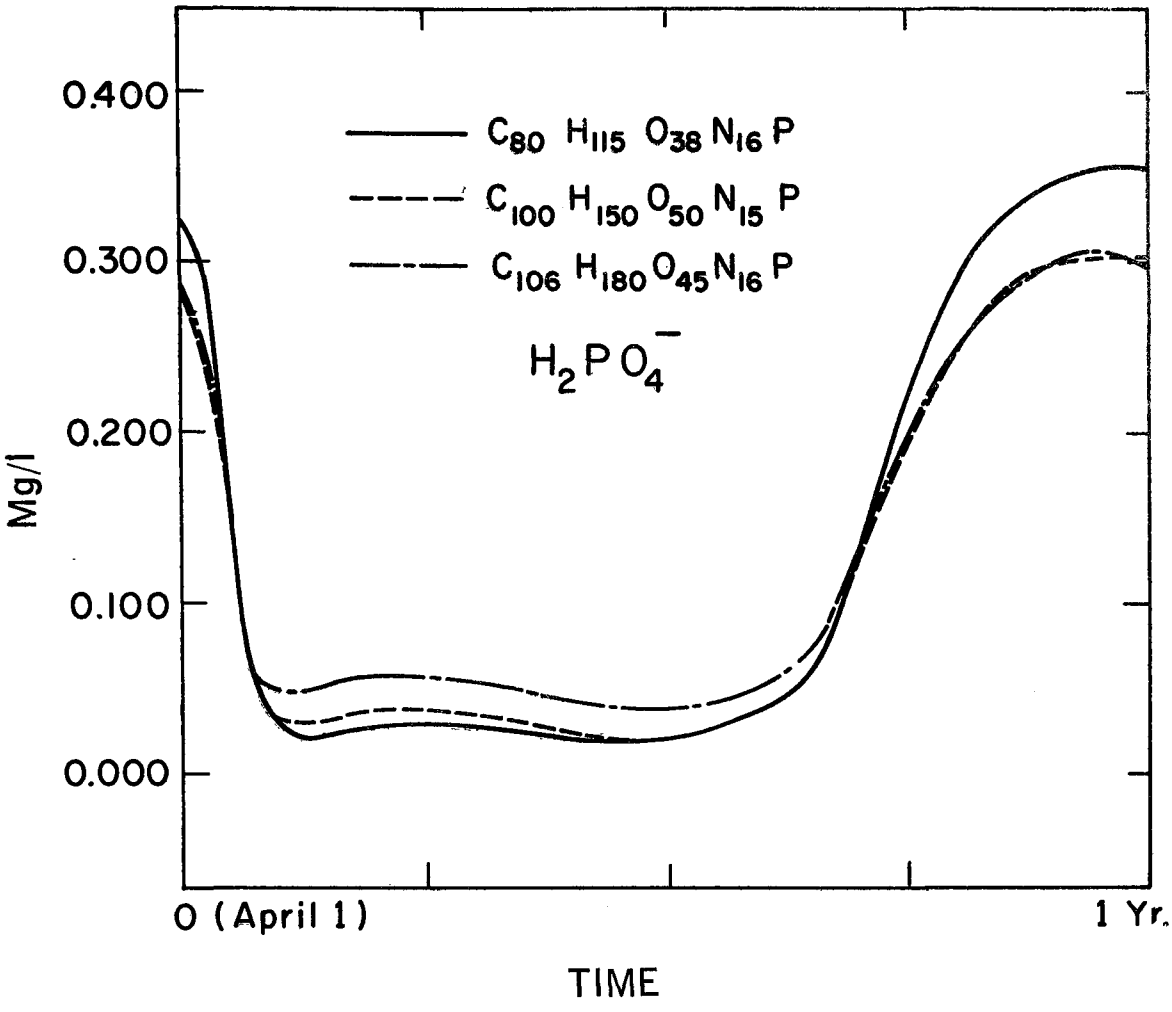

FIGURE 7.—Modeled seasonal variation of $H_2PO_4^-$ concentration.

Diurnal Model for the Upper Waters

The diurnal model follows essentially the same stoichiometry and kinetics as that used in the annual model. Changes were made in the model to follow the light and temperature variations over a twenty-four hour period. An equinoxial day was chosen for study giving twelve hour periods of light and darkness.

Temperature was assumed to reach a maximum around 4 p.m. and a minimum at 4 a.m. Thus it could be approximated with a sinusoidal function of time.

The algae production rates are mainly light dependent and this is reflected by setting the ingestion rate constant

$$k_2 = k_2^0(0.525 + 0.475 \sin(\phi - \phi_L))$$

where

ϕ_L in this particular case is

$$\frac{2\pi \times 90}{360} \text{ radians.}$$

and $\phi = 2\pi \times t/24.0$ radians where t is in hours.

If we assume an 8% variation in temperature over the day, we would have

$$T = T_{\max}(0.96 + 0.04 \sin(\phi - \phi_T))$$

T = temperature at time t

where

$T_{\max}$ = max temperature reached

$$\phi_T = \frac{150 \times 2\pi}{360} \text{ radians}$$

and ϕ has the same form as before.

Taking a linear variation of bacterial reproduction rate with temperature, its rate can be given by

$$k_5 = k_5^0 T / T_0$$

Similarly, the transport rates of O_2 and HCO_3^- into the system can be given by

$$k_8 = k_8^0 T / T_0$$

$$k_9 = k_9^0 T / T_0$$

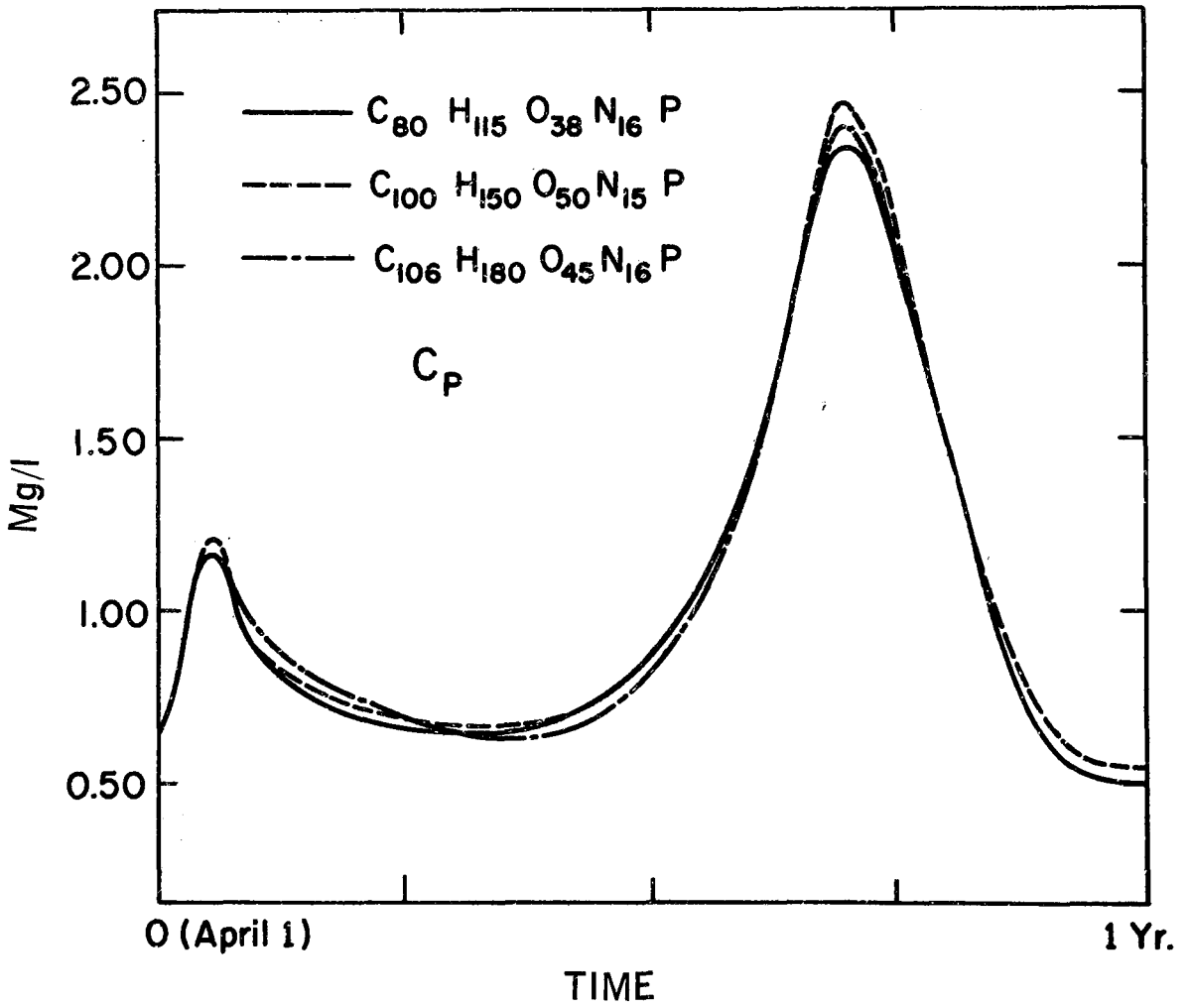

FIGURE 8.—Modeled seasonal variation of CP concentration.

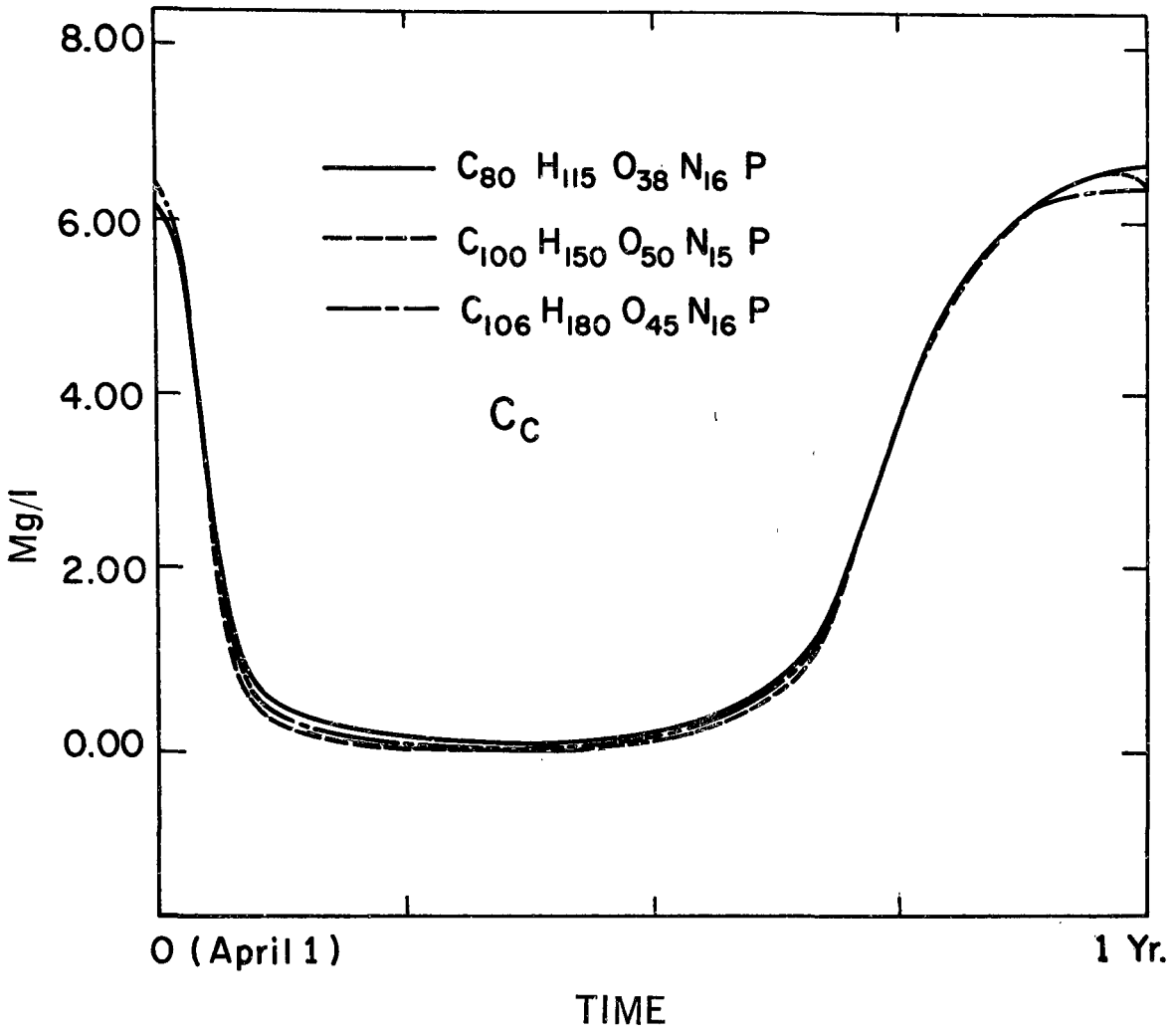

FIGURE 9.—Modeled seasonal variation of C_c concentration.

Saturation values of O_2 and HCO_3^- are also temperature dependent in an approximately linear fashion.

Thus

$$Y_{eq\cdot HCO_3^-} = Y_{eq\cdot HCO_3^-}{}^{0}(2T_M - T)T_0$$

$$Y_{eq\cdot O_2} = Y_{eq\cdot O_2} \times (2T_M - T)/T_0$$

$$T_M = T \quad \text{at} \quad \phi = \phi_T$$

In order to bring the model to an intransient mode changes had to be made in some of the parameters used in the annual model. However, these changes were kept within experimentally observed bounds. The composition of the bacteria, algae, and carbon-phosphorus material was not varied.

The constants used in the simulation were:

$$k_1 = 25.0$$

$$k_2 = 3.0(0.525 + 0.475 \sin(\phi - \phi_L))$$

$$k_3 = 0.05$$

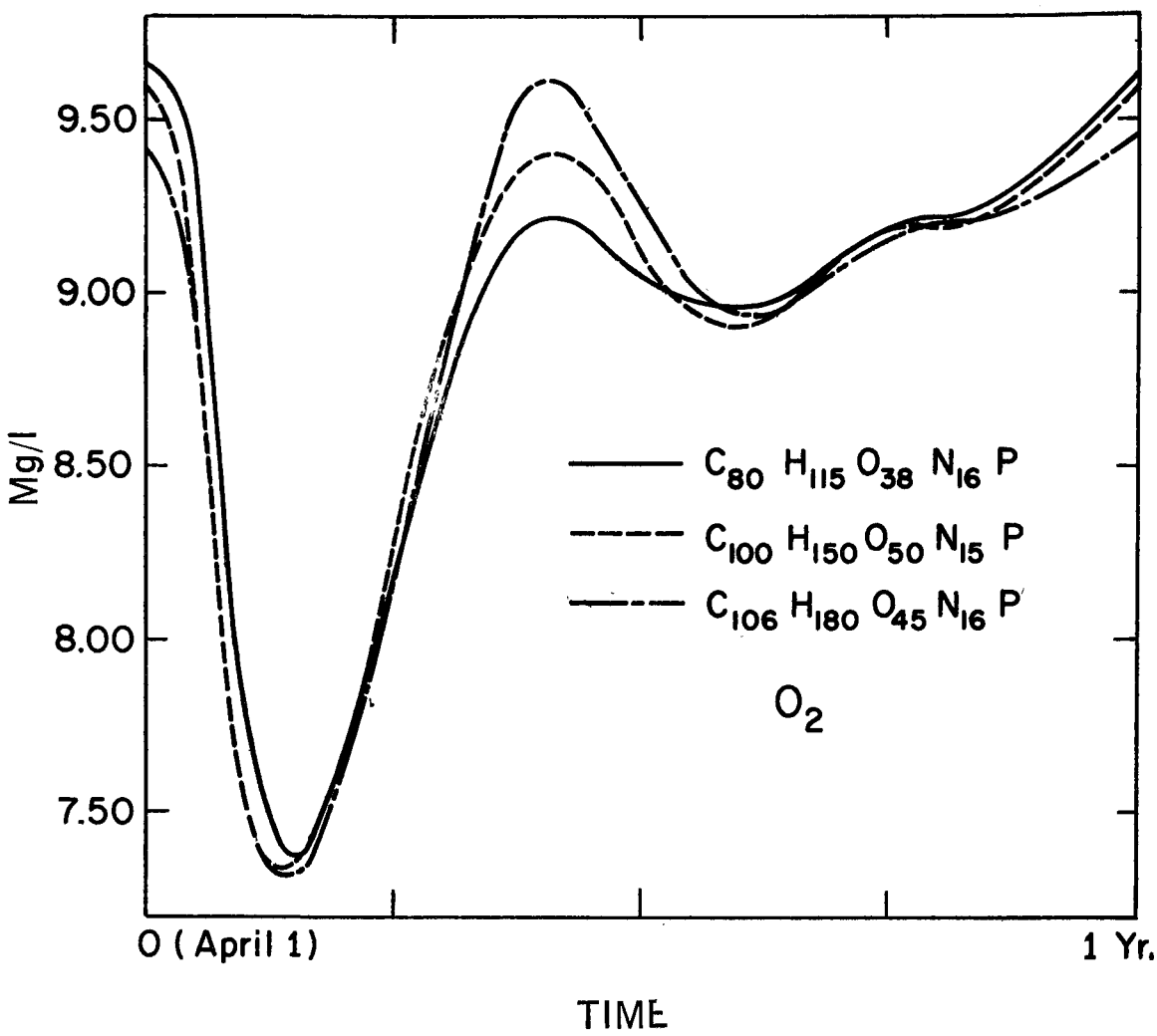

FIGURE 10.—Modeled seasonal variation of O_2 concentration.

$$k_4 = 0.10$$

$$k_5 = 6.00(0.96 + 0.4 \sin(\phi - \phi_T))$$

$$k_6 = 1.00$$

$$k_7 = 0.1$$

$$k_8 = 1.00(0.96 + 0.04 \sin(\phi - \phi_T))$$

$$k_9 = 0.2(0.96 + 0.04 \sin(\phi - \phi_T))$$

$$\eta_1 = 1.5$$

$$\epsilon_2 = 1.0$$

$$\eta_2 = 0.7$$

$$Y_{eq \cdot HCO_3^-} = 0.44(0.96 - 0.04 \sin(\phi - \phi_T))$$

$$Y_{eq \cdot O_2} = 9.1(0.96 - 0.04 \sin(\phi - \phi_T))$$

Results

The system was run with intervals of half-an-hour using a Runge-Kutta subroutine. In order to obtain better stability, it would be necessary to cut down on the interval size, but this leads to larger computing time. The system was found to be sensitive to various parameters predominant among them being the ingestion and lysing rates of the bacteria and algae, and decomposition rates of the organics. The transport rates into the organism had less of an effect while change in the mass transport rates of O_2 and HCO_3^- showed very little disturbance in the system.

Oscillatory behavior was also noticed in the system with a period ranging from 10–30 days depending on the step size, the machine accuracy and the parameters used.

In the model simulated, the algae (Fig. 11) reached a peak early in the day with a subsequent die-off. The concentrations are mass concentrations and this reflects the high ingestion rate into the system.

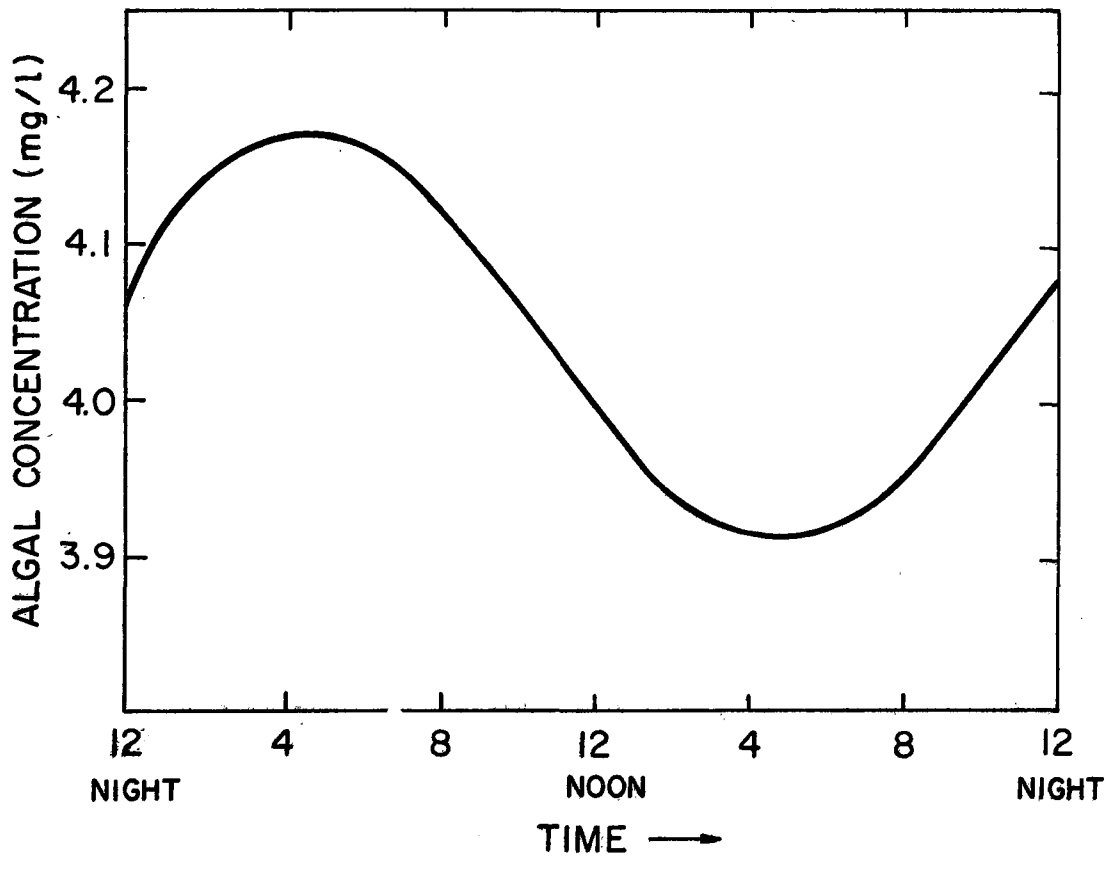

FIGURE 11.—Modeled diurnal variation of algal concentration.

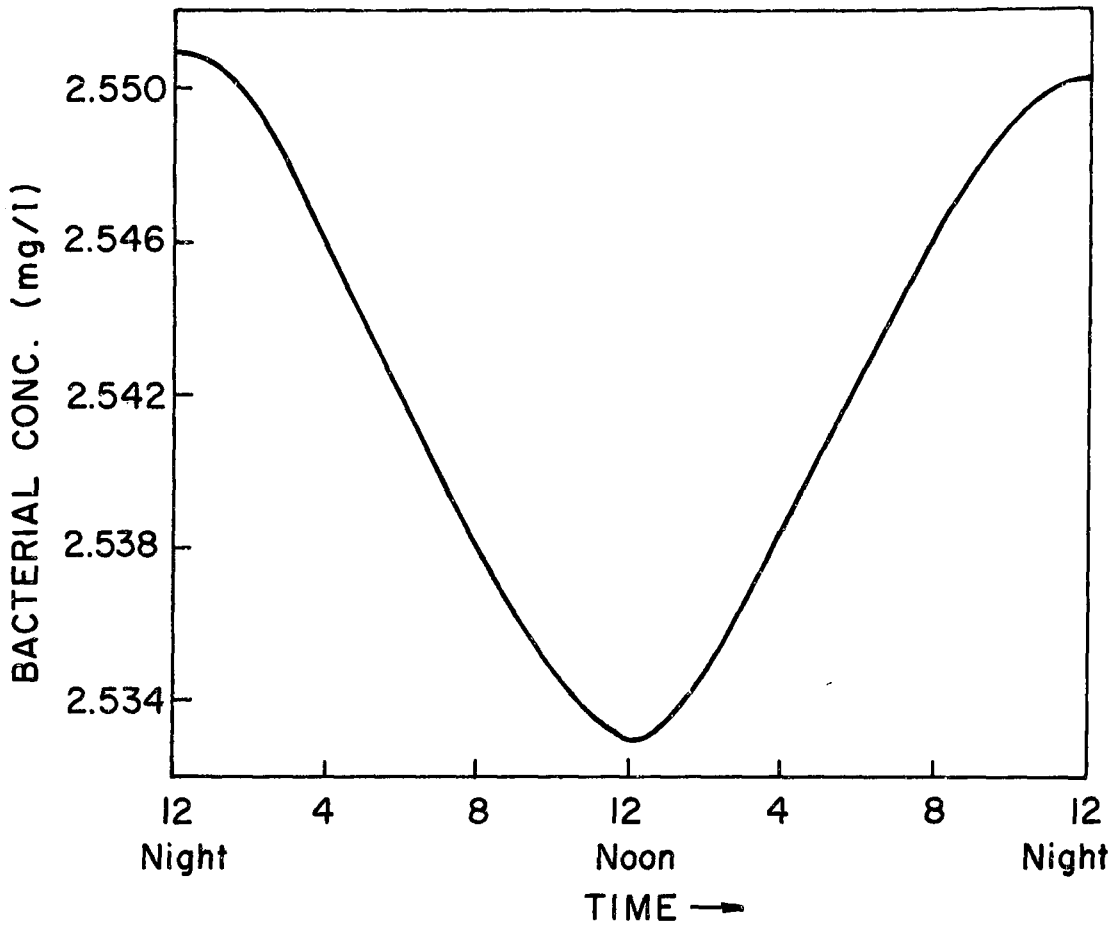

FIGURE 12.—Modeled diurnal variation of bacterial concentration.

Bacteria (Fig. 12) on the other hand, reach a peak at night due to the higher availability of oxygen produced by the algae during the day. Then HCO_3^- given off by the bacteria is again taken in by the algae. The dissolved oxygen (Fig. 14) peaks at the end of the daylight period showing the accumulation that has been occurring through the day. It drops down during the night due to the consumption by the bacteria.

Both the nutrients $H_2PO_4^-$ and HCO_3^- (Fig. 13) peak at around the same time and follow essentially the same pattern in the system. These peaks which occur during the day show the sum total of their components produced by the bacteria less that taken up by the algae.

Expansion of the Model

There are two basic directions in which the model is to be expanded. The cycling of nitrogeneous nutrients is to be included into the basic model. The water model can also be amplified to include the bottom waters.

INCLUSION OF NITROGEN

The nitrogen compounds thought to be most important in the growth of algae and bacteria are NH_4^+, NO_3^- and N_2. The nitrite form of nitrogen was not included because of the relatively rapid conversion of NO_2^- to NO_3^-. In order to model different stages of decomposition of organics in the system, four types of organic components are included. The first, designated as CPN, is formed on death of bacteria or algae and has the same composition as the organisms. CPN may decompose giving off $H_2PO_4^-$ to yield CN or may give off NH_4^+ to yield CP. CN and CP then decompose with removal of NH_4^+ and $H_2PO_4^-$ respectively yielding a carbonaceous compound C. Three different forms of algae are postulated to correspond to each of the nitrogeneous nutrients. They are A_1 which uses

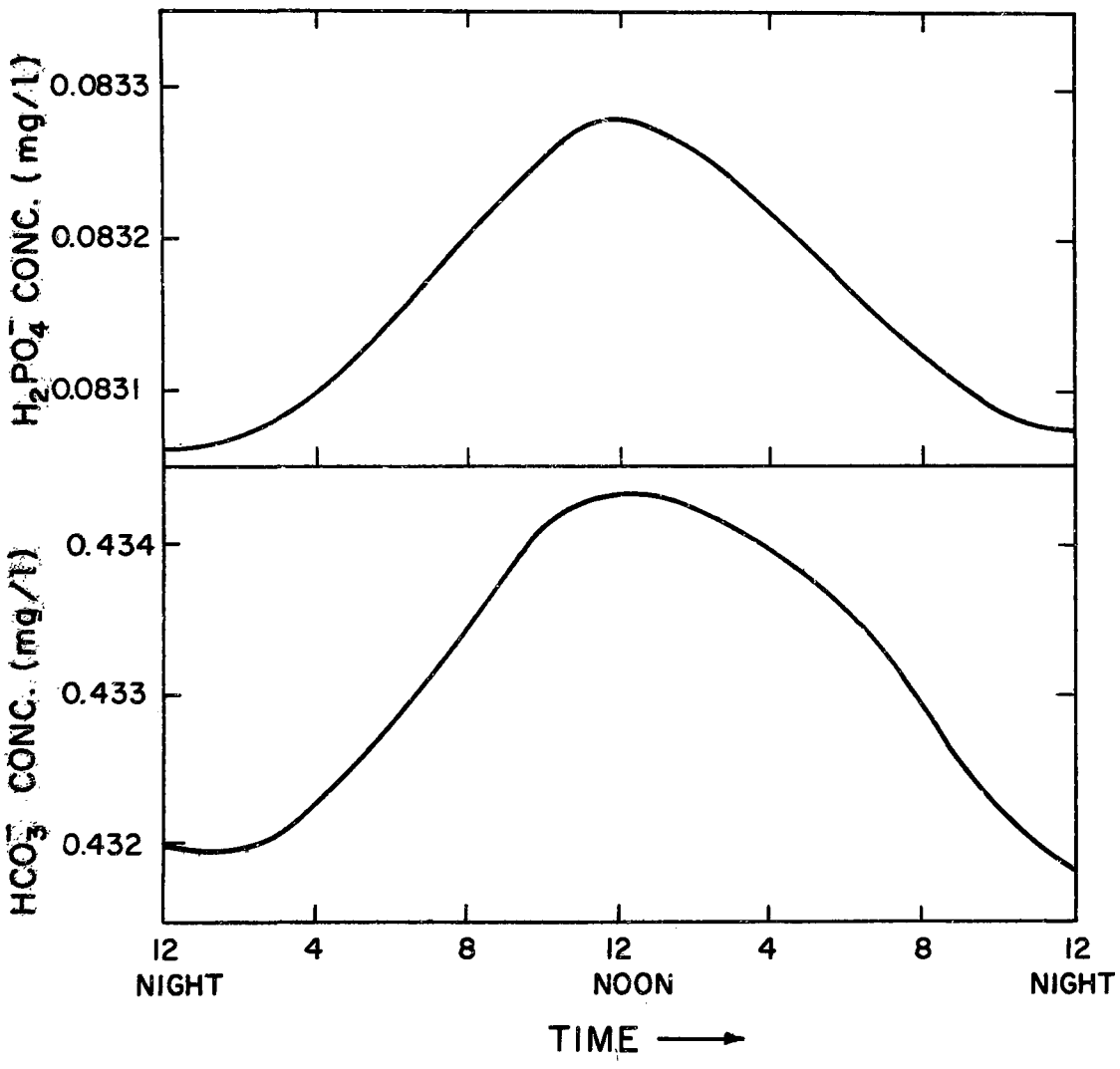

FIGURE 13.—Modeled diurnal variation of $H_2PO_4^-$ and HCO_3^- concentrations.

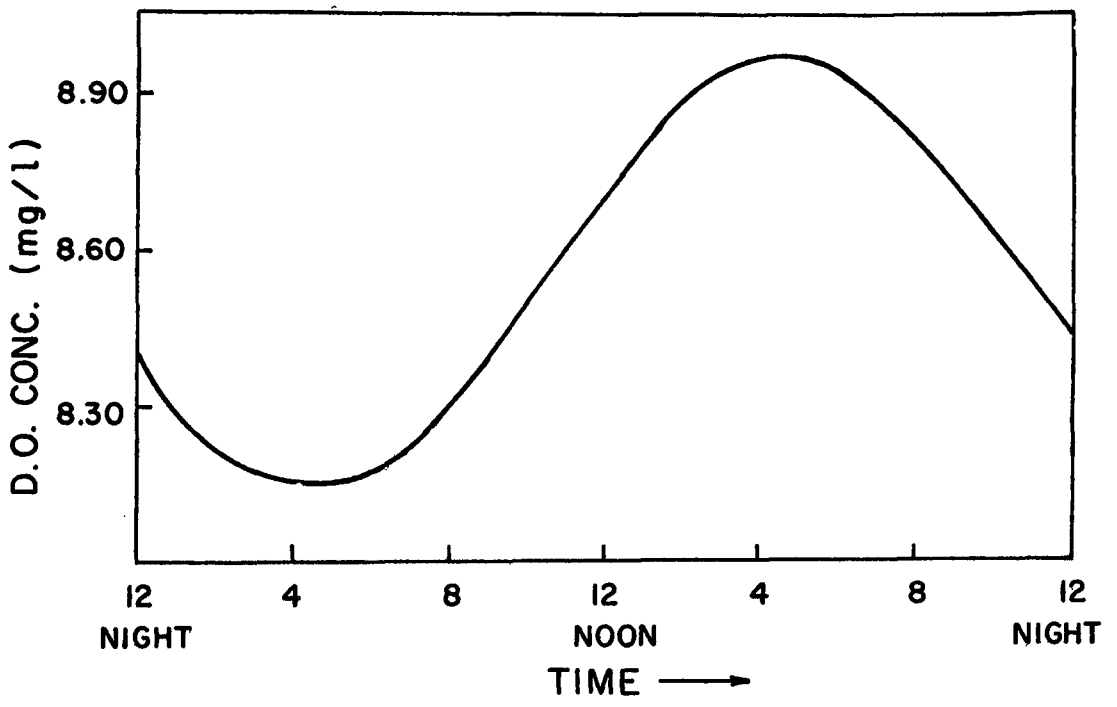

FIGURE 14.—Modeled diurnal variation of O_2 concentration.

NH_4^+ as a nutrient, A_2 which utilizes N_2, and A_3 which takes NO_3^- as its nutrient. Each of them has its corresponding ingestion state A_{21}, A_{22}, and A_{23}.

The bacteria too are of three kinds: the first, B_1, oxidizes NH_4^+ to NO_3^-, the second, B_2, utilizes both NO_3^- and O_2 to decompose the carbonaceous materials into the nutrients while the third, B_3, uses solely NO_3^- as an oxygen source and evolves N_2 as a product. They too have their corresponding secondary stages, B_{21}, B_{22}, and B_{23}.

The various forms of algae and bacteria will be predominant according to the availability of the nitrogeneous nutrients and the growth rates will also be dependent on their concentrations.

Kinetics

The kinetics used are the same as in the surface model.

The diffusive transfer of N_2 into the system is given by

$$r_N = k_N(Y_{eq \cdot N_2} - Y_{N_2})$$

r_N = mass accumulation of N_2 per unit volume of lake per unit time

k_N = mass transfer coefficient for N_2. It is time variant

$Y_{eq \cdot N_2}$ = water mass concentration of N_2 in equilibrium with the atmosphere.

The decomposition of organics is by homogeneous chemical reactions.

$r_{P_1} = k_{P_1} Y_{CPN}$

$r_{N_1} = k_{N_1} Y_{CPN}$

$r_{N_2} = k_{N_2} Y_{CN}$

$r_{P_2} = k_{P_2} Y_{CP}$

r_{P_1} = mass of CPN per unit volume of lake decomposing to CP

r_{N_1} = mass of CPN per unit volume of lake decomposing to CN

r_{N_2} = mass of CN per unit volume of lake decomposing to C

r_{P_2} = mass of CP per unit volume of lake decomposing to C

k_{P_1}, k_{N_1}, k_{N_2}, k_{P_2} = first order reaction constants

Y_{CPN}, Y_{CP}, Y_{CN} = mass concentrations of CPN, CP, CN in lake.

The rate functions describing the absorption of nutrients from water have essentially the same form as in the simpler model. The nitrogeneous components have been included as a factor in the rate expressions.

For the algae

$$r_{A1a} = k_{A11} Y_{A_1} Y_{H_2PO_4^-} Y_{HCO_3^-} Y_{NH_4}$$

$$r_{A2a} = k_{A12} Y_{A_2} Y_{H_2PO_4^-} Y_{HCO_3^-} Y_{N_3}$$

$$r_{A3a} = k_{A13} Y_{H_2PO_4^-} Y_{HCO_3^-} Y_{NO_3^-}$$

For the bacteria.

$$r_{B1a} = k_{B11} Y_{B_1} Y_{O_2} (N_1 Y_{CP} + N_3 Y_c) Y_{NH_4^+}$$

$$r_{B2a} = k_{B12} Y_{B_2} Y_{O_2} (Y_{CPN} + N_1 Y_{CP} Y_{NO_3^-} + N_2 Y_{CN} + N_3 Y_c Y_{NO_3^-})$$

$$r_{B3a} = k_{B13} Y_{B_3} Y_{NO_3^-} (Y_{CPN} + N_1 Y_{CP} + N_2 Y_{CN} + N_3 Y_c)$$

r_{A1a}, r_{A2a}, r_{A3a} = masses of A_1, A_2, A_3 formed per unit time per unit volume of lake.

r_{B1a}, r_{B2a}, r_{B3a} = masses of B_1, B_2, B_3 formed per unit time per unit volume of lake.

The Y's are the mass concentrations of the various components in the rates. N_1, N_2, N_3 are preference factors for the other organic components over CPN. The k's are rate constants.

The disappearance by ingestion of the components A_{21}, A_{22}, A_{23} and B_{21}, B_{22}, B_{23} are assumed to be first-order in their mass

concentrations. The first-order law is also used for the lysing rates of the algae and bacteria.

e.g., $$r_{A1i} = k_{A21} Y_{A21}$$

and $$r_{B1i} = k_{B21} Y_{B21}$$

would be the ingestion rates of components A_{21} and B_{21}. The rate constants can be set according to the relative activities of the different components in the system.

Stoichiometry

In order to conserve the nutrients, the stoichiometry used is important and is required for the absorption, ingestion, and lysing processes of the organisms and the decomposition of the organic components.

The stoichiometry associated with the decomposition of the organic components which are assumed to have the composition $C_aH_bO_cN_dP$ are given by the following formulae

$$(C_aH_bO_cN_dP)_{PN} \rightarrow (C_aH_{b-4d}O_cP)_{CP} + dNH_4^+$$

$$(C_aH_bO_cN_dP)_{CPN} \rightarrow (C_aH_{b-2}O_{c-4}N_d)_{CN} + H_2PO_4^-$$

$$(C_aH_{b-4d}O_cP)_{CP} \rightarrow (C_aH_{b-4d-2}O_{c-4})_C + H_2PO_4^-$$

$$(C_aH_{b-2}O_{c-4}N_d)_{CN} \rightarrow (C_aH_{b-4d-2}O_{c-4})_C + dNH_4^+$$

For algae the absorption stoichiometry is given by

$$(C_aH_bO_cN_dP)_{A_1} + n_1[aHCO_3^- + dNH_4^+ + H_2PO_4^- + ((b-a-2-4_d)/2)H_2O] \rightarrow A_{21}$$

$$(C_aH_bO_cN_dP)_{A_2} + n_1\left[\frac{d}{2}N_2 + H_2PO_4^- + aHCO_3^- + ((b-a-2)/2)H_2O\right] \rightarrow A_{22}$$

$$(C_aH_bO_cN_dP)_{A_3} + n_1[dNO_3^- + aHCO_3^- + H_2PO_4^- + ((b-a-2)/2)H_2O] \rightarrow A_{23}$$

For the bacteria the absorption stoichiometry is given by

$$(C_aH_bO_cN_dP)B_1+(\epsilon_2+(\eta_2-\epsilon_2)S_{B_1})\begin{bmatrix}CP+dNH_4^+\\ C+dNH_4^++H_2PO_4^-\end{bmatrix}$$

$$+(1-S_{B_1})(\eta_2-\epsilon_2)((3a+3d+4+(b-a-2)/2-c)/2)O_2\rightarrow B_{21}$$

$$(C_aH_bO_cN_dP)_{B_2}$$

$$+(\epsilon_2+(\eta_2-\epsilon_2)S_{B_2})\begin{bmatrix}CPN\\ CP+d(NO_3^-+2H_2O-\frac{5}{2}O_2)\\ CN+H_2PO_4^-\\ C+d(NO_3^-+2H_2O-\frac{5}{2}O_2+H_2PO_4^-)\end{bmatrix}$$

$$+(\eta_2-\epsilon_2)(1-S_{B_2})((3a+4+3d+(b-a-2)/2-c)/2)O_2\rightarrow B_{22}$$

$$(C_aH_bO_cN_dP)_{B_3}$$

$$+(\epsilon_2+(\eta_2-\epsilon_2)S_{B_3})\begin{pmatrix}1\\ 1+\alpha\\ 1\\ 1+\alpha\end{pmatrix}\begin{bmatrix}CPN\\ CP+d(NO_3^-+2H_2O)\\ CN+H_2PO_4^-\\ C+d(NO_3^-+2H_2O)+H_2PO_4^-\end{bmatrix}$$

$$+\tfrac{1}{3}S_{NO}(\eta_2-\epsilon_2)(1-S_{B_3})[(3a+4+(b-a-2)/2-c)/3NO_3^-]$$

$$-\frac{5}{3}\begin{pmatrix}0\\ 1\\ 0\\ 1\end{pmatrix}\left(\frac{S_N}{S_{B_3}}\right)(\epsilon_2+(\eta_2-\epsilon_2)S_{B_3})[\tfrac{5}{3}dNO_3^-]$$

$$\rightarrow B_{23}-\begin{pmatrix}0\\ 5\\ 0\\ 5\end{pmatrix}(\tfrac{1}{3}S_{NO}{}^{-1})\left(\frac{S_N}{S_{B_3}S_{NO}}\right)(\epsilon_2+(\eta_2-\epsilon_2)S_{B_3})[\tfrac{5}{6}dN_2]$$

$$+(\tfrac{1}{3}S_{NO}{}^{-1})(\eta_2-\epsilon_2)(1-S_{13})[(3a+4+(b-a-2)/2-c)/2N_2]$$

where

$$S_N = \frac{62d}{AM}; \quad S_{NO} = \frac{(I.W)_{NO_3^-}}{(A.W)_O} = \frac{62}{16}; \quad = \frac{5S_N}{S_{B_3} S_{NO}}$$

The ingestion stoichiometry for the algae is given by the following expressions.

$$A_{21} \rightarrow (1+\epsilon_1)(C_aH_bO_cN_dP)_{A_1} + (1-S_{A_1})\eta_1(3a+4+(b-a-2-4d)/2-c)O_2$$

$$A_{22} \rightarrow (1+\epsilon_1)(C_aH_bO_cN_dP)_{A_2} + (1-S_{A_2})\eta_1[(3a+4+(b-a-2)/2-c)/2]O_2$$

$$A_{23} \rightarrow (1+\epsilon_1)(C_aH_bO_cN_dP)_{A_3} + (1-S_{A_3})\eta_1[(3a+3d+4+(b-a-2)/2-c)/2]O_2$$

For bacteria they are:

$$B_{21} \rightarrow (1+\epsilon_2)(C_aH_bO_cN_dP)_{B_1} + (\eta_2-\epsilon_2)[aHCO_3^- + dNO_3^- + H_2PO_4^- + (b-a-2)/2H_2O]$$

$$B_{22} \rightarrow (1+\epsilon_2)(C_aH_bO_cN_dP)_{B_2} + (\eta_2-\epsilon_2)[aHCO_3^- + dNO_3^- + H_2PO_4^- + (b-a-2)/2H_2O]$$

$$B_{23} \rightarrow (1+\epsilon_2)(C_aH_bO_cN_dP)_{B_3} + (\eta_2-\epsilon_2)\left(aHCO_3^- + H_2PO_4^- + (b-a-2)/2H_2O + \frac{d}{2}N_2\right)$$

In the above equations several stoichiometric constants are used which are defined as follows:

$$S_{A_1} = \frac{\text{mol. wt. of organism } A_1}{\text{wt. of corresponding nutrients}} = \frac{AM}{ANH}$$

$$S_{A_2} = \frac{\text{mol. wt. of organism } A_2}{\text{wt. of corresponding nutrients}} = \frac{AM}{AN2}$$

$$S_{A_3} = \frac{\text{mol. wt. of organism } A_3}{\text{wt. of corresponding nutrients}} = \frac{AM}{AN}$$

$$S_{B_1} = \frac{\text{mol. wt. of organism } B_1}{\text{wt. of corresponding nutrients}} = \frac{AM}{AN}$$

$$S_{B_2} = \frac{\text{mol. wt. of organism } B_2}{\text{wt. of corresponding nutrients}} = \frac{AM}{AN}$$

$$S_{B_3} = \frac{\text{mol. wt. of organism } B_3}{\text{wt. of corresponding nutrients}} = \frac{AM}{AN2}$$

where

$$AM = 12a + b + 16c + 14d + 31$$

$$AN2 = 61a + 14d + 97 + 9(b - a - 2)$$

$$ANH = 61a + 18d + 97 + 9(b - a - 2 - 4d)$$

$$AN = 61a + 62d + 97 + 9(b - a - 2)$$

Extension to the Bottom Waters

The next logical stage in the modelling is the inclusion of the bottom waters in the system. This would be most essential in the cases of lakes which show stratification and varying characteristics through their depth.

The lake would be modelled as consisting of two parts, the upper waters and the lower waters. Both would be considered as well-mixed bodies not affecting each other physically. The physical parameters of each of the systems would be different, e.g., they would have different temperatures and light densities.

Both anaerobic and aerobic bacteria have to be included in the bottom waters as also other reaction products like CH_4 and organic acids. Transfer of components may occur across the interface. This may occur in either of two ways: (1) Convective transport can be modelled with mass transfer coefficients multiplied by the difference in concentrations. This would be the mode in which the nutrients like O_2, HCO_3^-, $H_2PO_4^-$, the nitrogenous compounds and the dissolved organic compounds may be transferred. (2) Gravitational transport occurs in the cases of settling of the dead organisms and the rising of gaseous bottom products. There may also be settling included into the bottom muds and hence out of the system.

The same kinetics and stoichiometry would hold for the upper waters and the aerobic components of the bottom waters. However, they are not very well known for the other components and this would present an obstacle.

SUMMARY

Nutrient cycling through the microbial populations is an important factor in the dynamics of aquatic ecosystems and attempts to develop mathematical models of this part of the ecosystem should lead to a better understanding of the eco-

system as a whole. In the construction of such ecosystems three major fundamental relationships are used: The conservation of each chemical element that cycles through the ecosystem must be preserved, the kinetics of the microbial and chemical reactions which occur in the ecosystem should be realistic, and the stoichiometry of these reactions must be handled in detail. This paper presents a summary of various research activities designed to accomplish this goal.

The kinetics of microbial cell growth are developed from a mechanism which postulates that this growth is accomplished by two consecutive processes, the transport of the metabolite from the exterior of the cell to the interior and the chemical conversion of these chemicals into cellular protoplasm. Only the rate of the transport process is assumed to be dependent upon the external nutrient concentrations, while the chemical conversion rate is assumed to be dependent upon the light and temperature of the media.

Since the exact elemental composition of the microbial populations is not known, these parameters were investigated by simulating a particular ecosystem. This ecosystem was composed of the algae and the bacteria microbial components along with the associated nutrients of carbon, phosphorus, and oxygen. The seasonal simulation indicated that the composition of the microbes significantly altered the dynamic response of the ecosystem, although it did not change some expected system properties such as the increase of algae in the summer. Qualitative comparisons of the mathematically predicted response of the ecosystem with known occurrences in real aquatic ecosystems are presented.

This same ecosystem containing the cycling of carbon, phosphorus, and oxygen was simulated for diurnal variations of the water quality. The diurnal response obtained from the mathematical model resembled that found in a nearly stagnant lake since this more closely approaches the assumption that there were no nutrient inputs. As would be expected, all the nutrients followed the same diurnal cycle indicating that nutrient cycling was a contributing factor to the dynamics of the ecosystem.

Finally, a discussion of the necessary model modification to

include the cycling of the nitrogen in its various forms and the inclusion of microbial organisms on the bottom of the water body. The existence of several nitrogen forms in natural waters complicates the stoichiometry of microbial and chemical reactions involving this nutrient. The relatively unknown stoichiometry of anaerobic microorganisms and the stationarity of attached algae in flowing streams are two of the major problems to be solved when the bottom organisms are included in the microbial nutrient cycling model.

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Questions and Comments Following Mr. Verhoff's Talk

QUESTIONS FROM THE FLOOR: I notice you talk a fair amount about aerobic cycles. Have you purposely neglected the anaerobic cycle or did you just dismiss them for the sake of simplicity?

DR. VERHOFF: We are at the present developing or have included in the model the nitrogen as ammonia, nitrate, and nitrogen gas. We've ignored the nitrite since it can be assumed that it goes to nitrate. Ignoring it doesn't cause problems because every time we add something we have to add bacteria to that particular operation and that just adds two more components to the model. But with regard to anaerobic conditions, I couldn't find any data which specified the stoichiometry with regard to nitrogen, phosphorus, carbon, hydrogen, or oxygen, and what really happens under anaerobic conditions. That is the problem. We've ignored them at present to get some feel for problems we will have with modeling but we certainly do intend to include them. I should say that we've done some modeling with anaerobes but at more gross level. We do intend to include them for sediment conditions with exchange of nutrients between the overlying aerobic water and the anaerobic bottom sediments.

QUESTION FROM THE FLOOR: Where empirical data have differed from what your model predicted, are there any particular areas that you've gone back and reevaluated? For example, your model predicted a late summer or early fall peak

in algae. The two examples that you showed, Lake Constance and some other lake, one had two peaks which were much earlier than fall and one had a broad peak which also occurred much earlier. Did you go back and reevaluate the model?

DR. VERHOFF: We can make that kind of check. But there was something else going on in those lakes. We never before saw those types of instabilities that Lake Lenore showed in the particular model dealt with. I can't tell you exactly why that occurred. It might have something to do with the nitrogen cycle. We do not have the nitrogen model developed yet to the point where can see those kinds of instabilities.

QUESTION FROM THE FLOOR: Where are the grazing zooplankton included in your model?

DR. VERHOFF: The grazing zooplankton operate like bacteria contributing to the recycle of the nutrients back to the water. However, I think there should be a separate consideration of zooplankton in certain ecological conditions because they definitely graze the algae down to nothing.

QUESTION FROM THE FLOOR: You indicated that the transport of nutrients was governed by intercellular nutrient concentrations rather than extracellular concentrations. Do you have any evidence of this?

DR. VERHOFF: No. I think it's extracellular nutrient concentrations that control transport. We separate the transport process from the reaction process because we think the external extracellular nutrient concentrations governs the rate at which transport occurrs and we think temperature and sunlight governs the rate which the reactions occur.

QUESTION FROM THE FLOOR: Did you intimate that more than one nutrient can be growth limiting at one time?

DR. VERHOFF: I think that's the case.

QUESTION FROM THE FLOOR: What evidence do you have of this? In any of the work we've done we've never seen any more than one nutrient growth limiting at one time.

DR. VERHOFF: It depends on what you mean by growth limiting. Some are normally more limiting than another, if you think you're phosphate limiting you add some phosphate. Without changing the growth rate too much. There are few data on mixed systems, which are the kind of data that I need.

I think O'Connor and DiToro developed a model for two limiting nutrients, or how to handle two limiting nutrients.

QUESTION FROM THE FLOOR: I'm not referring to modelling. I mean actual laboratory or field data.

DR. VERHOFF: There are a great deal of data available on limitation of phosphorus and of nitrogen. Very little, I should say, where both are nutrients low with very slow growth rates. O'Connor and DiToro summarized the data that they could find available when they were trying to develop phytoplankton growth models for the San Joaquin Valley. They summarized the data that they were able to use. They didn't separate the transport process from the reaction scheme and used two Monod expressions multiplied together. There was also a paper delivered at a American Institute of Chemical Engineers meeting at Chicago in 1970 which I've never been able to find in print. Some investigators looked at bacteria and two limiting nutrients however I do not remember which two limiting nutrients they used. They tried two Monod expressions multiplied together in an effort to model the two limiting nutrient system and found that it didn't work. I don't contend that we have the answer on how to handle two limiting nutrients because the transport schemes that are going on which I think play the key role are extremely complex.

QUESTION FROM THE FLOOR: When you speak of two limiting nutrients, are you speaking of two limiting nutrients for the same group of organisms or are you splitting them up? Say one for algae versus one for bacteria?

DR. VERHOFF: I would have to say that we're talking about a group of organisms because we don't model individual organisms per-se. If we were to look at bacteria as a group and talk about two limiting nutrients, we would have to be talking about groups of organisms. For example, the carbonaceous material is limiting the one group and phosphorus limiting to the other. This situation should be modified.

QUESTION FROM THE FLOOR: Well is this a possible situation?

DR. VERHOFF: Yes, it certainly is.

QUESTION FROM THE FLOOR: What I'm saying is that for a particular alga or for a alga population there cannot be more than one limiting nutrient at one time, nor for bacteria.

DR. VERHOFF: In that case I think there is one general limiting nutrient. The normal way of determining it is to add a certain amount of that particular nutrient to the batch growth or other culture system and whichever addition shows the best response is what is called the limiting nutrient.

QUESTION FROM THE FLOOR: I don't agree with some of the assumptions you have made before you closed the "black box". I think this tends to run counter to some of our understanding of the nutrient changes occurring in a heterogenous community of organisms. One of the things I'm interested in is the basis for your conclusions that you can separate out contributions for the turnover of nutrients of algae and bacteria. I wonder if you can open up the "black box" and indicate the basis for your decision that you can distinguish between various heterogenous communities of both algae and bacteria, forgetting of course not only the herbivores but the protozoa and the fungi in the same communities.

DR. VERHOFF: The problem every modeller faces is how to separate a system to make it, at least half-way mimic reality. In chemical engineering we run into exactly the same problem. Whenever a chemical reaction occurs, there are various kinds of intermediates that occur and we have to separate out those intermediates whether we know for sure they're there or not, and whether we know they're independent of what's going on. It's just a process of modelling. You have to be able to separate all components and look at individual transport processes.

QUESTION FROM THE FLOOR: I'm talking of end products not the intermediates: the algal biomass and the bacterial biomass.

DR. VERHOFF: You mean separate them experimentally?

QUESTION FROM THE FLOOR: Well, in your conclusions you apparently have been able to separate them.

DR. VERHOFF: In the experimental data that I presented on algae and bacteria that is what the experimenters contended. I realize that there is tremendous difficulties in this area and that instrumentation could be very helpful that could provide precise separation. One example of separation that we would like to see and I think is along the line of your question is how do you distinguish reactions even if you do separate all the bacteria.

QUESTION FROM THE FLOOR: I question to what extent one ought to accept the validity of the model in giving these biological solutions?

DR. VERHOFF: We modellers realize that our models can be off and any suggestion as to how to handle or how to do this is important and is going to be a great contribution to describing what is actually happening. Models are only so good.

QUESTION FROM THE FLOOR: Presumably from your model you are treating these various groups of organisms as a single population that exists throughout the year. So one would think from your model that this is the way the system would behave if you have a single population of algae, a single population of bacteria, and so forth. But this is not in fact what happens. There are definite qualitative changes. These qualitative changes not only change the composition of the community but also the stoichiometry. They also change the kinetics of the community. And so, with these qualitative considerations put on top of your model how can we see any sort of pattern whatsoever? The band of variation around your model could be 400%.

DR. VERHOFF: That's very true but at the present time all we're looking for from these models are general phenomena. With regard to including everything in a model, and one would like to include everything right down to the species, including all kinds of interactions. I think an example of the difficulty one encounters in trying to include everything, is exemplified by what occurred at an IBP modelling seminar in 1969 connected with deserts. One of the persons there insisted that any of the models that was developed for the desert must include the effects of the circling hawk on the motion of the ground squirrel. Now this is carrying it to an extreme but we just can't include it and we can't include very easily species changes. We just have to look at what effect general nutrient cycling between organisms that tend to degrade and organisms that tend to synthesize substrate. That's about the only conditions we can help elucidate with a model.

QUESTION FROM THE FLOOR: Can you give us an order of magnitude of goodness of fit or of your model as applied to situations that you have tested against?

DR. VERHOFF: In the case discussed today we really didn't intend to achieve 50% fits or anything like that. This discussion is of general phenomena that are observed from models compared with the general phenomena that are observed in nature I didn't mean to imply that there was any kind of fit statistically. All of the data that we have were scaled from some phenomena to show a peak here, a valley there. We've done some comparison independent of this particular model and depending on how general or how crass we want to be with the model we get predictions of nutrients not algae. We are not going to attempt to predict cover algal blooms populations.

QUESTION FROM THE FLOOR: There are three elements that you have to consider: generality, precision, or you can be realistic. I think it's possible to have all three of these in one model. So if you're a non-modeller, and if you're opposed to the modelling concept, you can always find something to argue about in a model. The modeller has to decide what he is opting for, is he going for a general model, a precise model, or what? And of course the non-modeller can say, "Well your model isn't realistic." That's the nature of the beast.

DR. VERHOFF: We can find out if these various instabilities can be attributed to the nutrient cycling and the kinetics that are actually occurring in nature. That's the hope. If these instabilities are due to other things, and they have been proposed as have being caused by other things, such as feed-back organisms, production of poison by algae affecting bacteria, etc. then a modeller that doesn't include this kind of phenomena surely won't explain it.

Bacterial Growth Yields

BY W. J. PAYNE

Department of Microbiology
University of Georgia
Athens, Georgia 30601

ABSTRACT

For prototrophic growth of microorganisms in minimal media (with carbon, but no mineral nutrients limiting), yields in terms of available electrons per mole of substrate utilized (i.e., Y_{ave}^- values) were found experimentally to approximate 3.14 grams dry weight of cells per available electron. It was therefore expected that this value is characteristic of prototrophic growth, in general, when growth occurs at the expense of ordinary organic substrates. However, growth on methane and linear hydrocarbons appears not to be governed similarly. A mean Y_{ave}^- of 2.08 was calculated from 32 values for growth on hydrocarbons. But, extending from the Y_{ave}^- values for ordinary substrates to predict cell yields for either auxotrophic or prototrophic growth, it was predicted that a Y_{kcal} value of approximately 0.118 grams dry weight of cells may be expected for each kilocalorie of energy taken in any way from a culture medium by the growing cells. Thus, consistent with expectations, the mean for 94 Y_{ave}^- values calculated from growth yield data found in the literature is 3.07. This represents expenditure on the average of 39 percent of the substrate energy during growth and incorporation of 61 percent into cell structure. The mean for 63 Y_{kcal} values is 0.121. A figure used in determining Y_{kcal} values is the mean energy content of bacterial cells. This figure was predicted from elemental and macromolecular struc-

tural analysis data to lie between 5.3 and 5.46 kilocalories per gram, ash-free dry weight and was then estimated by bomb calorimetry to average 5.38. Finally, it is suggested that statement of Bail's M concentration in terms of Y_{ave}- or Y_{kcal} may be preferred over expression in terms of numbers of cells per unit volume.

INTRODUCTION

In the past, alliances between engineering and microbiology were likely to be uneasy. The engineer who uses microorganisms is likely to strive for constancy upon which he can base a continuous operation that can be modeled—then set in motion and left to run with minimal upkeep; and in many cases the microorganisms appear to behave quixotically. I say "appear" because, the closer we examine their seeming vagaries, the more obvious it becomes that microorganisms respond precisely and reliably to environmental influences. In the domain of growth yields particularly, it is clear that microorganisms will respond predictably to easily determined properties of growth substrates. One outstanding value of this observation lies in the prospect it offers of meeting the producing engineer's need for reliable yield data to base cost feasibility projections upon—when he decides that certain reduced organic material may be converted to edible or otherwise useful microbial products. Moreover, this is a "classical thermodynamics" approach that considers properties only at the beginning and the end of a system's functioning and thus seems more promising for engineering purposes than "statistical thermodynamics" derived from following one reaction to the next through biochemical pathways.

Significant interest among microbiological engineers today lies with production of single cell protein (SCP) by microbial utilization of waste hydrocarbons; but in the introductory portion of their report on the growth of *Candida utilis* on waste acetate left over from synthesis of cellulose acetate, Cama and Edwards (*10*) pointed out that a significant tonnage of SCP is produced annually from sulfite liquor wastes from the wood pulp industry. Some of the SCP is used in foods prepared for human consumption. They further suggest that whey, starchy

and cellulose-containing wastes, and citrus and other plant wastes are additional substrates available for microbial conversion to SCP. Edwards and Finn noted, moreover, that, in general, chemical process wastes have been neglected as transformable substrates, despite several obvious advantages of their use (*12*).

It seems clear that, if microbial transformation of reduced organic wastes from agriculture and industry can be shown to be more advantageous than just disposal, both economic and environmental gains will be achieved. The more so-called wastes that are converted microbiologically to useful products, the less there are left to pollute the water ways of the world. Toward that end, the purpose of this paper is to show that potentially useful predictive factors have been derived experimentally and tested for consistency with published figures on growth yields from various sorts of cultures.

METHODS

Interest in our laboratory in the predictability of growth yields stemmed from demonstration by Prochazka and Payne (*27*) that bacterial growth could be correlated directly with physico-chemical methods of analysis of the biodegradability of detergents and their intermediate degradation products—and growth is easily assayed. We were aware of the hypothesis put forward by Bauchop and Elsden (*4*) based on their studies of the growth of fermentative bacteria that an average of 10.5 grams dry weight of bacteria were produced for every mole of adenosine triphosphate (ATP) synthesized during growth. This is the well known Y_{ATP} value currently under close consideration and question in several laboratories as to its constancy or variance (*25*). But, leaving that question aside, we noted that Battley proposed, after lengthy but little known investigations of growth yields of *Saccharomyces cerevisiae* (*3*), (i) that energy obtained by either respiratory or fermentative pathways should serve equally well for biosynthesis and (ii) that aerobic, prototrophic bacteria whose only products of growth in minimal culture medium are cells and CO_2 should be used in model studies of efficiency of energy conversion by growing cultures.

These were attractive arguments, and we set out with them in mind. As a beginning, yields from growth of our soil pseudomonads based on molar consumption of substrate (Y_{sub} values), molar consumption of carbon (Y_{carb} values), and molar consumption of oxygen (Y_{O_2} values) were determined for the growth of the pseudomonads on limiting quantities of a variety of simple substrates that served simultaneously as sole sources of carbon and energy in minimal, mineral media. Mineral nutrients were not limiting, and NH_4^+ served as nitrogen source. Cultures were incubated at 30 C with aeration. Acting on advice from Elsden (private communication), the cells were harvested at the moment that the slope of the oxygen uptake curve turned sharply over to horizontal. At this time, the spent culture fluid was analyzed for unused substrate or organic products. Y_{sub} values were thus based on substrate actually utilized rather than the quantity provided at the beginning of the experiments. Maintenance energy for such cultures is considered to be zero.

RESULTS AND DISCUSSION

It was soon obvious from our results that Y_{sub}, Y_{carb} and Y_{O_2} values were widely divergent for growth of the pseudomonads on various substrates. A search for normalizing factors in our data was thus begun, following at first in much the same vein as other workers concerned with ATP-related yields, despite the realization that actual measurements of ATP production would be operationally impossible. But, basing our calculations on Hernandez and Johnson's (*18*) suggestion, the Y_{O_2} value for growth on acetate was assumed to correspond to synthesis of 2 moles of ATP for every mole of oxygen consumed (i.e., an assumed P/O ratio of 1 for acetate). With this assumption, an apparent Y_{ATP} value of 10.7 was obtained for acetate—very near Bauchop and Elsden's Y_{ATP} of 10.5 for anaerobic growth. Moreover, when the Y_{O_2} values for growth on the other substrates that we used (*21*) and that Hajipetrou et al. (*15*) used were all then made proportional to the Y_{O_2} value for growth on acetate, and fractional molar ATP-to-oxygen ratios were assumed, then apparent Y_{ATP} values very near 10.7 were obtained for the 20 different substrates utilized by our soil pseudomonads

and by *Aerobacter aerogenes*. This set of data could be significant in that the constancy of the values may reflect screening out, by our treatment of the data, of energy lost by mixed function oxidase activity or other nonphosphorylation-linked oxygen consumption. But, two currently untestable assumptions are included in the calculations. The potential worth of this approach thus could not be estimated, and it was abandoned.

Rather than continue to be concerned with quantity of ATP synthesized, we examined instead the oxidation-reduction state of the carbon atoms of the growth substrates utilized for possibly normalizing influences; and at this point an obvious but useful datum came to light (i.e., the number of available electrons per molecule of substrate). Every organic compound can be characterized by the number of these electrons, for they are the ones that are transferred to oxygen when a mole of the compound is completely burned to CO_2 and H_2O. Kharasch recorded figures for a great variety of compounds in 1925 (*19*). For example, glucose has 24 available electrons per mole, acetic acid has eight and dodecanol has 72. The value of this property became apparent when we divided the Y_{sub} values for growth on each of the 10 substrates we employed by the number of available electrons per mole of each substrate. This provided Y_{ave^-} values in units of grams dry weight of cells per equivalent of available electron (*21*). These Y_{ave^-} values clustered around a mean of 3.14. Figure 1 illustrates the simple calculation of

General consideration:

$$Y_{ave^-} = \frac{Y_{sub}}{ave^-} = \frac{\text{g cells/mole substrate}}{ave^-/\text{mole substrate}} = \frac{\text{g cells}}{ave^-}$$

For succinic acid: $Y_{sub} = 42.3$

$ave^- = 14$

$Y_{ave^-} = 3.02$ for *Pseudomonas* $C_{12}B$

For tetraethylene glycol: $Y_{sub} = 129.9$

$ave^- = 40$

$Y_{ave^-} = 3.25$ for Bacterium TEG-5

FIGURE 1.—Calculation of Y_{ave^-} values for aerobic, prototrophic heterotrophs.

Y_{ave}^- values for growth of pseudomonads $C_{12}B$ and TEG-5 on succinic acid and tetraethylene glycol, respectively. It can be seen that the yield per mole of succinic acid consumed was 42.3 grams dry weight. A mole of succinic acid contains 14 available electrons. Y_{ave}^- is thus 3.02, and the calculation of Y_{ave}^- for the ether glycol is similarly uncomplicated.

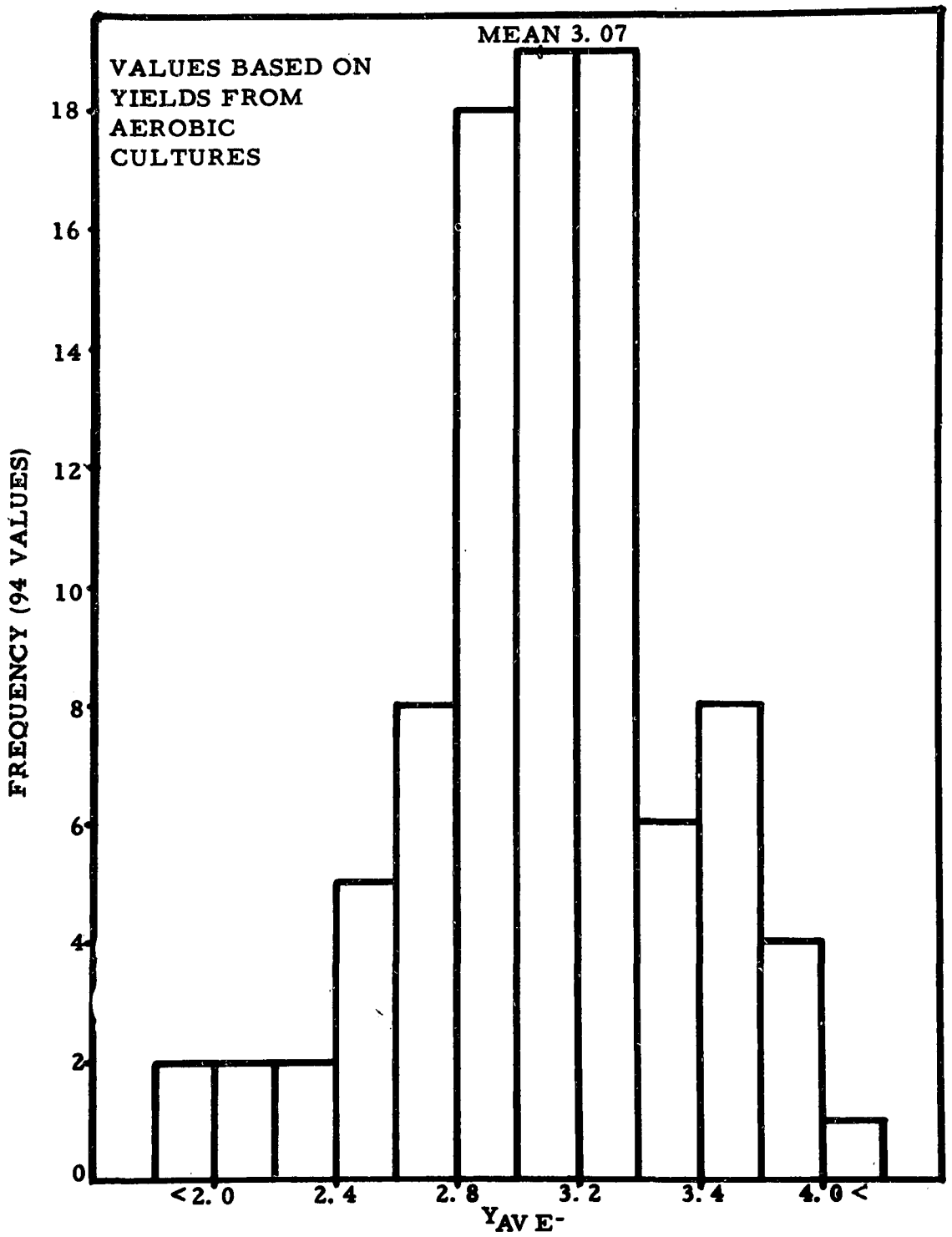

FIGURE 2.—Y_{ave}^- values for growth on various substrates calculated from data published in the microbiological and engineering literature (25; also see Table 1).

With these results in hand, it seemed reasonable to hypothesize that, irrespective of species or substrate utilized, the growth of prototrophic heterotrophic bacteria or yeasts will yield approximately 3.14 grams dry weight of cells per available electron in the substrate used as sole source of carbon and energy—if the conditions of our experiments are met or approximated. To test the hypothesis, an extensive search of the literature was initiated, and as the frequency diagram in Fig. 2 reveals, the hypothesis need not be rejected. This graph represents a compilation of data from a review of this topic presented in 1970 (25) plus Y_{ave}^- values from Table 1 that have come to our notice since that time.

Thus, both the mean Y_{ave}^- value of 3.07 from the earlier review (25) and 3.10 for data obtained from these 15 additional sources over the past two years are close to the predicted mean of 3.14, as is the overall mean of 3.07 for all the values we have been able to find. Three quarters of the values lie within 0.5 g of the mean even though none of the experiments were performed in any of the various laboratories with this type of analysis in mind. Apparently, prototrophic microorganisms have the capacity to budget the electrons of an organic substrate in minimal medium in predictable manner between biosynthesis and oxidation, and control of this distribution by an "investment-expenditure" gene has been hypothesized (25).

TABLE 1.—Y_{ave}^- Values Not Previously Recorded in (25)

Rieche et al. (29) growing *Torulopsis utilis* on acetic acid	2.84
Same studies (29) growing *T. utilis* on glucose	3.72
Vernerova and Belonsheva (34) growing *Candida tropicalis* on acetic acid	2.25
Tsuchiya and Drake (personal communication) growing *Escherichia coli* on glucose	2.93
Cama and Edwards (10) growing *Candida utilis* on acetate in batch culture	2.55
Same studies (10) growing *C. utilis* on acetate in continuous culture	3.36
Perry and Edwards (26) growing *Pseudomonas fluorescens* on maleic acid at 25 C	3.29
Edwards, Kinsella and Sholiton (13) growing *P. fluorescens* on maleic acid at 30 C	3.46
Same studies (13) growing *P. fluorescens* on malic acid at 35 C	2.86
Bongers (7) growing *Hydrogenomonas eutropha* on succinic acid	3.64
Same studies (7) growing *H. eutropha* on fumaric acid	3.24
Ahern, Turner and Mohan (1) growing *Candida tropicalis* on glucose	2.89
Gaudy, Obayashi and Gaudy (14) growing a *Flavobacterium* species on glucose	3.35
Harrison and Loveless (16) growing *Klebsiella aerogenes* on glucose	3.20
Same studies (16) growing *Escherichia coli* on glucose	2.90
	Mean 3.10

One reservation and a significant deviation should be noted here. For this prediction to hold, the organic substrates utilized must have at least one oxidized function in the molecule. Growth on methane or linear hydrocarbons provides smaller Y_{ave}- values with a mean of 2.08 (Fig. 3). Represented here are values derived from growth of *Candida* and *Torlua* species of yeasts, mixed bacterial cultures, *Micrococcus cerificans*, and unidentified species of *Mycobacterium*, *Nocardia* and *Pseudomonas*. None are derived from our own studies, but they are reported here because of the significantly different mean ob-

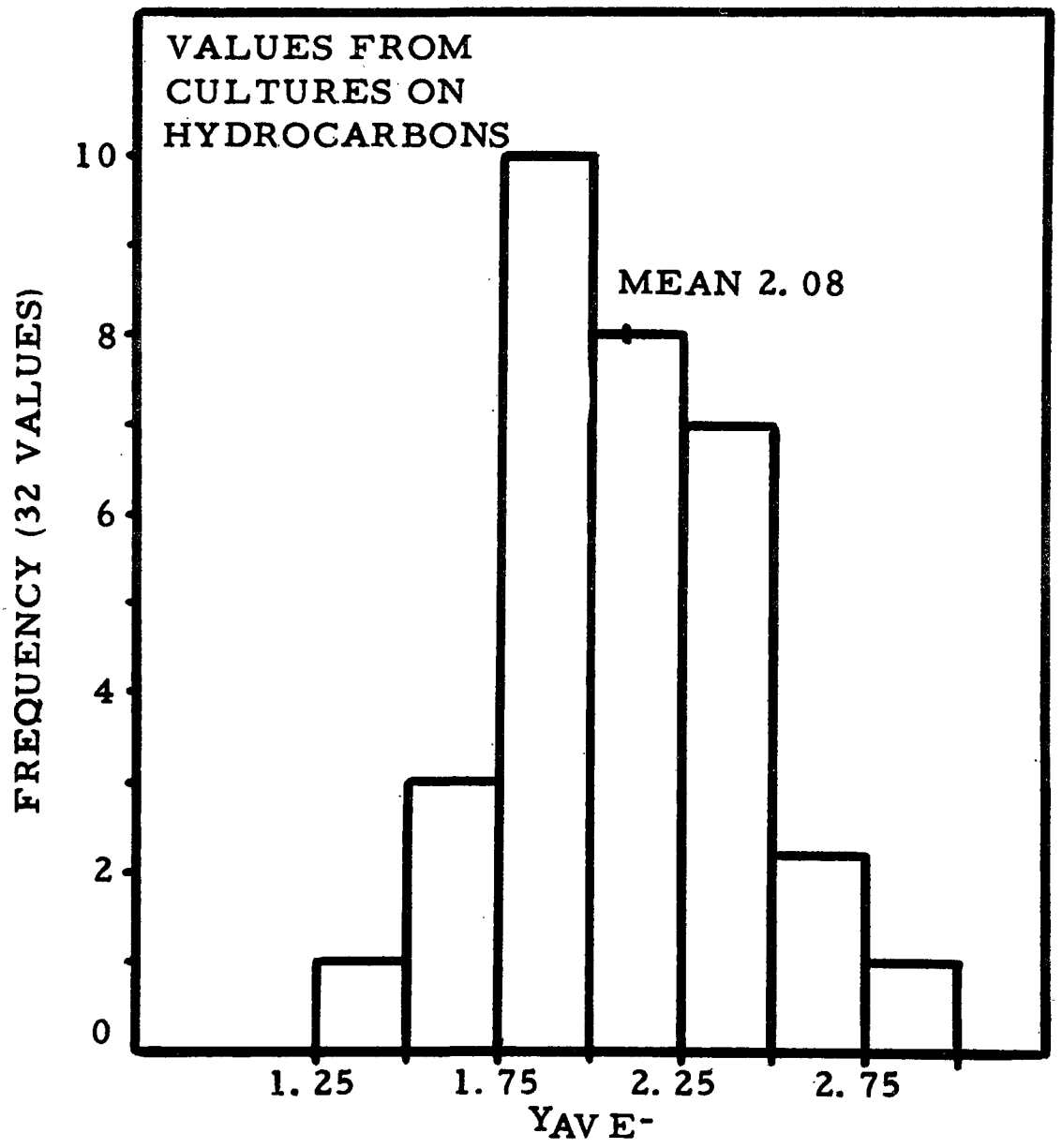

FIGURE 3.—Y_{ave}- values for growth on hydrocarbons calculated from data published in the microbiological and engineering literature (1, 6, 25, 32).

We predict that:

$$Y_{kcal} = \frac{Y \text{ (g dry wt of cells)}}{E_a + E_d} = \frac{Y \text{ (in g)}}{E_c \text{ (in kcal)}}$$

Numerically, therefore:

$$Y_{kcal} = \frac{Y_{ave^-}}{E_c} = \frac{3.14 \text{ g/ave}^-}{26.5 \text{ kcal/ave}^-} = 0.118 \text{ g/kcal}$$

Thus, the yield in grams dry weight per kilocalorie of the combined energy assimilated and dissimilated in a bacterial culture will approximate 0.118.

FIGURE 4.—Derivational basis for the value, Y_{kcal}.

served. Only four values have been added to the list (*1*, *6*, *32*) since the earlier review (*25*). No physiological explanation for the difference is currently at hand, but an interesting suggestion for dealing with it in the treatment of data was recently put forward by Bell (*5*). He derived an empirical mathematical factor to be subtracted from the number of hydrogen atoms in the hydrocarbon substrate molecules prior to analysis of yield data. This provided less variant Y_{carb} values. Bell considers Y_{carb} figures preferable to Y_{ave^-} values, for he holds carbon content rather than energy content of substrate to be the dominant factor regulating prototrophic growth.

Whether the influences of carbon and energy contents of organic substrates on growth are indeed separable as Bell suggests is yet to be determined. Meanwhile, we have asked if Y_{ave^-} values might not be used to gain insights into growth in more complex systems. The answer appears to be, yes. As indicated in Fig. 4, the ratio of yield of cells per available electron to kilocalories of energy that each available electron represents is proposed as an approximation of the yield of cells that may be expected per kilocalorie of energy taken in any way from a culture medium by microorganisms growing either aerobically or anaerobically. This value is represented as the Y_{kcal}, and its calculation is based on the average energy potential of the available electron in organic material considered generally. This latter figure was approximated as 26.5 kcal/ave$^-$ by dividing the molar heats of combustion of a wide variety of organic compounds, as specified by Kharasch (*19*), by the number of available electrons per mole of each—and then averaging.

SAMPLE CALCULATION

Pseudomonas C12B

$$Y_{kcal} = \frac{Y_{sub}}{E_a + E_d} \text{ for aerobic growth on benzoate}$$

$$= \frac{86.8 \text{ g/mole}}{[86.8 \text{ g/mole} \times 5.3 \text{ kcal/g}] + [106 \text{ kcal/mole} \times 3.46 \text{ mole/mole}]}$$

$$= \frac{86.8 \text{ g/mole}}{460 \text{ kcal/mole} + 366 \text{ kcal/mole}}$$

$$= 0.105 \text{ g/kcal}$$

FIGURE 5.—Procedure for determining a Y_{kcal} value from experimental data from an aerobic growth yield.

Actual calculation of Y_{kcal} approximations were undertaken as indicated in Fig. 5. The molar growth yields from a number of studies were related directly to the sums (represented as E_c) of the quantities of energy taken both by assimilation and dissimilation from the culture media employed. To calculate E_a (the energy assimilated) Y_{sub} values were multiplied by the average calorific content of bacterial cells. This value is taken as 5.3 kilocalories per gram ash-free dry weight (*28*). To calculate E_d (the energy dissimilated), the number of moles of oxygen consumed per mole of substrate utilized during growth was multiplied by the energy equivalent of four available electrons, for one mole of oxygen requires four electrons for reduction.

SAMPLE CALCULATION

Streptococcus fecalis

$$Y_{kcal} = \frac{Y_{sub}}{E_a + E_d} \text{ for fermentative growth on glucose}$$

$$= \frac{22 \text{ g/mole}}{[22 \text{ g/mole} \times 5.3 \text{ kcal/g}] + [673 \text{ kcal/mole} - 2(326 \text{ kcal/mole})]}$$

$$= \frac{22 \text{ g/mole}}{117 \text{ kcal/mole} + 21 \text{ kcal/mole}}$$

$$= 0.159 \text{ g/kcal}$$

FIGURE 6.—Procedure for determining a Y_{kcal} value from experimental data from a fermentative growth yield.

Since each available electron in an organic compound represents approximately 26.5 kilocalories, four available electrons represent 106 kilocalories. It can thus be seen that aerobic growth on benzoate provided a Y_{kcal} of 0.105.

Calculation of Y_{kcal} values for fermentative growth (Fig. 6) required only a different method for estimating E_d values. These may be derived from fermentation balance data and are

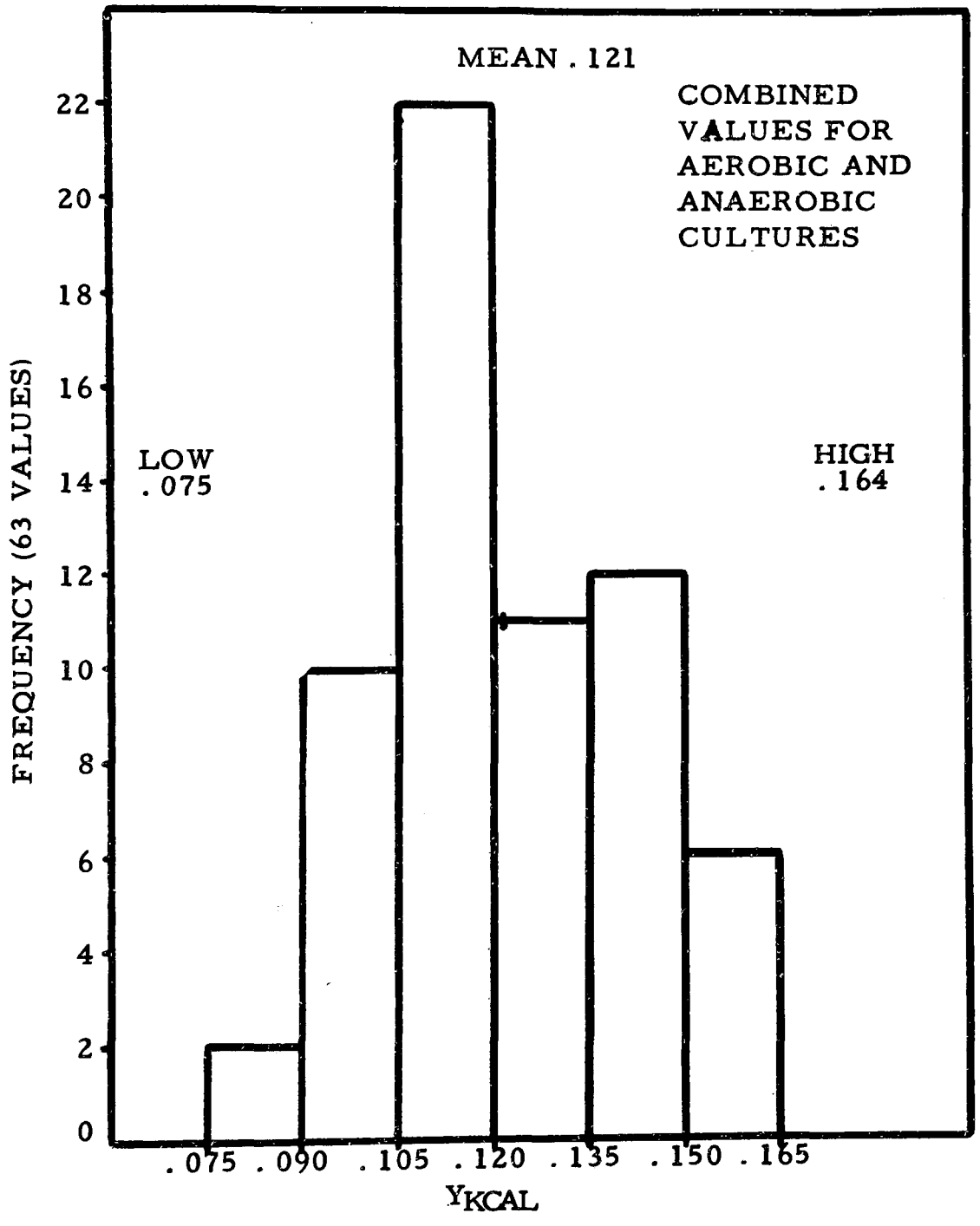

FIGURE 7.—Y_{kcal} values calculated from figures published in the microbiological and engineering literature (24, 25).

simply the difference in molar terms between the heat of combustion of the original substrate and the heat of combustion of all the fermentation products—between a mole of glucose and two moles of lactic acid in this example. Values for fermentative growth calculated out slightly higher than those for aerobic growth. Means were 0.130 and 0.116, respectively. An overall mean (Fig. 7), when both were considered together, was then 0.121, which is remarkably near the predicted value of 0.118. Only one value has been added since an earlier review (*25*)—0.110 for growth of *Saccharomyces carlsbergensis* on glucose in a complex medium (*24*).

By algebraic rearrangement, the expression $Y_{kcal} = Y_{sub}/E_c$ can be stated as $E_c = 8.47 \times Y_{sub}$ (i.e., the energy in kilocalories per mole removed in any way from a culture medium is related to the yield in grams ash-free dry weight per mole by the constant 8.47). It seems likely that, in one of these forms, this expression may be usefully applied as a base for prediction of yield and cost feasibility for projected production runs.

An additional word should be said about the value we employed to express the calorific content of cells. The experimental estimate obtained by bomb calorimetry was 5.38 kilocalories per gram ash-free dry weight (*28*) as the average for a number of types of bacteria and yeasts. This agrees closely with the value projected from the elemental composition of bacteria (Fig. 8). Neglecting ash content, bacterial cells were determined by elemental analysis to comprise a hypothetical polymer of $C_4H_8O_2N$ with a formula weight of 102 (*22*). This predicts, the number of available electrons in this formulation, a heat

Energy Content Predicted from
Elemental Analysis of *Pseudomonas* C12B

$C_4H_8O_2N$ (neglecting ash); F. W. 102

Thus,

21 ave$^-$/F. W. at approximately 26.5 kcal each = 556.5 kcal/102g
= 5.46 kcal/g;

(experimental value, 5.38 kcal/g)

FIGURE 8.—Estimation of probable calorific content of bacteria cells from elemental analysis and average energy equivalence of each available electron.

$$\Delta H = \Delta F + T\Delta S$$

Molar heats of combustion 1 *number of available electrons per mole* = *Average energy equivalent of each ave⁻ in organic material generally* = 26.5 kcal/mole⁻ ΔH

$$\frac{\text{Average yield}}{3.07\ \text{g/ave}^-} \times \frac{\text{Average cellular energy}}{5.3\ \text{kcal/g}} = \frac{\text{Energy in average yield of cells}}{\Delta F\ 16.3\ \text{kcal/ave}^-}$$

$$\frac{\text{Thus, energy lost to the system by dissimilation}}{T\Delta S\ 10.2\ \text{kcal/ave}^-} =$$

In sum, aerobic growth represents approximately 61% conservation at the expense of 39% loss of substrate energy.

FIGURE 9.—Rationale for estimating energy budgeting of aerobic prototrophic growth of microorganisms.

content of 5.46 kilocalories per gram. Another close approximation was obtained by Mennett and Nakayama (*23*) who determined the macromolecular composition of *Pseudomonas fluorescens*, then based their calculations on the average heats of combustion recorded in the literature for proteins, nucleic acids, polysaccharides and lipids. Their estimate was 5.3 kilocalories per gram. Use of this value for calculating Y_{kcal} thus seems justified.

From the foregoing studies, a strictly controlled energy budget for aerobic, prototrophic microbial growth has been postulated (Fig. 9). Reasoning that microbial cultures proceed in accord with the second law of thermodynamics, we've taken the average energy content of the available electron in nature, 26.5 kilocalories, to represent the culture's enthalpy or ΔH. Growth then yields an average of 3.07 grams of cells per available electron at approximately 5.3 kilocalories per gram. Thus, 16.3 kilocalories of energy are conserved (representing free energy or ΔF). By difference, approximately 10.2 kilocalories of energy are randomized by the system (representing the entropy term, TΔS). When the distribution of the energy represented by available electrons in the original growth substrate is thus accounted for, conservation into cellular structure averages 61 percent and loss 39 percent.

Such figures are consistent with Terroine and Wurmser's (*33*) estimate (as far back as 1922) of 60 percent energy conservation by *Aspergillus niger*. Moreover, in 1950 Siegel and Clifton (*30*)

found that *Escherichia coli* growing in glucose minimal medium oxidized 40 percent of the glucose carbon and assimilated 60 percent at any time from lag to log growth phase. During the 1960's Herbert and his colleagues (*17*) obtained similar values for growth of *Aerobacter* on glycerol. MacKechnie and Dawes (*20*) did likewise for *Pseudomonas aeruginosa* growing on glucose, gluconate and 2-oxo-gluconate at 30 and 37 C. Busch and Myrick (*9*) found a similar, reliable distribution of 59 percent of the carbon into cells and 41 percent into CO_2 when mixed sewage and soil cultures were grown on glucose. And, Danforth (*11*) observed an identical distribution of the carbon of acetate by heterotrophically growing, bleached *Euglena.*

One other persuasive bit of evidence exists that is consistent with the notion that the "investment-expenditure" control mechanism for optimal microbial growth couples the amount of energy that we can measure as biomass accumulated specifically and predictably with the quantity of energy expended in the effort. We can accept Bail's (*2*) original suggestion of an M concentration for a bacterial culture, measured in numbers of viable cells per unit volume, as an indication of the constancy of biomass accumulation for any bacterial species grown under standardized conditions. But, a significant study was carried out in 1954 by Smith and Johnson (*31*) with a pigmented and a non-pigmented strain of *Serratia marcescens.* They observed that, under identical culture conditions in identical volumes of minimal medium, a larger population of the pigmented than the colorless bacteria were obtained at M concentration for each. This was not in keeping with Bail's hypothesis, for the bacteria were monospecific. Smith and Johnson (*31*) noted on microscopic examination, however, that the colorless cells were larger than the pigmented. When we analyzed their yield data 16 years later in terms of the concept of yield per available electron per mole of substrate utilized (*25*), there was no difference in the Y_{ave}^- values for the pigmented and non-pigmented cells. M concentration thus appears to be more appropriately expressed in grams dry weight than in numbers of cells.

Finally, what appears to be the simplest possible growth system has been examined, and has revealed data in good accord with our predictions. It is described separately here

because all the values presented to this point were derived from growth yields of heterotrophic microorganisms. But, Bongers (*8*) found that for the autotrophic growth of *Hydrogenomonas* in a mineral salts medium gassed with an optimal mixture of hydrogen, carbon dioxide and oxygen, Y_{sub} (i.e., Y_{H_2}) values of 6.2 to 6.6 were obtained. Hydrogen contains 2 ave^- per mole. Thus, by division, Y_{ave^-} values for *H. eutropha* growing autotrophically on hydrogen range from 3.1 to 3.3, bracketing the predicted value of 3.14.

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I am grateful for the many contributions made by Drs. W. R. Mayberry and G. J. Prochazka to this work.

Questions and Comments following Dr. Payne's Talk

QUESTION FROM THE FLOOR: Do you know how far you depart from the growth yield values as you depart from the optimum conditions?

DR. PAYNE: I haven't made such calculations; however, when we examined a paper reporting the influence of temperature on yield, we took the optimum values. In many papers one has to convert from one set of units to another and the data is usually given in grams of organisms per gram of substrate or grams of organisms per gram mole of substrate. I simply took the optimum value that the experimenter reported and did not analyze the various numbers given.

QUESTION FROM THE FLOOR: If you got into a freshwater situation with substrate concentrations of 10^{-6} or 10^{-8} molar, would you expect the same degree of efficiency and energy utilization?

DR. PAYNE: I can't answer that because referring back to my own data, we took non-limiting concentrations of carbon substrate. We don't know what the situation would be when there is micro-micro quantities of substrate. The only thing I can bring to bear on that is the work by Siegel and Clifton, back in 1950. They showed that at any time during the growth of *E. coli* substrate glucose taken up by these organisms would be divided with 40% of it oxidized and 60% assimilated. Whether that would pertain to micro molar quantities, I don't know.

The Sulfur Cycle

J. LE GALL

C.N.R.S., Marseilles, France

INTRODUCTION

Although the general title of this series of communications is restricted to the influence of microbial populations upon the aquatic environment, it is necessary to discuss first what we shall refer to later as the "Global Sulfur Cycle". By global sulfur cycle, we mean a model for the transfer of sulfur on a planetarial scale from the seas to the atmosphere to the lands and so on (see Kellog *et al.*, (*15*)). The definition of the Sulfur Cycle given in most textbooks is generally confined to biological aspects and particularly to the description of the bacterial groups which are involved in the reduction or oxidation steps of elemental sulfur. Such a representation gives but a partial picture of the different processes by which sulfur is being cycled on our planet. If we really want to understand and eventually control the flux of the natural elements which are necessary for our survival, we need a clear picture of all natural cycles and their interconnections, including all sources and forms of a given element, together with the amounts involved, on a planetary scale. As far as sulfur is concerned it is now possible to find such data in the literature. In the present paper we shall discuss the models that have been proposed and point out the uncertainties that still exist. Then we shall turn to the biological sulfur cycle and give a few examples of the interactions among the different groups of microorganisms which are involved in the cycle with the environment. Examples are so numerous that it is not possible to give an exhaustive list, but by pointing out

some characteristic examples, we shall try to make clear to the reader how delicate the balance between the environment and the sulfur bacteria is and what can be the consequences of man's bad management of our natural resources.

The Global Sulfur Cycle

Models representing the global sulfur cycle have been published. Figure 1 shows a simplification of the model presented by Kellog, *et al.* (*15*). The authors propose that in order to compensate the sulfur intake living organisms must produce 282×10^6 tons of "reduced" sulfur per year, that form of sulfur

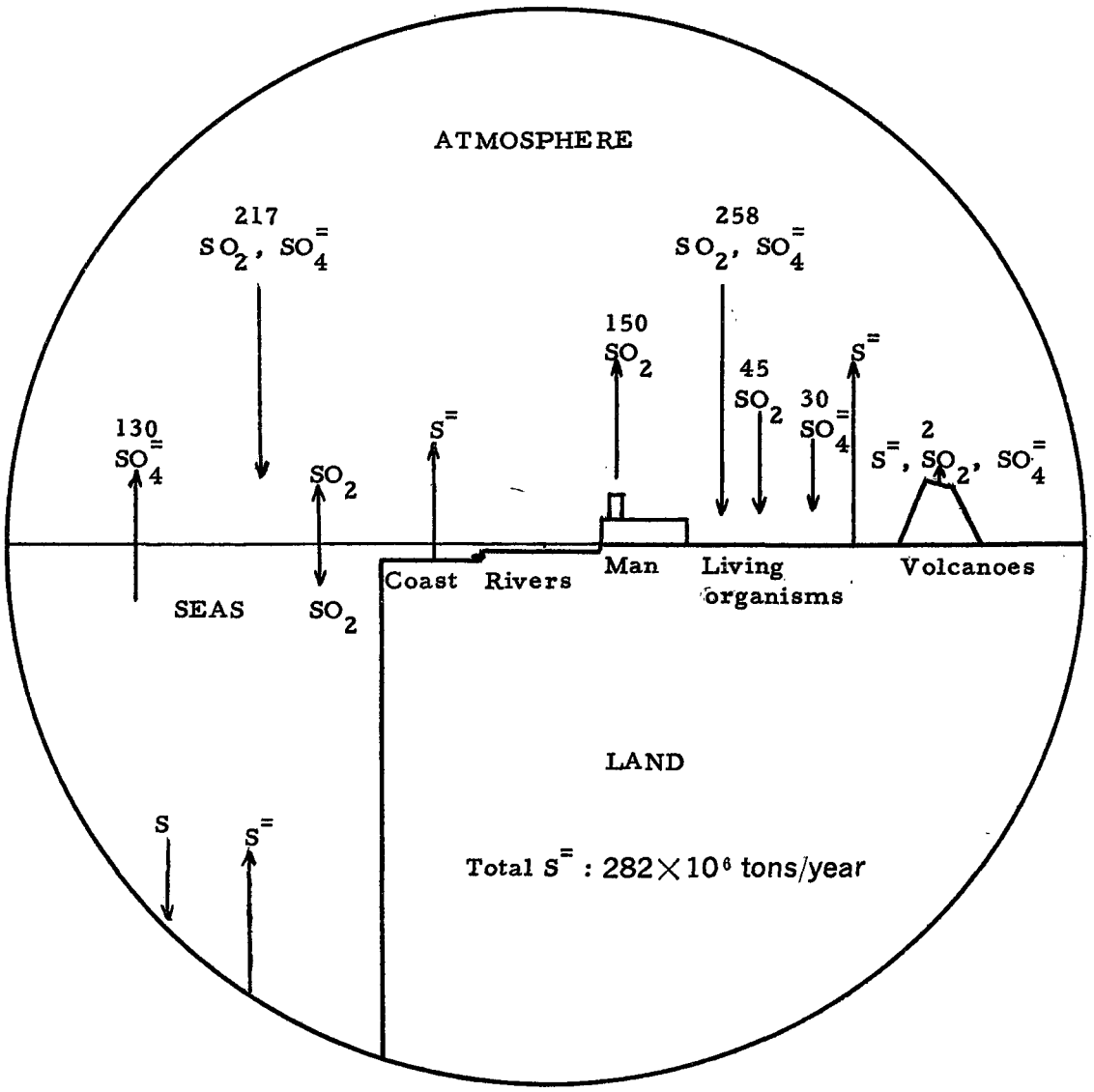

FIGURE 1.—The global sulfur cycle (from Kellog *et al.*, (15)).

Figures are given in million of tons per year. In order to balance the cycle, 282×10^6 tons per year of "reduced" sulfur must be produced by living organisms. For a discussion on the nature of the "reduced" form of sulfur, see text.

being sent into the atmosphere. It is their conclusion that man is contributing more and more to this figure and is presently sending 150×10^6 tons per year of sulfur, mainly in the form of sulfur dioxide (SO_2) into the atmosphere and within a few years he will be overmatching nature. Two questions are to be posed then:

(1) What is the form of sulfur which is sent by living organisms into the atmosphere and what are the organisms which are responsible for this process?

(2) What is the effect of man's contribution upon the natural balance of sulfur?

The answer to the first question is not yet known with certainty. It was generally assumed that hydrogen sulfide was playing that role. But since this compound was not detectable, even in trace amounts, Lovelock *et al* (*20*) concluded that other compounds could be the actual form under which sulfur is present in the atmosphere. Indeed, they found that the concentration of dimethyl sulfide (CH_3–S–CH_3) was sufficient so that this organic sulfur compound could be the actual form of sulfur which is balancing the cycle. The question was then to determine the origin of this compound. Challenger (*9*) reported that many living systems are able to produce dimethyl sulfide and, in particular, marine algae. Lovelock *et al* (*20*) found that samples of *Laminaria* produced dimethyl sulfide at a rate of $50\text{–}400 \times 10^{-12}$ g $\times g^{-1} \times h^{-1}$ of fresh wet tissue. Leaves from cotton, spruce, oak and pine trees also were capable of producing the same compound. The possible role of bacteria in the production of organic sulfur was not examined by the authors, however, it should not be overlooked since Baas-Becking and Ferguson-Wood (*5*) demonstrated in 1955 that sulfate reducing bacteria and thiobacilli were able to produce such compounds. In conclusion, one has to point out that, surprisingly enough, we still don't know with certainty which are the compounds balancing the sulfur cycle and the contribution of the different groups of living organisms in this balance.

We now must try to answer the second question: Kellog *et al* (*15*) conclude that man's contribution to the atmospheric sulfur is already too great and will still increase in the future. But it is important to note that this does not mean that the sulfur thus

sent into the atmosphere has the time to be evenly diluted. Increasing air pollution above industrialized areas has a tendency to increase the total concentration of sulfur in rainfalls, thus creating an artificial "sulfur cycle" limited to these areas. Data exist supporting the idea: for example in Virginia (*3*) the sulfur deposited at Norfolk, a very heavily industrialized area was of 33.5 pounds/acre/year, when at Halifax, a rural area, it was only 12.7 pounds/acre/year. In fact, sulfur deficiencies appear more and more in very wide areas of the earth in addition to previously well-known areas where sulfur deficiencies were always present such as Africa, Australia, New Zealand, etc . . . severe sulfur deficiencies are developing. The reasons for this have been developed by Tisdale (*26*):

—continued cropping and increasing yields have decreased the sulfur content of the soils;
—the change of patterns of fuel consumption (from coal to oil) has decreased the sulfur deposited in rainfalls;
—and finally the use of modern fertilizers and pesticides which contain little or no sulfur also has decreased the sulfur content of soils.

As a consequence sulfur deficiencies appear in the United States. Examples can be given from Georgia (*2*) and Virginia (*3*). In France a similar situation has been reported in the Yonne County (*25*). Man's role in the imbalance of the global sulfur cycle is then clear, we are sending more and more sulfur into the atmosphere, but at the same time we are decreasing the sulfur content of soils without rendering "our" sulfur available for crop production. In addition, since from Kellog's model (*15*) it is evident that coastal areas are important in the balance, our tendency to over-urbanize and, at the same time, to destroy the ecological balances of these regions (in particular marshes) also contributes to the decrease of organic sulfur in the atmosphere.

The Biological Sulfur Cycle

A simple way of representing the sulfur cycle as it occurs in soils and waters is given on Figure 2. Although the lower part of the cycle (namely the synthesis and decomposition of

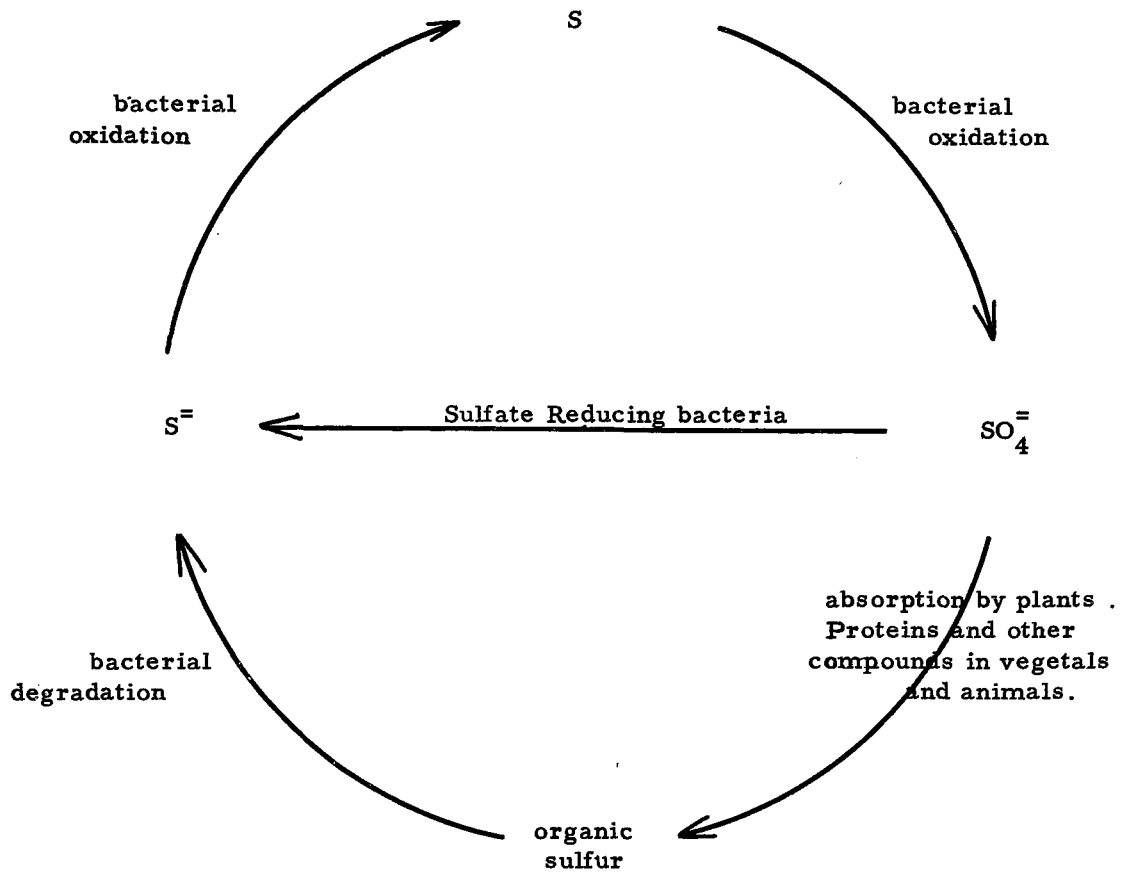

FIGURE 2.—The biological sulfur cycle.

organic sulfur-containing substances) is a vast and interesting field, it is not the purpose of this paper to discuss its various aspects. Our interest will be mainly centered on what may be called the "dissimilatory" sulfur cycle which is carried out by bacteria.

Two groups of sulfur oxidizing bacteria are aerobic and chemoantotrophic organisms, namely the Thiobacilli and the Beggiatoaceae. Thiobacilli oxidizes sulfur according to the following reaction:

$$2H_2O + 2S^\circ + O_2 \rightarrow 2H_2SO_4$$

so, the result of their proliferation is a sharp drop of pH which can have important consequences upon the environment, as will be discussed later.

The other groups of sulfur oxidizing bacteria, the purple and green photosynthetic bacteria, are almost exclusively anaerobes and derive their carbon from CO_2 with reduced sulfur as electron source:

$$CO_2 + 2H_2S \rightarrow (CH_2O) + 2S^\circ + H_2O$$

Since the reduction of sulfur is a strictly anaerobic process, one can see that the sulfur cycle can turn with little or no atmospheric oxygen. In fact, such an environment, where low oxygen tensions are predominant, have been called a "sulfuretum" by Baas Backing (*4*) with the following definition "The natural ecological community of these bacteria (the sulfur bacteria) is a miniature cycle in itself and will be called Sulfuretum."

The ecological system thus created is completed by the aerobic sulfur oxidizers, some algae and even higher organisms such as protozoa and nematodes able to tolerate low oxygen tensions. A recent work by Fenchel and Rield (*13*) has shown that even more organisms are implicated in sulfureta and that they cover large areas under the oceans, ranging from high tide level to the sea bottom.

Such a notion of an anaerobic or semi-anaerobic world living close to our aerobic world has a very basic importance if one takes Oparin's view that oxygen was absent from our atmosphere when life first appeared, then the organisms present in the sulfureta can represent living relics of the very first stages of evolution.

The agents responsible for the reduction of sulfates and other oxidized sulfur compounds are called sulfate reducing bacteria. They are all strict anaerobes and have been classified by Postgate and Campbell (*24*) and Campbell and Postgate (*8*) into two genera: *Desulfovibrio* and *Desulfatomaculum.*

They utilize oxidized sulfur on a dissimilatory level leading, in contrast to the assimilatory pathway (which is used by organisms for the biosynthesis of organic sulfur compounds such as amino acids), to the accumulation of large amounts of hydrogen sulfide (*23*).

The typical metabolism of the different species belonging to the genus *Desulfovibrio* is as follows:

$$\text{Carbon Substrate} + SO_4^{=} \rightarrow S^{=} + CO_2^{=} + \text{acetate}$$

They are not true autotrophs since they require organic substrates for growth. A review of the various aspects of their metabolism is in press (*19*).

It is mainly the sulfate reducing bacteria which are responsible for the accumulation of hydrogen sulfide in nature. It has been

calculated by Deuser (*12*) that in the Black Sea, where concentrations of H_2S up to 1 g per liter can be found, only 2.5 percent of this compound comes from organic matter.

During their metabolic activity, the sulfur bacteria can produce sulfur compounds which are potentially active reagents, such as sulfuric acid or hydrogen sulfide, and thus able to have severe consequences upon the environment. Under normal conditions, the sulfur cycle will turn smoothly and will be unnoticed, although ubiquitous. But if for some reason the cycle becomes unbalanced, then one of the terminal products may accumulate, sometimes in a useful way, sometimes with catastrophic consequences. The following section will deal with some of these economic consequences of the proliferation of sulfur bacteria.

Economic Activities

Table 1 gives a list of the possible interactions of sulfur bacteria with man's activities. As impressive as it is, this list is far from being complete. We have chosen a few examples that will mainly serve to illustrate their actual importance.

OIL TECHNOLOGY

Sulfate reducing bacteria are almost always associated with oil deposits although their actual role, either in the oxidation of oil or in oil synthesis, is not yet known with certainty.

As it has been pointed out by Floodgate (*14*), in contrast to

TABLE 1.—Some Economic Activities of the Sulfur Bacteria.

—weathering of rocks
—attack of concrete (dam)
—attack of Ankor-Vat temple
—formation, oxidation of metal sulfide ores (acid mine waters)
—formation of sulfur deposits
—pollution of artificial ports (marinas)
—oil technology: sulfur, corrosion in oil wells
—sulfur in town and natural gas
—lethality of various gases
—mortality of fish
—mortality of man
—etc . . .

the aerobic mechanism, the evidence for a possible anaerobic pathway of hydrocarbon degradation remains meager. Furthermore, as has been pointed out by Alexander (*1*), as a consequence of their dissimilatory use of sulfate, in order to be of importance in the degradation of hydrocarbons, they would have to reduce huge amounts of sulfate, which is not usually present in that high a concentration. However, Kusnetov (*18*) states that, when marine water is pumped into the stratum to maintain stratal pressure, the oil becomes the source of energy to sulfate reducing bacteria, and H_2S is sometimes formed at the rate of 0.2 mg/liter/24 hours. The explanation for this apparently contradictory statement probably resides in the fact that another organism attacks the oil and provides some organic material for the growth of the sulfate reducers. The presence of such a syntrophy has been demonstrated by Kuznetsov and Gorlenko (*16*) who found that a *Pseudomonas* was able to provide substrates for sulfate reduction in the Romashkino oil field. Kusnetzov's observations provide at least an excellent example in which an unwanted and unexpected imbalance has been created, leading to the attack of a useful compound.

Whether or not sulfate reducing bacteria play an important role in oil formation is still an open question. Oppenheimer (*22*) demonstrated the presence of "hydrocarbon-like" compounds in mixed cultures of sulfate reducing bacteria. When a pure culture of sulfate reducing bacteria was used, the organisms contained 0.8 percent of hydrocarbon-like material and, after heat treatment, 4 percent of the same material.

The formation of oil is most probably a very intricate process involving several types of organisms. In an interesting paper, Ciereszko and Youngblood (*10*) showed that sediments of decomposed gorgonia yielded an n-alkyl disulfide fraction containing a mixture of:

$$R_{16}\text{–S–S–}R_{16};\ R_{16}\text{–S–S–}R_{18};\ R_{18}\text{–S–S–}R_{18}.$$

Desulfurization of these compounds would give the corresponding hydrocarbons. The disulfides were probably derived from gorgosterol ($C_{30}H_{50}O$). It is obvious that a close examination of the ecosystem responsible for the decomposition of the gorgonia, and in which sulfate reducers are most probably involved, would be of the greatest interest.

Formation of Sulfur Deposits

Several examples of the formation of sulfur deposits have been given. Kusnetzov (*17*) has described the mechanism of this formation in Russia. In that particular example, hydrogen sulfide originates from an oil field, in the water, and produces 0.2 mg H_2S/liter/24 hours. Ground water and H_2S containing oil and water are transported by gas pressure near the surface. There the water contains oxygen and *Thiobacillus thioparus* in large quantities. It is estimated that about 50 percent of H_2S is oxidized to sulfur and that *T. thioparus* contributes up to 20 percent, the rest being chemical oxidation.

The observations by Butlin and Postgate (*7*) that certain lakes in Lybia produce elemental sulfur (in that case the oxidizing agents are the green photosynthetic bacteria *Chromatium* or *Chlordirum*) up to 100 to 200 tons of crude sulfur per annum, has led them to propose that sulfur could be precipitated out of sewage water. This works on a pilot scale in London, India and also in Czechoslovakia where sulfur is produced from industrial wastes (*6*). In Russia, Murzaev (*21*) calculated that the sewage water of Moscow could make a useful contribution to the sulfur supplies of USSR.

Acid Mine Water

The role of sulfur oxidizing bacteria in the formation of acid mine water is now well established (*11*). It is due to the oxidation of pyrites by members of the genus *Thiobacillus* which results in the formation of sulfuric acid producing a drop of pH (with values in the order of 2.8).

Tuttle *et al* (*27, 28*) have proposed the utilization of wood dust and possible sewage water to correct the pH of acid mine water. In their reported experiments, they were able to raise the pH from 2.84 to 3.38. The decomposition of wood provides substrates for the sulfate reducing bacteria and the production of sulfides raises the pH. Unfortunately, this procedure does not solve the problem entirely since the sulfides should be removed before they have a chance to be reoxidized.

CONCLUSION

We have seen that many questions are still unanswered concerning the sulfur cycle. More research is needed in order to determine what is the actual role of plants and microorganisms in the balance of the global sulfur cycle and in particular in the biosynthesis of sulfur compounds. More information is also

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needed concerning the relations between the different groups of microorganisms. This has been clearly illustrated by the example of hydrocarbon degradation and in acid mine water treatment.

When all this information is available we may be able to understand and eventually control one of the natural cycles which are necessary for our survival on this planet.

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Questions and Comments Following Dr. LeGall's Talk

QUESTION FROM THE FLOOR: Would you predict that with the massive use of aluminum sulfate and tertiary treatment which has been proposed for some plants in the United States that some problems will arise with regard to the sulfate cycle?

DR. LEGALL: It all depends on whether or not there is a tendency for the water containing the sulfate becoming anaerobic. As long as you have dissolved oxygen plus sulfate no reduction of sulfate occurs but problems develop when you have anaerobic conditions. These anaerobic conditions can be created by organisms besides *Desulfovibro*; for example by algae growing, as they remove oxygen during part of the daily cycle. And I would wonder about any disturbance of the sediments in doing the same sort of thing.

QUESTION FROM THE FLOOR: What do you feel would be the environmental consequence of nitrification inhibitions. In other words, to the other cycles?

DR. LEGALL: Nothing at all. There were a number of statements, about the disasterous affect of nitrification inhibitors. Dr. Hardy said that the nitrogen reserve is about 10 million years so if we inhibit nitrification with a compound which has a life of 10 million years and cover the earth with 1% of this inhibitor then we have 1 billion years of reserve.

Global Nitrogen Cycling: Pools, Evolution, Transformations, Transfers, Quantitation and Research Needs

R. W. F. HARDY and R. D. HOLSTEN

Central Research Department
E. I. du Pont de Nemours & Co.
Wilmington, Delaware 19898

INTRODUCTION

One of the earliest designations for nitrogen was azote, meaning without life; however, it is a combination of biological as well as abiological chemical transformations and purely physical transfers that constitute the global nitrogen cycle. Our presentation considers the nitrogen cycle with emphasis on: nitrogen compounds, nitrogen pools, nitrogen transformations, nitrogen transfers, global nitrogen cycle and limitations and research needs in this sequence. Each nitrogen transformation will be defined and examined for biological and abiological reactions, organisms, biochemistry and physiology relevant to ecological studies especially of aqueous environments, methodology, occurrence and rate. An understanding of the biochemistry and physiology of each transformation is an essential prerequisite for effective investigations of these reactions in the "gemische" of natural environments. In one striking case fundamental biochemical research led to a significant new analytical application. Difficulties in measurement undoubtedly impose the greatest limitation on environmental work. New analytical approaches are essential in order to provide adequate measurements for the development, testing and application of realistic model systems. For example, ^{15}N mass spectrometric analyses

are widely applied in nitrogen cycle studies, but the method involves assumptions which are not always valid (*13*). We can think of no greater contribution the present paper can make than to stimulate research to develop novel and facile assays to enable extensive quantitation of the various nitrogen transformations in the biosphere.

The breadth of the nitrogen cycle makes it difficult, if not impossible, for any two scientists to be intimately familiar with all phases of it. Therefore, we will emphasize the input reaction, dinitrogen fixation, which is our particular research specialty, and denitrification, the major output reaction on which we can speak only as reviewers of the literature; brief outlines of the other transformations will be presented here but will be considered in detail by the following paper.

Several of the topics—nitrogen pools, possible evolution of biological nitrogen transformations and quantitative estimates of global nitrogen cycling are based on a recent monograph "Nitrogen Fixation in Bacteria and Higher Plants" by Burns and Hardy (*24*). Other recent general reviews of the nitrogen cycle (*9, 28, 33, 40, 45, 83, 104, 156, 195*) are also available as are several that specifically emphasize the aquatic environment (*4, 17, 56, 101, 102, 120, 122*).

TABLE 1.—Oxidation numbers of nitrogen

	Nitrogen Compounds		
	Environmental Significance		
Oxidation number	Main	Possible intermediate	Other
6+			NO_3
5+	HNO_3		N_2O_5
4+	NO_2		
3+	HNO_2		N_2O_3
2+	NO		$H_2N_2O_3$
1+	N_2O	$(HON)_2$	NO_2NH_2
0	N_2		
1−		NH_2OH_2; N_2H_2	HN_3
2−		N_2H_4	
3−	NH_3; RNH_2		

NITROGEN COMPOUNDS

Compounds containing nitrogen in oxidation states from −3 to +6 inclusive are known (Table 1). The main nitrogen compounds in the environment are ammonia and organic amines (−3), molecular nitrogen or dinitrogen, the latter designation introduced by chemists and increasingly used because it distinguishes N_2 from N (O), nitrous oxide (+1), nitric oxide (+2), nitrous acid or nitrite (+3), nitrogen dioxide (+4) and nitric acid or nitrate (+5). In addition other oxidation states are represented by proposed intermediates in biological transformations *e.g.*, hydrazine (−2), hydroxylamine and diazene or diimide (−1). Other nitrogen compounds are known but their global abundance is negligible and their role in nitrogen cycling insignificant.

NITROGEN POOLS

The five major pools of global nitrogen (*93*, *120*, *168*) are diagramed in Figure 1 (*24*). The largest pool, containing 97.8 percent of all nitrogen, is nitrogen occluded in primary rocks, mainly as dinitrogen. The second largest pool, making up 2.0 percent of the total, is in the atmosphere. Quantitatively, N_2, with an average turnover time of about 17 million years is by far the dominant species; however, the traces of other gases—N_2O, NO, NO_2, and NH_3—and aerosols—NH_4^+ and NO_3^-/NO_2^-—with turnover times as short as several minutes are highly significant in nitrogen cycling within the atmosphere and between the atmosphere and the land-sea pool. The third largest pool occurs in sedimentary rock and the fourth in deep sea sediments. Finally, the smallest pool is the land-sea pool. The major components of the land-sea pool listed in order of descending quantitative significance are N_2 dissolved in the sea, soluble inorganic nitrogen mainly as nitrates in the sea, dead organic matter in the land and sea, insoluble inorganic matter in the land, biomass in land and sea and soluble inorganic nitrogen in land. It is obvious that this pool contains only an infinitesimal quantity of global N, but with respect to cycling of nitrogen and environmental concern, the land-sea and the atmospheric pools are of primary concern.

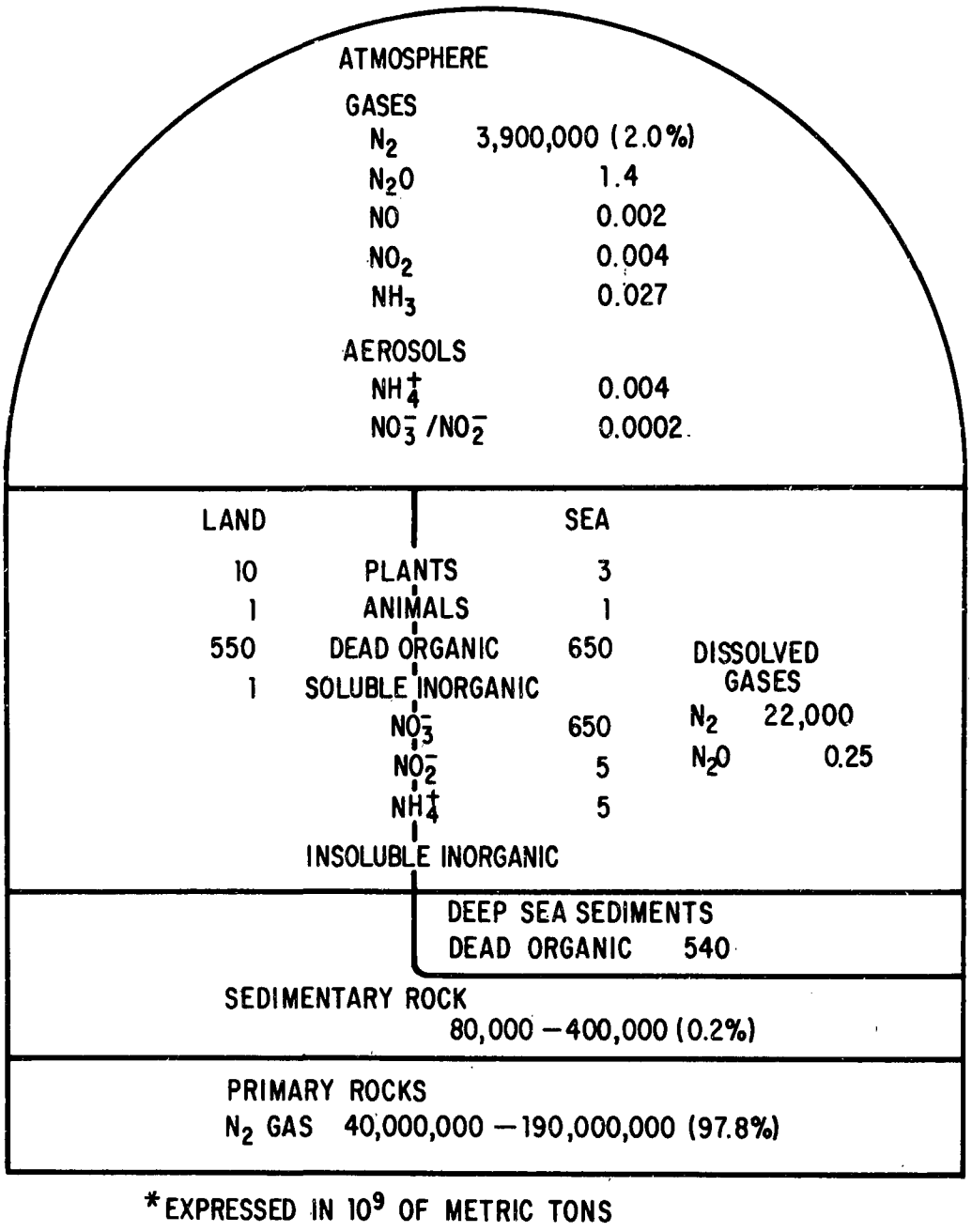


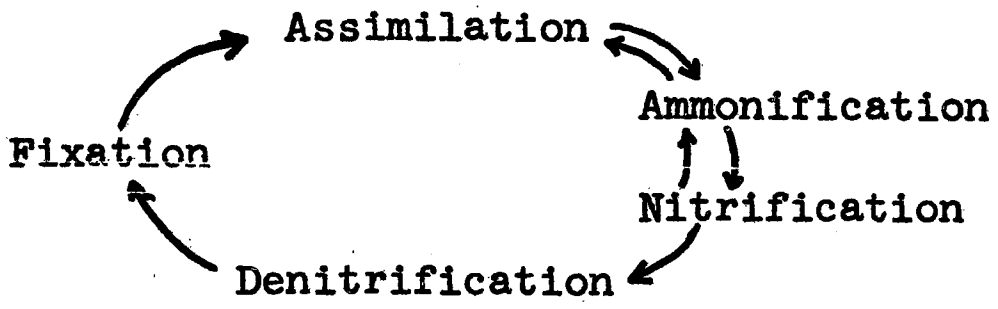
FIGURE 1.—Inventory of the pools of global nitrogen from (*24*).

NITROGEN CYCLES

Chemical Transformations and Physical Transfers

What systems contribute to the cycling of nitrogen? There are basically two types—chemical transformations of either a biological or an abiological nature and physical transfers (Figure 2). Biological chemical transformations include fixation of dinitrogen to ammonia, assimilation of ammonia into organo-

CHEMICAL TRANSFORMATIONS



PHYSICAL TRANSFERS

Volatilization ⇌ Precipitation

Leaching and Runoff

Volcanic Release

Sedimentation

FIGURE 2.—Chemical transformations and physical transfers contributing to nitrogen cycle.

nitrogen compounds, ammonification of organonitrogen to ammonia, nitrification or oxidation of ammonia to nitrate and denitrification of nitrate mainly to dinitrogen. Abiological chemical transformations (Table 2) are well known in the atmosphere (*24*, *156*) and abiological denitrification occurs in soils. The physical transfers include two counter-directional movements: (1) volatilization-precipitation and (2) sedimentation-solubilization, and two unindirectional movements: (1) leaching and runoff which contributes to the transfer of nitrogen from land to sea and (2) volcanic release which introduces N_2 from primary rocks to the atmosphere.

Evolution of Biological Transformations

A logical but hypothetical sequence (*24*) for the evolution of the various types of biological transformations of nitrogen is shown in Fig. 3. Abiological transformations are not considered.

TABLE 2 (*24*).—Abiological chemical transformations of nitrogen in the atmosphere

Ozonization of N_2

$N_2 + O_3 \rightarrow N_2O + O_2$

Lightning

e.g., $O^+ + N_2 \rightarrow NO^+ + N$

$NO^+ + H_2O \rightarrow 2H^+ + NO_3^-$

Photolysis of N_2O

$N_2O \rightarrow N_2 + O$

$N_2O \rightarrow NO + N$

Ozonization of NO

$NO + O_3 \rightarrow NO_2 + O_2$

Oxidation of NO

$2NO + O_2 \rightarrow 2NO_2$

Hydration of NO_2

$3NO_2 + H_2O \rightarrow 2HNO_3 + NO$

Oxidation of NH_3

$NH_3 + [O] \rightarrow \rightarrow \rightarrow NO_3^-$

Hydration of NH_3

$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$

Acid aerosol neutralization of NH_3

$NH_3 + HNO_3 \rightarrow NH_4NO_3$

$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4$

The sequence is based upon a series of challenges and responses. A challenge by the primordial or most recently accumulated from of nitrogen compound leads to the evolution of a biological reaction to meet the challenge and at the same time provide an advantage through satisfaction of a biological need. Ammonia, generally accepted as the major nitrogen compound in the reduced primordial environment presented a challenge that was first met by development of assimilation, the conversion of ammonia to organonitrogen compounds such as amino acids. This anabolic biological reaction is essential for all living things, and therefore is proposed as the initial biological transformation of nitrogen. Subsequent biological transformations are less ubiquitous. Ammonification, which reverses assimilation by release of organonitrogen as ammonia, arose in response to the challenge of the accumulated organonitrogen compounds. The next challenge is suggested to have developed from the concurrent accumulation of ammonia and O_2, the former from primordial sources and the ammonification reaction and the latter initially from abiological reactions and subsequently from photo-

synthesis. Nitrification evolved to oxidize ammonia to nitrate, and the reaction possessed the added advantage of making energy available to the organism able to do this. The accumulation of nitrate initiated two responses. Possibly the first to evolve was denitrification, the conversion of nitrate to dinitrogen since it provided a new nitrogen compound as well as some energy. The other, nitrate assimilation or reduction of nitrate to ammonia, provided living organisms with an alternate source of nitrogen for organonitrogen compounds. Finally, the challenge of dinitrogen accumulation in the atmosphere as a product of denitrification was met by the response of N_2 fixation which, of course, provided a third source of nitrogen for organonitrogen compounds. This hypothetical evolutionary sequence adequately accounts for the known biological nitrogen utilizing capacities of organisms and their ability to interconvert N_2, NO_3^-, NH_3 and RNH_2.

Challenge	Response	Nitrogen utilization capacity of organisms
Ammonia assimilation, NH_3	$NH_3 \rightarrow RNH_2$	$NH_3 \rightarrow RNH_2$
Ammonification, RNH_2	$RNH_2 \rightarrow NH_3$	$NH_3 \rightleftarrows RNH_2$
Nitrification, NH_3, O_2	$NH_3 \xrightarrow{[O]} NO_3^- +$ energy	$NH_3 \rightleftarrows RNH_2$ $\downarrow$ NO_3^-
Denitrification, NO_3^-	$NO_3^- \xrightarrow{e} N_2 +$ energy	$NH_3 \rightleftarrows RNH_2$ $\downarrow$ $NO_3^- \rightarrow N_2$
Nitrate assimilation, NO_3^-	$NO_3^- \xrightarrow{[H]} NH_3$	$NH_3 \rightleftarrows RNH_2$ $\uparrow$ $NO_3^- \rightarrow N_2$
Nitrogen fixation, N_2	$N_2 \xrightarrow{[H]} NH_3$	$NH_3 \rightleftarrows RNH_2$ $\uparrow\downarrow$ $NO_3^- \rightarrow N_2$

FIGURE 3.—A possible evolutionary sequence of biologically-catalyzed nitrogen transformations from (*24*).

NITROGEN TRANSFORMATIONS

Ammonia and Nitrate Assimilation

Ammonia or nitrate assimilation is the conversion of NH_4^+ or NO_3^- to biomass such as amino acids. Typical biological reactions are:

$$NO_3^- \xrightarrow{8e} NH_4^+ \qquad \text{I}$$

$$NH_4^+ + R\underset{\displaystyle O}{\overset{\displaystyle \|}{C}}COOH \xrightarrow{2e} R\underset{\displaystyle NH_2}{\overset{\displaystyle |}{C}}COOH + H_2O \qquad \text{II}$$

The ability to assimilate ammonia and/or nitrate is common to all non-animal organisms. Preference for ammonia *vs.* nitrate varies with organisms. Assimilation of these compounds in temperate lakes varies with the season showing a spring and late summer maxima (*48*, *102*). Sea algae have been shown to have a low K_T for NH_3 which enables them to take up ammonia effectively even at the low concentrations found in the sea (*46*). Studies of coastal marine environments indicate that nitrogen, not phosphorus, is the limiting component for algal growth (*162*). The ability to assimilate NO_3^- or NH_3 is ubiquitous, occurring in soil, lakes, sea and sediment.

Ammonification (8)

Ammonification is the release or mineralization of organic nitrogen to ammonia. A biological reaction is:

$$H_2\underset{\displaystyle NH_2}{\overset{\displaystyle |}{C}}COOH + {}^3\!/_2 O_2 \rightarrow 2CO_2 + H_2O + NH_3$$

$$\Delta F' = -176 \text{ Kcal} \qquad \text{III}$$

Bacteria and fungi are the primary agents of ammonification in soil while bacteria or possibly zooplankton are the major decomposers in aquatic environments (*94*, *102*). Considerable and rapid re-cycling of nitrogen by assimilation and ammonification probably occurs (*46*, *199*). The balance of assimilation *vs.* ammonification is influenced by the C:N ratio with mineralization dominant at a low ratio and assimilation at a high ratio.

Ammonification usually exceeds assimilation at temperatures near freezing and under anaerobic environments (*8*, *102*). Organonitrogen in sediments is more stable than plant and animal remains, possibly due to complex formation. Ammonification also occurs in all environments—soil, lakes, sea and sediment.

Nitrification (2, 3, 28, 31, 41, 102, 115, 132, 187, 193, 194)

Oxidation of nitrogen from a reduced state such as NH_4^+ to a more oxidized state such as NO_3^- is referred to as nitrification. Biological reactions include:

$$NH_4^+ + 3/2 O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

$$\Delta F' = -65 \text{ Kcal} \qquad \text{IV}$$

$$NO_2^- + 1/2 O_2 \rightarrow NO_3^-$$

$$\Delta F' = -18 \text{ Kcal} \qquad \text{V}$$

Autotrophs are the most important organisms with members of the genus *Nitrosomonas* catalyzing reaction IV and those of *Nitrobacter* catalyzing reaction V. Heterotrophs such as fungi may be responsible for nitrification at acidic pH's. The ratio of moles of oxygen consumed to ammonia oxidized is 2, and it is well recognized that nitrification of ammonia can create a major biological oxygen demand in aquatic systems (*36*, *183*). Nitrification enzymes have a high affinity for O_2 with activity occurring down to a pO_2 of about 1 percent so that diffusion of O_2 into the system may be the major limitation. High levels of ammonia inhibit *Nitrobacter* and may make the reaction proceed in stepwise manner. Nitrification occurs at temperatures as low as 0° with optimum activity at about 30°–40° and in the case of *Nitrosomonas* and *Nitrobacter* at pH's of 6 to 8. Hydroxylamine and urea are alternate substrates. A number of inhibitors of nitrification are recorded in the patent literature. One, 2-chloro-6-(trichloromethyl) pyridine has been extensively studied and is reported to selectively inhibit *Nitrosomonas* (*66*). It may be a useful experimental tool. Nitrification occurs in soil, lakes, sea and sediment.

Denitrification (15, 16, 20, 39, 102, 131, 139)

DEFINITION AND REACTIONS

Denitrification is the reduction of NO_3^-/NO_2^- to N_2 and nitrogen oxides. Examples of biological reactions are:

$$C_6H_{12}O_6 + 6NO_3^- \rightarrow 6CO_2 + 3H_2O + 6OH^- + 3N_2O$$

$$\Delta F' = -545 \text{ Kcal/mole glucose} \qquad \text{VI}$$

$$5C_6H_{12}O_6 + 24NO_3^- \rightarrow 30CO_3 + 18H_2O + 24OH^- + 12N_2$$

$$\Delta F' = -570 \text{ Kcal/mole glucose} \qquad \text{VII}$$

$$5S + 6KNO_3 + 2CaCO_3 \rightarrow 3K_2SO_4 + 2CaSO_4 + 2CO_2 + 3N_2$$

$$\Delta F' = -132 \text{ Kcal/mole S,} \qquad \text{VIII}$$

while a not yet understood abiological denitrification reaction, unfortunately named chemodenitrification (*14*, *20*, *53*), (all reactions whether biological or abiological are chemical), occurs:

$$NO^-_2 \xrightarrow{?} N_2, NO, NO_2 \qquad \text{IX}$$

ORGANISMS

A variety of facultative anaerobic bacteria catalyze biological denitrification. Some, such as *Pseudomonas*, use sugar or organic acids as reductant, while others such as *Thiobacillus* use S and others such as *Micrococcus* use H_2. Examples of denitrifiers include:

Pseudomonas denitrificans
P. perfectomarinus
P. aeruginosa
Thiobacillus denitrificans
Micrococcus denitrificans
Corynebacterium nephridii

BIOCHEMISTRY

The biochemistry of denitrification is becoming increasingly well defined. Payne and colleagues (*7*, *37*, *139*, *140*, *141*) have used *P. perfectomarinus* to provide an *in vitro* system for elucidation of the pathway of dentrification. A four-step sequence

of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ has been clearly demonstrated using separated fractions to catalyze each step (*141*). Nitrite accumulates until NO_3^- is completely reduced. This control is mediated by inhibition of the conversion of $NO \rightarrow N_2O$ (*140*) by NO_3^-. The product mixture of N_2O to N_2 will vary with conditions, but in at least one case, *C. nephridii*, N_2O is the terminal product (*150*). Synthesis of the denitrifying enzymes and their activity is highly sensitive to O_2 but insensitive to NH_3 or NO_3^- (*55*). Denitrification couples NO_3^- in place of O_2 to the oxidation of a variety of substrates, *e.g.*, sugars, organic acids, S, H_2, with energy production. In *P. perfectomarinus* the substrate required is asparagine (*151*) which is converted to malate and oxidized to NADP, to NAD and FAD.

Both assimilatory and dissimilatory nitrate reductases are known (*139*). Oxygen sensitivity of the dissimilatory reductases differentiates them from the assimilatory reductases that function in nitrate assimilation. There are two types of dissimilatory nitrate reductases. Reductase A catalyzes the first step of denitrification while reductase B catalyzes the respiratory reductase of coliforms, *Achromobacter*, *Rhizobium japonicum*, bacilli, *Neurospora*, *Proteus mirabilis*, *Hemophilus parainfluenzae*, and *Spirillum itersonii*. Both are O_2-sensitive, but reductase A reduces chlorate and transfers electrons from NADH while B does not (*142*, *143*).

PHYSIOLOGY

Denitrification is restricted to anaerobic environments containing less than 0.2 ml of O_2/l (*60*). The rate in soil is sensitive to moisture and increases with degree of saturation. Organic matter stimulates denitrification by provision of reductant and consumption of O_2. Added electron donors *e.g.*, S or CH_3OH, increase dentrification rate (*5*, *119*). The rate is very rapid at neutral to alkaline pH's and very slow at pH's below about 5; the rate increases rapidly from 2 to 25°C and has a high optimum of 60–65°C (*15*, *41*, *134*). The K_m for NO_3^- is low and the rate in soil is relatively independent of concentration. Denitrification and assimilation of NO_3^- may occur in the same environment (*30*, *103*, *118*). In aquatic and soil systems nitrification in a more aerobic surface layer may provide NO_3^- for denitrification in a

less aerobic subsurface layer (*137*). In the equatorial Pacific Ocean, nitrite produced from nitrate accumulates in regions with very low populations of denitrifying bacteria and is presumably a product of other bacteria (*57, 81*). Denitrification rates in sediments are reported to be inversely related to C/P ratio (*29*).

METHODOLOGY

Development and application of more effective techniques for measurement of denitrification are urgently needed since the greatest data limitation in nitrogen cycle studies is the lack of quantitative measurements of gaseous nitrogen losses (*124*). Most global assessments of denitrification are simply the unaccounted for difference between measured gains and losses due to other transformations and transfers. (See Figure 8, for example.)

Denitrification is measured by loss of NO_3^- or formation of gaseous nitrogen products. Indirect measurements based on NO_3^- disappearance are the least reliable since assimilatory nitrate reduction and denitrification occur simultaneously in some environments (*30, 103, 108*). A more direct measurement is based on formation of the volatile products of denitrification, N_2 and N_2O. However, fixation and denitrification also both occur under anaerobic conditions (*98*) and measurements of N_2 will give only net N_2 changes.

Special open and closed chambers (*124, 157, 158, 167*) are being used for *in situ* measurements in soil, e.g., modifications of the Van Bavel air-sampling well, air lysimeters, aeromatic apparatus and volatilization chambers. *In situ* measurements that cause minimal disturbance of the eco-system are preferred for denitrification as well as other nitrogen measurements since disturbances often lead to increased or altered activity.

Methods of analysis are listed in Table 3. Mass spectrometry is used to measure ^{15}N abundance in volatile or non-volatile compounds after an appropriate incubation with $^{15}NO_3^-$. It is also used to measure N_2/Ar and/or $^{15}N/^{14}N$ ratios to determine excess N_2 in aquatic samples (*10, 82, 85, 86, 155*). Recently a gas chromatographic separation method utilizing Porapak Q to separate N_2O, NO and N_2 has been used to measure denitrifi-

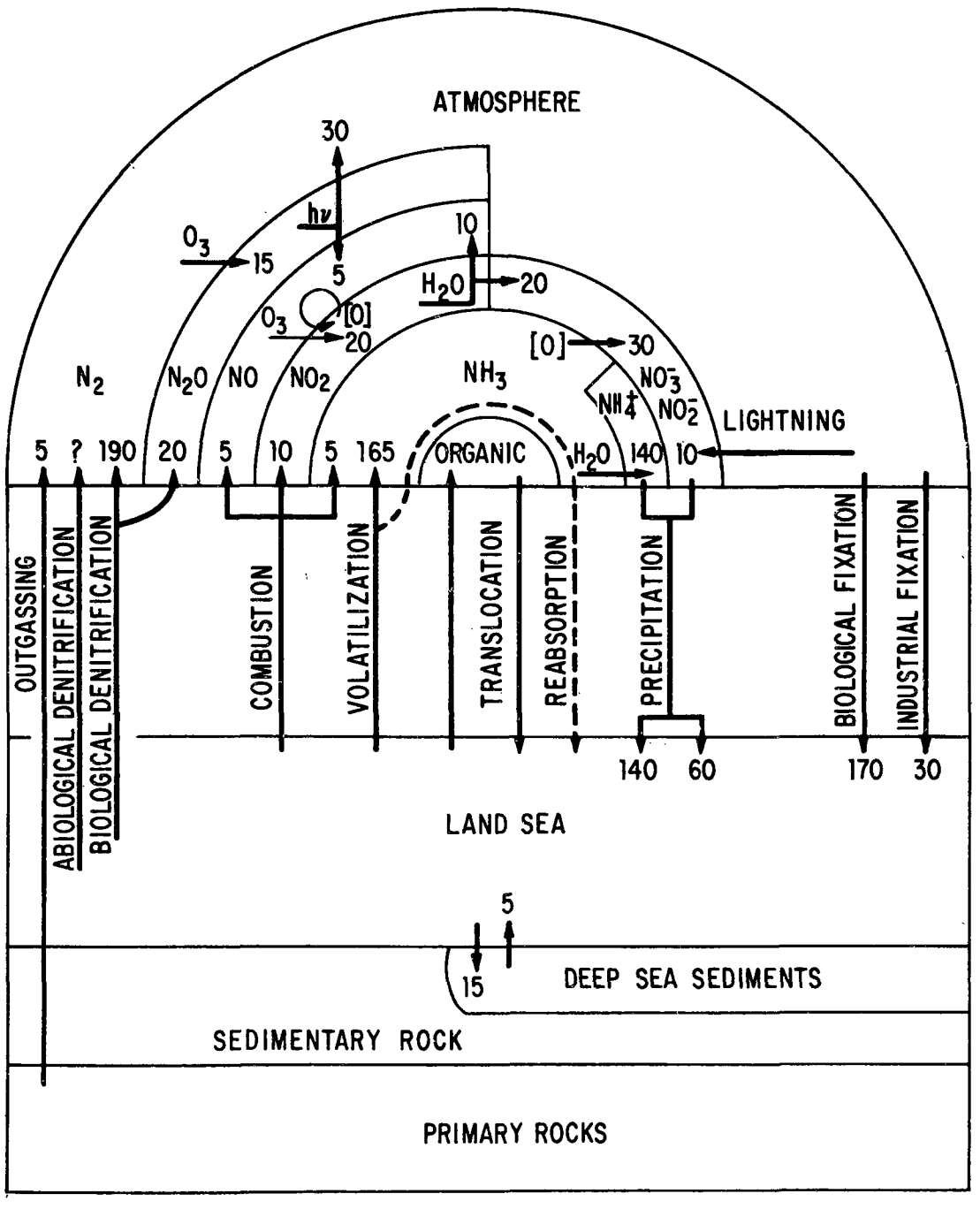


FIGURE 8.—Global Nitrogen Inter-Pool Transfers from (*24*).

cation in laboratory experiments (*7*). Other detection systems e.g., a He detector in place of a thermal conductivity detector, would increase sensitivity by about 10^3. Gas chromatography could be used in series with mass spectrometry to eliminate errors in measurement of N_2 and N_2O mixtures introduced by breakdown of N_2O to N_2 occurring in the mass spectrometer.

TABLE 3.—Denitrification assays

Manometric
Kjeldahl (*15*)
Mass Spectrometric
Gaseous loss of ^{15}N using $^{15}NO_3^-$ (*30, 63*)
Excess N_2 in sea based on N_2/Ar (*10, 82, 85, 86, 154, 155*)
^{15}N-Enrichment in sea (*82, 155, 10, 85, 86, 154*)
Gas Chromatographic
Separation of N_2O, NO and N_2 on Porapak Q and detection by thermal conductivity (*7*)
More sensitive gas chromatographic detection system, e.g., He detector
Combination of Gas Chromatography and Mass Spectrometry
Infra-Red Spectrometric (*130*) N_2O

The application of gas chromatography to measurements of denitrification and N_2 fixation was first proposed at about the same time. In a recent conversation, Dr. Payne noted that only seven papers have been published on denitrification using this technique (e.g., *7, 32, 123, 140, 189*) in contrast to the over 200 on gas chromatographic assays of N_2 fixation (74). Additional use of gas chromatography in place of mass spectrometry in denitrification assays deserves more serious consideration.

OCCURRENCE AND RATE

Denitrification occurs in soil, lakes, sediment and sea. A tabulation of representative measurements in aquatic ecosystems is presented in Table 4. Only a few measurements are recorded and there is a need for more intensive and extensive analyses. Recorded activity is about 15–20 μg N denitrified/l·day in lakes. Sediments are several times as active with a rate of 12.4 Kg/ha·yr recently reported for Lake Mendota sediment. Denitrification occurs in a variety of sea locations; the two reported rates for the tropical Pacific Ocean are similar to those for lakes.

N_2 FIXATION (24, 25, 38, 71, 72, 73, 75, 76, 79, 102, 116, 129, 145, 148)

Definition and Reactions

N_2 fixation is any reaction of N_2 that yields nitrogen covalently bonded to any other atom. Biological and abiological fixation reactions are summarized in Table 5. There is only one

TABLE 4.—Denitrification measurements

Occurrence	Rate	
Soil		
Many reports e.g., Broadbent and Clark, 1965		
Lakes and sediments		
Subarctic lake	15 μg N/L·day	(63)
Subarctic lake mud	90 μg N/L·day	(63)
Brackish Japanese lake	A.D.*	(82)
Lake Kizaki	A.D.	(109)
Lake Mendota	8–26 μg N/L·day	(19)
Lake Mendota sediment	12.4 Kg/ha·yr	(30, 103)
Reservoir mud, USSR	A.D.	(112)
Sea		
Oxygen-minimum layer of eastern tropical Pacific	7.2 μg N/L·day	(60)
Island bay in equatorial Pacific	12–18 μg N/L·day	(62)
Sea water	A.D.	(61)
Cariaco Trench—Caribbean	A.D.	(155)
—Norwegian fjord	A.D.	(155)
Northeastern tropical Pacific	A.D.	(184)
Coast of Peru	A.D.	(57)
Central Pacific	A.D.	(80)

* A.D. = activity detected.

biological reaction, but several abiological reactions of which lightning and ozonization occur naturally while the remainder are synthetic reactions. The Haber-Bosch process, developed in 1914, has become increasingly significant. This reaction is the basis of the relatively cheap fertilizer nitrogen essential to support the increased crop yields of developed countries and will be of equal importance for expanding the green revolution in undeveloped countries. The Haber-Bosch fixation process requires extreme conditions of pressure and temperature for effective conversion of N_2 to NH_3. Moreover, the low production cost possible in large plants generating 1000 or more tons per day are accompanied by almost equivalent costs for transportation incurred in distributing from such widely separated production centers. Novel synthetic fixation reactions have recently been described in which N_2 is converted to NH_3 under ambient conditions. In one process the conversion occurs in an aqueous environment (*163, 165*) under conditions similar to those of the biological reaction. It may lead to an improved system for providing additional nitrogen. For example, utilization of mem-

TABLE 5.—Biological and abiological fixation reactions of molecular nitrogen (*72, 76*)

Biological reaction

$$X\,ATP + 6e + 6H^+ + N_2 \xrightarrow[25°,<1\ atm]{Nitrogenase} 2NH_3 + x\,ADP + x\,Pi \quad \text{X}$$

Abiological reactions

Lightning*

$$O^+ + N_2 \rightarrow NO^+ + N \quad \text{XIa}$$

$$NO^+ + H_2O \rightarrow 2H^+ + NO_2^- \quad \text{XIb}$$

Ozonization*

$$N_2 + O_3 \rightarrow N_2O + O_2 \quad \text{XII}$$

Haber-Bosch*

$$N_2 + 3H_2 \xrightarrow[450°,\ 200\ atm.]{Fe,\ Mo} 2NH_3 \qquad \Delta F. = -7.95\ kcal/mole \quad \text{XIII}$$

Dinitrogen Transition Metal Complexes**

$$N_2 + M \rightarrow M \cdot N_2 \xrightarrow[25°,\ 1\ atm.]{?} N_2H_4, NH_3 \quad \text{XIV}$$

Homogeneous Aprotonic**

$$N_2 + RMgX \xrightarrow[25^c\ 1\ atm.]{Ti} NH_3 \quad \text{XV (192)}$$

Homogeneous Protonic*

$$N_2 + BH_4^- \xrightarrow[25°,\ 1\ atm.]{Mo \cdot Thiol} NH_3 \quad \text{XVI (163)}$$

* Significant natural or industrial inputs

** Exploratory research systems—possible future inputs

branes for N_2-enrichment of air and for retention of the catalyst effective in aqueous systems may provide a system for the fixation of N_2 in irrigation water at the time of plant need, and thereby more closely couple nitrogen fertilizer production to crop location and need (*136*).

Organisms

Which organisms are capable of fixing N_2? We have suggested that this process is the most recently evolved major biological nitrogen transformation. N_2-fixing organisms possess a covenant analogous to that of the chosen. They have been given the unique ability to utilize the sea of atmospheric N_2 surrounding them to fill their fixed nitrogen needs. Possibly only one percent of living organisms are diazotrophs. We recently coined the

word diazotrophs to designate N_2-fixing organisms. They are distributed somewhat randomly throughout the bacterial and blue-green algal orders, families and genera (Table 6) (*24*). Examples of bacterial diazotrophs found in aquatic ecosystems include the broadly distributed anaerobe, *Clostridium*, photosynthetic bacteria, *Rhodospirillum* and *Chlorobium*, and a facultative anaerobe, *Klebsiella*; algal diazotrophs include *Anabaena*, *Aphanizomenon* and *Nostoc* in lakes and *Calothrix* and *Trichodesmium* in seas (e.g., *21*, *89*, *169*, *170*, *175*).

The breadth of N_2-fixing relationships extend from asymbiotic to obligatory symbiotic with various associative relationships in between (Figure 4). Asymbiotic diazotrophs fix N_2 independent of other organisms and include bacteria and blue-green algae. Both natural and synthetic asymbiotic diazotrophs are now known with the recent production of N_2-fixing *Escherichia coli* (*42*). The obligatory symbiotic diazotrophs are exemplified by the legume root nodules based on genetic involvement of the legume and *Rhizobium* component, each of which is ineffective in N_2 fixation by itself. The associative symbioses (*113*) also involve two partners, but one is an asymbiotic diazotroph. In the associative relationships morphological changes by the non-diazotroph may provide an environment more favorable to the diazotroph e.g., fungal and algal relationship in lichens (*127*). The importance of these associative symbioses are only currently being recognized and they may well contribute a major amount of fixed nitrogen. For example, a marine associative

TABLE 6.—Molecular nitrogen-fixing organisms (*24*)

Bacterial Diazotrophs	
Orders	3/10*
Families	11/47
Genera	26/200
Examples: *Clostridium, Rhodospirillum, Klebsiella, Chlorobium*	
Blue-Green Algal Diazotrophs	
Orders	2/3
Families	6/8
Genera	23/165
Examples: *Anabaena, Aphanizomenon, Nostoc, Calothrix, Trichodesmium*	

* Ratio of orders, families or genera with diazotrophs to total orders, families or genera.

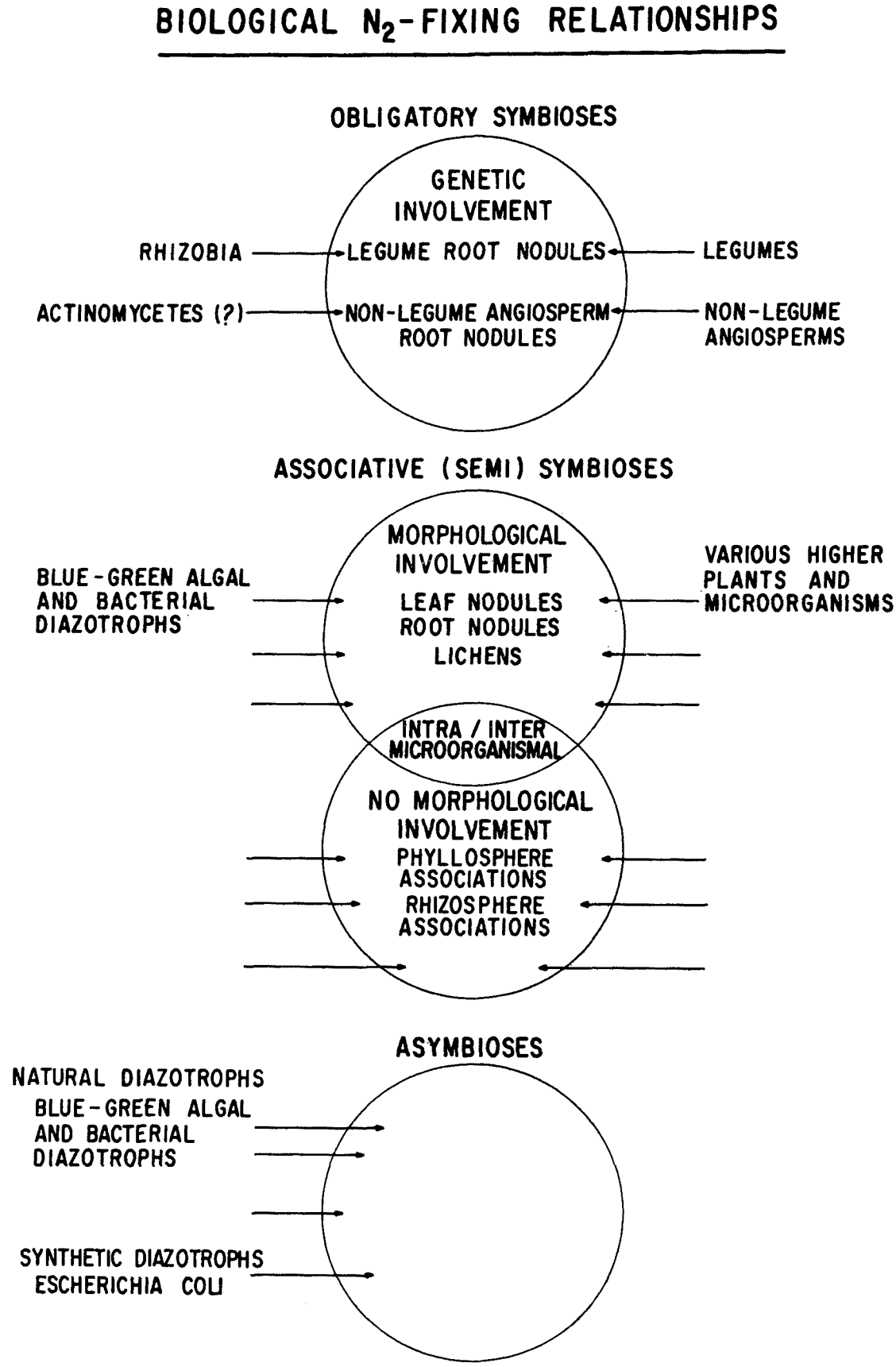


FIGURE 4.—Biological N_2-fixing relationships from (*24*).

symbiosis has recently been found to fix up to 500 Kg/ha·yr (*138*).

Biochemistry

During the 1960's the major aspects of biological N_2 fixation were elucidated. At the beginning of the decade essentially nothing was known at the molecular level; while at the end the nature of the enzyme and its reactions had been defined. A new assay of great application for ecological measurements was developed from this new biochemical knowledge. Spectacular advances in biochemistry are not anticipated during the present decade. On the other hand progress in unravelling the biology of N_2 fixation is expected to dominate this decade.

What aspects of the biochemistry of N_2 fixation are relevant to aquatic studies? Selected highlights are listed in Table 7 and discussed below. Nitrogenase, the enzyme that catalyzes the biological fixation of dinitrogen is composed of a Mo-Fe protein and an Fe protein, each named on the basis of their metal content (Fig. 5). They are essentially similar in all organisms and are both extremely sensitive to O_2, and additionally the Fe protein is cold labile. Oxygen-sensitivity influences the distribution and activity of nitrogenase. It requires energy in the specific form of ATP and a reductant provided by

TABLE 7.—Biochemistry of nitrogen fixation

(*24, 25, 72, 73, 76, 79, 116, 129, 145*)

Enzyme	Nitrogenase $\rightleftarrows$ Mo-Fe protein + Fe protein
	—Similar in all organisms
	—Cold labile
	—O_2-Sensitive
Requirements	—Energy—ATP(up to 15–20 ATP/$N_2 \rightarrow 2NH_3$)
	—Reductant—Ferredoxin or Flavodoxin
Reaction	—Electron activation
	—Substrate reduction
Alternate Substrates	—$N \equiv N$, $N \equiv O$, $C \equiv N$ and $C \equiv C$ bonded small molecules such as N_2, N_2O, N_3^-, RCN, RNC and RCCH as well as H_3O^+
Intermediates	—None, enzyme-bound diazene and hydrazine proposed
Product	—NH_3, no feedback inhibition
Activation Energy	—$>20°$ 14 kcal/mole
	—$<20°$ 35–50 kcal/mole
pH optima	—~7

the strongly reducing electron carrier proteins, ferredoxin or flavodoxin. The isolated enzyme has a large energy requirement of 15–20 ATP/N_2 fixed. The nitrogenase reaction (Figure 6) is composed of two steps: (1) electron activation and (2) substrate reduction. The former is the limiting reaction of nitrogenase and its activity is independent of the latter. The "sensuous" nitrogenase by analogy with "The Sensuous Woman" is an unusually versatile enzyme coupling with and reducing a heretofore unequalled variety of small N, O or C, triple-bonded or potentially triple-bonded substrate partners. All reductions cleave the NN, NC or NO bonds except in the case of CC where the triple bond is reduced only to the double bond. There are no enzyme-free intermediates between N_2 and the product, NH_3, although diazene and hydrazine are proposed as enzyme-bound intermediates. Ammonia does not inhibit nitrogenase activity. The temperature response of nitrogenase is expressed as activation energy and above 20° an approximate doubling in rate occurs for a 10° increase while below 20° the change per 10° is much greater. The pH optimum of nitrogenase is near neutrality.

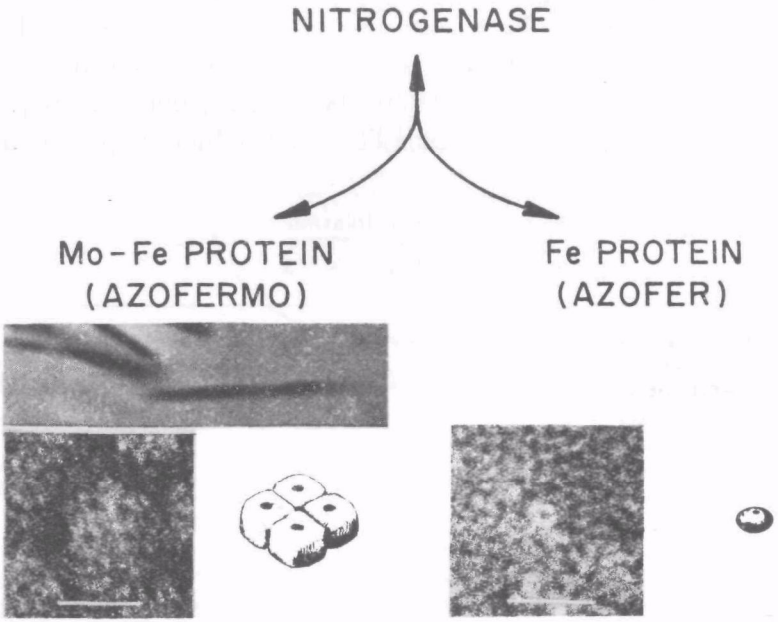


FIGURE 5.—Mo-Fe and Fe protein components of nitrogenase with light micrographs of crystals of Mo-Fe protein and electron micrographs and models of each protein from (*75*, *72*).

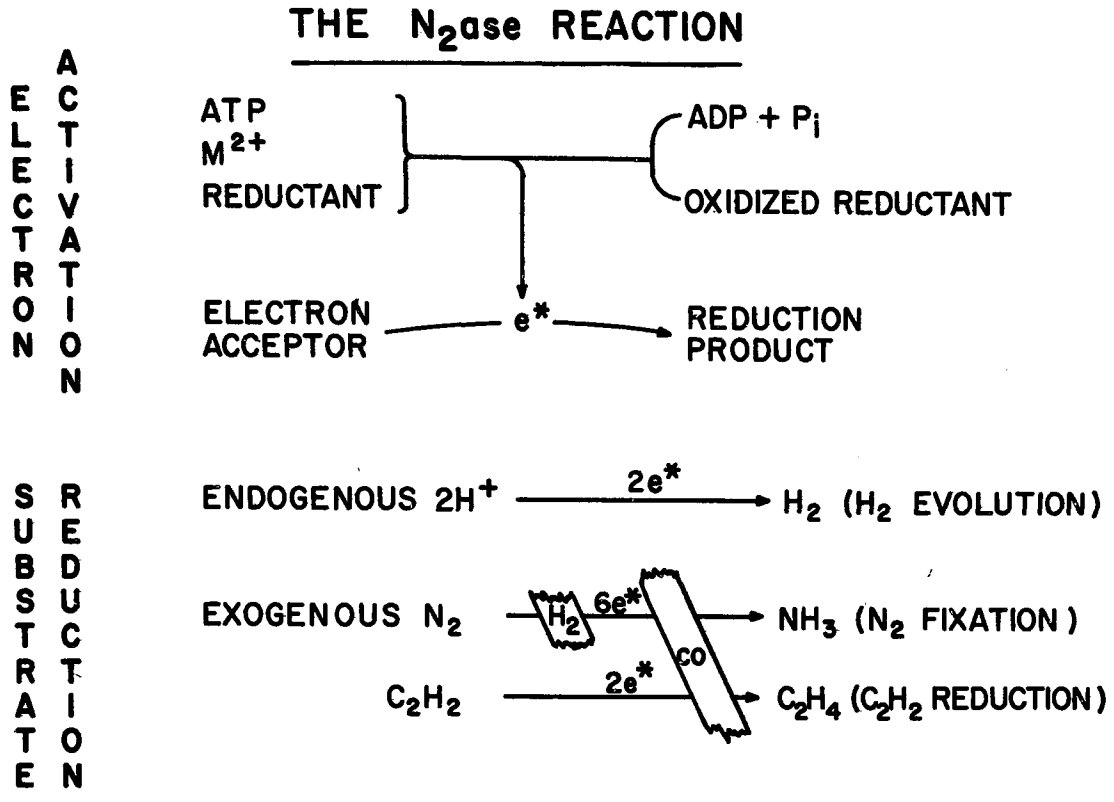


FIGURE 6.—The nitrogenase reaction showing electron activation and substrate reduction with either reduction of H^+ or of added N_2 or C_2H_2 to give H_2 evolution, N_2 fixation and C_2H_2 reduction respectively from (*74*).

Biology

The biology of N_2 fixation relevant to environmental studies is tabulated in Table 8. Oxygen concentration is a key variable (*87*). Anaerobic, facultative anaerobic and photosynthetic bacteria fix N_2 only in the absence of O_2 while aerobic bacteria and blue-green algae are more active at subatmospheric oxygen concentrations. Since nitrogenase is oxygen-sensitive (*196*), protection against O_2 by enhanced respiration, conformational change or membranes in aerobic diazotrophs must occur. Ammonia is a repressor, while N_2 is not essential for nitrogenase synthesis (*135*). The K_m of N_2 for nitrogenase is about 0.05 atm so that the enzyme is essentially saturated by the N_2 in air. Diazotrophs consume about 1 g of carbohydrate per 10–20 mg of N_2 fixed. Nitrogenase has only been definitively demonstrated in lower organisms without organized nuclei (*126*). Substantial amounts of fixed nitrogen are not excreted but may be transferred to associated organisms. The optimum temperature

TABLE 8.—Biology of nitrogen fixation (*116, 129, 145*)

pO_2—Type of organisms
—Activity of aerobic diazotrophs
—Protection against O_2
Ammonia repression
$Km(N_2)$—0.05 atm.
High CH_2O utilization/N_2 fixed.
Transfer *vs.* excretion of fixed N
Temperature—Optimum 20°–35°
—Limit 5°–55°
Nitrogenase restricted to prokaryotic organisms
Heterocysts
Algal activity and available P elevation.
Activity in eutrophic *vs.* non-eutrophic aquatic systems.

is 20–35°C although activity may be detected as low as 5°C and as high as 55°C, in hot springs; the optimum pH varies and is dependent on the particular organisms.

Aquatic Ecology

Algal fixation is correlated with abundance of heterocystous forms (*142, 172*); however, fixation can occur in non-heterocystous filaments as well as unicellular forms (*111, 161, 197*). The activity maxima of N_2-fixing algae in aquatic systems follows elevation of available phosphorus (*188*). Fixation is greater in eutrophic than non-eutrophic environments (*177*). It is suggested that fixed nitrogen does not markedly decrease activity in aquatic environments (*102*).

Measurement

Methods for measurement of N_2 fixation are listed in Table 9. These have recently been evaluated in a review of the C_2H_2–C_2H_4 assay (*74*). This assay, first proposed by Hardy and Knight in 1966 (*78*), is based on the nitrogenase-catalyzed reduction of C_2H_2 to C_2H_4 coupled with gas chromatography. The method has gained wide acceptance with over 300 reports from at least 15 different countries in 5 continents already published.

Steps of the C_2H_2–C_2H_4 assay are tabulated in Table 10 (*70, 73, 74, 77, 106, 146, 149, 152, 174, 175, 176, 180*). Sample preparation for aquatic systems involves transfer of a sample,

TABLE 9.—Methods of measurement of N_2 fixation (*74*)

Growth and Morphology	
Biomass or O.D. increase on N_2-free medium	2*
Heterocysts (algae only)	2, 3
N-Based Methods	
Kjeldahl or Dumas	
N Increase	2, 3
Inoculated minus Uninoculated	2, 3
Legume N minus Cereal N	3
^{15}N-Enrichment from $^{15}N_2$	
Mass Spectrometry (*26*)	1, 2, 3
Optical Emission (*128*)	2, 3
^{13}N from $^{13}N_2$	
Radioactive Counting (*27*)	1, 2
N_2:Ar Ratios	
Mass Spectrometry	2
NH_3	
Titrimetry	1
Colorimetry	1
N_2:H_2 Uptake	
Manometry	1
Alternate Substrates	
C_2H_2–C_2H_4	
Gas Chromatography (*70, 77, 174*)	1, 2, 3
Colorimetry (*114*)	3
Nitriles and Isonitriles	
Gas Chromatography (*59*)	1, 2
H_2-Evolution	
Manometry	1
Gas Chromatography (*59*)	1
Mass Spectrometry	1

* Areas of use: 1, nitrogenase *in vitro*; 2, diazotrophs in culture; 3, field system

possibly following concentration, into a gas tight chamber made 10 percent in C_2H_2 and incubated *in situ* for a defined time at the temperature, illumination etc. of the population being measured. Sensitivity of the method is extremely high and therefore a short incubation is recommended to avoid altered activity due to derepression of nitrogenase synthesis during incubation. Although it is preferred on at least a theoretical basis that N_2 be removed so as to eliminate its competition with C_2H_2, in practice this is not essential since the error introduced by failure to remove N_2 is insignificant relative to the inherent variability of field measurements. Excess C_2H_2 should be avoided since growth of one organism is inhibited by such levels of C_2H_2 (*22*). The reaction can be terminated by gas removal and we find

TABLE 10.—Methodology of C_2H_2–C_2H_4 assay (*74*)

- Incubation
  - Sample Preparation
  - Incubation chambers—serum vials
    - —syringes
    - —plastic bags
    - —fermentors
    - —micro canopies
  - Gas Phase—aerobic *vs.* anaerobic
    - —removal of N_2
    - —purification of C_2H_2
  - Incubation Conditions—temperature
    - —time
    - —illumination
  - Termination of Reaction—removal of gas
- C_2H_4 Analysis
  - Fractionation—gas chromatography
    - —oxidation
  - Detection System
    - —flame ionization
    - —colorimetry
- Expression of Results
  - g $N_2[C_2H_2]$ fixed = moles of $\frac{C_2H_2 \rightarrow C_2H_4 \times 28}{3}$

that evacuated blood sampling tubes are ideal for transfer and/or quantitative storage of gas between incubation and analysis. Termination of reaction by acid addition has recently been demonstrated to introduce errors due to release of C_2H_4 from acid-treated rubber stopalls (*182*) and results reported by the several laboratories using this termination technique may be invalid and require re-examination. Analysis of C_2H_2 and C_2H_4 is usually performed by gas chromatography using a flame ionization detector. Less than one picamole of C_2H_4 can be detected. Recently a colorimetric assay (*114*) has been proposed but its relatively low sensitivity limits its application to high activity samples such as legumes and eliminates its use for aquatic systems.

The measurement of C_2H_2 reduction is converted to an N_2 fixation equivalent by the formula shown in Table 10. Moles of N_2 fixed is obtained by division of moles of C_2H_2 reduced to C_2H_4 by 3. This factor is based on the ratio of the requirement of 6 electrons for reduction of N_2 and 2 for C_2H_2. Twenty-six reports in which the C_2H_2 reduced:N_2 fixed conversion factor

was experimentally measured for nitrogenase and cultured diazotrophs validated the ratio of 3 (*74*). Only long term anaerobic incubations of soil varied substantially from the conversion factor of 3. We have recently introduced terminology to clearly indicate the source of N_2 fixation measurements obtained by C_2H_2–C_2H_4 assay (*74*). This terminology utilizes an italicized [C_2H_2] following N e.g.,

$$\text{mg N}[C_2H_2]\text{ fixed.}$$

Although the sensitivity, simplicity and facility of the C_2H_2–C_2H_4 assay recommend it for most field measurements, it must always be remembered that this is an indirect assay and new systems must be confirmed utilizing $^{15}N_2$ and measurements of ^{15}N-enrichment.

Occurrence and Rate

N_2 fixation occurs in soil, lakes, sediment and sea. The global rate of biological fixation was recently reestimated by Burns and Hardy (*24*). Table 11 indicates the rates and total

TABLE 11.—Estimate of Global biological N_2 fixation (*24*)

Land use	ha×10^{-6}	kg N^2 fixed per ha × yr	Metric tons per yr(×10^{-6})
Agricultural	4400		
Arable under crop	1400		
Legumes	250	140	35
Pulses	63		
Soybeans	34		
Groundnuts	18		
Other	135		
Non-legumes	1150		
Rice	135	30	4
Other	1015	5	5
Permanent meadows grasslands	3000	15	45
Forest and woodland	4100	10	40
Unused	4900	2	10
Ice covered	1500	0	0
Total land	14 900		139
Sea	36 100	1	36
Total	51 000		175

TABLE 12.—Basis for increase in estimates of biological N_2 fixation (*24*)

Increased reports of measurement of N_2 fixation in the biosphere due to improved assays e.g., C_2H_2–C_2H_4 assay (74).
Recognition of more effective N_2 fixation by aerobic bacterial and algal diazotrophs at pO_2 <0.2 atm. e.g., lichens (127, 178).
Documentation of associative symbioses in the phylloplane and rhizosphere e.g., *Paspalum-Azotobacter* association (43, 44, 159, 198).
Recognition of algae as highly significant factors in global N_2 fixation e.g., activity in marine environments and by nonheterocystous forms (23, 49, 111, 161, 197).
Demonstration of N_2 fixation in additional environments e.g., digestive tracts of ruminants
Evidence for new non-legume symbiotic N_2 fixation e.g., sagebrush (34, 54, 88).

amounts estimated for various environments which collectively yield a global rate of 175×10^6 metric tons of N_2 fixed/annum. This total is approximately equally distributed between cropland, grassland, forest and woodland and sea with the value for sea being the most uncertain. We feel this global esimate may err on the conservative side even though it is 75 percent greater than a previous widely quoted estimate made about a decade earlier (*45*). Some factors that may contribute to further increases in the size of the current estimate are listed in Table 12.

There have been a number of recent reports of measurements of N_2 fixation in lakes and sediments (Table 13) many of which have been made with the C_2H_2–C_2H_4 assay. With two exceptions, activity has been detected in all lakes and sediments tested including those located in arctic, temperate and tropical latitudes. One exception involved a newly established reservoir. Because of the high temporal variability of aquatic N_2 fixation rates it is essential that continuous sampling be made over periods of up to one year or more in order to obtain meaningful quantitation. This has been done for Lake Mendota in the U.S., Lakes Windermere and Esthwaite in England and Lake Erken in Sweden. Activities were 0.61 to 30.0 Kg N_2 fixed/ha·yr with a two-fold variation recorded for the same lake in successive years.

Some measurements of N_2 fixation in the sea have been made with $^{15}N_2$ and a few recent ones with the C_2H_2–C_2H_4 assay (Table 14). No data are yet available for intensive measurements over a long period. It is anticipated that projected testing will substantially add to the data on N_2 fixation in both the

TABLE 13.—Nitrogen fixation in lakes, sediments and rivers

Site	N_2 fixed*	
Lake Windermere, Eng. 1965	2.87 Kg/ha·yr	(58, 90)
Lake Windermere, Eng. 1966	1.07 Kg/ha·yr	(58, 90)
Lake Esthwaite, Eng. 1965	1.27 Kg/ha·yr	(58, 90)
Lake Esthwaite, Eng. 1966	0.61 Kg/ha·yr	(58, 90)
Lake Erken, Sweden	$_{30}$Kg/ha (5 months)	(68)
Lake Mendota, Wisc.	4.3	(17)
Lake Mendota, Wisc.	1.7–2.04	(64)
Lake Mendota, Wisc.	0.9 (51 days) or 2.4 Kg/ha·yr	(177)
Lake Mendota, Wisc. algal blooms	up to 1032	(175)
Lake Mendota, Wisc. sediment	A.D. (°)	(30)
Lake sediments, Wisc.		
—oligotrophic	0.5–2.5 μg/Kg·day	(117)
—moderately eutrophic	5.0–10 μg/Kg·day	(117)
Crystal Lake, Wisc.	N.A.D.	(160)
Green Bay, Wisc. 1971	0–49	(188)
Lake Mary, Wisc. 0 m	0.12	(18)
Lake Mary, Wisc. 10 m	0.38	(18)
Lake Mary, Wisc. 20 m	1.12 or 4.6 Kg/ha·yr	(18)
Lake Mize, Fla. 0 m	0–0.40	(18)
Lake Mize, Fla. 10 m	0.73–7.4	(18)
Lake Mize, Fla. 20 m	0.6–2.4	(18)
Lake Mize, Fla.	1.9–7.9 or 2.8–11.4 Kg/ha·yr	(105)
Sediments of lakes, Fla.	A.D. in 7 of 25	(105)
Sediments of Lakes, Guatemala	A.D. in 3	(105)
River, Pensacola, Fla.	A.D.	(50)
Sanctuary Lake, Pa.	24–144	(47)
Smith Lake, Alaska	up to 72	(12)
Lake Erie	A.D.	(92)
Lake Erie sediment	285 μg/Kg·day	(92)
Hot Spring streams	1–5 μg/plant N·day	(173)
Farm pond, ok.	A.D.	(185)
Keystone Reservoir, ok.	N.A.D.	(69)
Lake Carl Blackwell, ok.	A.D.	(186)
Fresh and Sea water and bottom mud	7.2–108 μg N/L·day	(181)
Lake George, Uganda	44 Kg/ha·yr	(91)
Fish culture ponds, Japan	A.D.	(99)
USSR reservoir	A.D.	(112)

* Expressed as μg N_2 fixed/L·day unless otherwise indicated; A.D. = activity detected; N.A.D. = no activity detected.

Pacific and Atlantic Oceans. The high activity of *Trichodesmium* blooms coupled with their extensive size suggests that this alga may represent a major source of fixed nitrogen (*46*). High activity has also been found for the bacteria associated with *Thalassia* in marine environments (*138*).

TABLE 14.—Nitrogen fixation in sea

Site	N_2 fixed	
Tropical ocean—*Trichodesmium sp*.	up to 0.32 Kg/ha•day	(49)
Pacific ocean—bacteria associated with phytoplankton	A.D.*	(122)
British rocky shore—*Calothrix*	25 Kg/ha•yr	(97, 171)
Florida current—planktonic *Trichodesmium sp*.	7.7 μg/mg protein•day	(23)
Waccasassa Estuary, Fla.		
—water	.035–0.11 μg/L•day	(21)
—sediment 0–2 cm	0 μg/kg•day	(21)
2–5 cm	15–144 μg/Kg•day	(21)
5–20 cm	0.3–4 μg/Kg•day	(21)
Caribbean marine angiosperms		
Thalassia	100–500 Kg N/ha•yr	(65, 138)
Syringodium	A.D.	(138)
Diplanthera	A.D.	(65, 138)
Ruppia	A.D.	(65)
Cymodocea	A.D.	(65)
Temperate marine angiosperms		
Zoostera	A.D.	(138)

* A.D. = activity detected

Rate of Abiological Fixation

The Haber-Bosch process is the only significant contributor of synthetically or industrially fixed nitrogen. This process currently provides 30–31 $\times 10^6$ tons of fertilizer nitrogen and it is estimated that this need will increase to about 50 $\times 10^6$ tons within a decade (*6*). Other non-physiological reactions including lightning, combustion and ozonization are estimated to fix about 10, 20, and 15 $\times 10^6$ tons of nitrogen each year (*24*).

PHYSICAL TRANSFERS OF NITROGEN

Leaching

The transfer of nitrogen from land to sea via runoff and leaching is of increasing concern since the transferred nitrate may be a major contributor to eutrophication in lakes and rivers (*1, 35, 100, 110, 190, 191*). It is estimated that about 15 $\times 10^6$ tons per annum are transferred from the land to the sea (*24*).

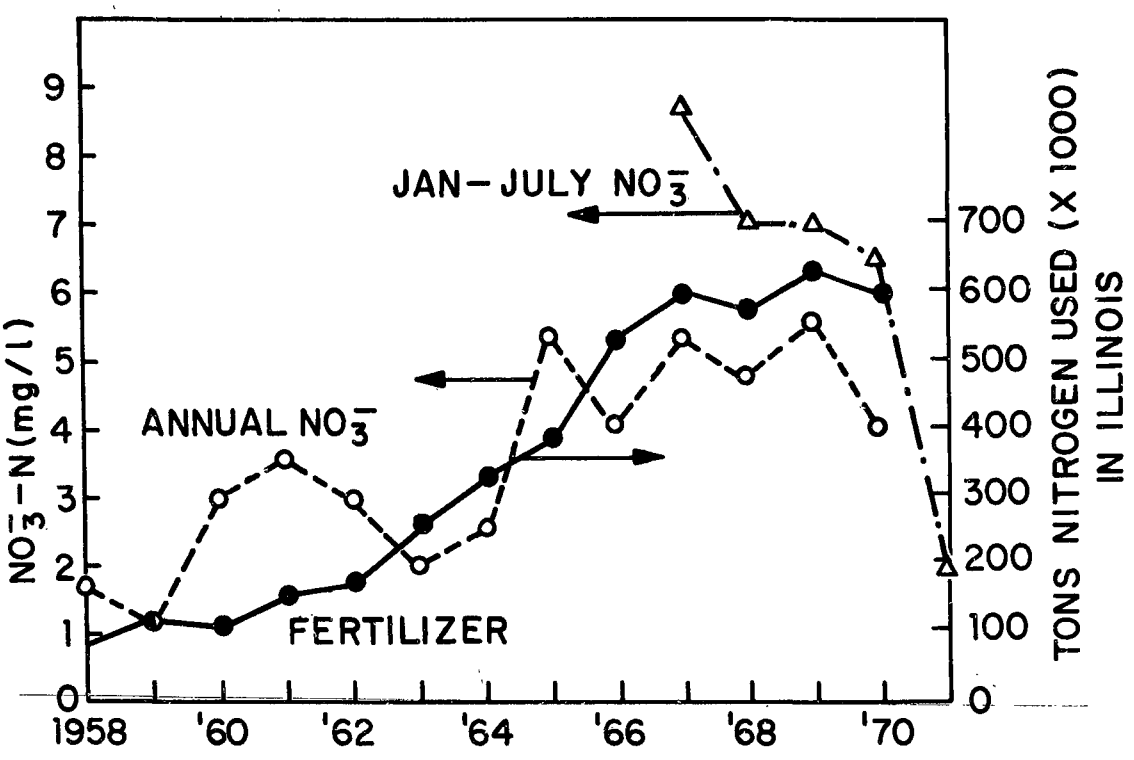


FIGURE 7.—Trends in annual use of fertilizer nitrogen in Illinois and concentration of NO_3^-–N in the Kaskaskia River at Shelbysville Illinois from (*1*). Data for January–July are plotted since 1967 to include 1971 data for which only 7 months were available.

The source of leached nitrogen—biological fixation, abiological fixation by industrial, lightning or other processes or mineralization—has not been established. Some attribute much of the nitrogen leached from agricultural soils to fertilizer nitrogen. The parallel between the increasing nitrate concentration of rivers and the accelerating rate of fertilizer nitrogen used during the last decade initially supported this hypothesis. However, the correlation may have broken down in recent years, as shown in Figure 7 with nitrate concentration decreasing but fertilizer nitrogen use remaining at least constant (*1*). One group has attempted to determine the source through measurements of abundance of ^{15}N in streams (*107*). They suggest that the $^{15}N/^{14}N$ ratio of streams more closely parallels that of fertilizer nitrogen than other nitrogen sources found in agricultural soils. This work has been severely criticized (*84*). In addition to the sensitivity limitations of this technique, the unfortunate use of virgin soil in place of an appropriate control such as unfertilized but tilled agricultural soil appears to invalidate the result. It is clear that additional work is needed to define the source of

leached nitrogen which is fundamental to the control of nitrogen-based eutrophication.

Sedimentation and Solubilization

Nitrogen is transferred from the sea to the deep sea sediment by sedimentation and returned to the sea by solubilization. Estimates indicate that 15×10^6 tons of nitrogen are sedimented each year and 5×10^6 tons are solubilized, providing a net balance in favor of sedimentation (*24*).

Volatilization and Precipitation

Ammonia nitrogen at the rate of about 165×10^6 tons per year is volatilized and about 140×10^6 tons of ammonia nitrogen and 60×10^6 tons of NO_3^-/NO_2^- nitrogen are returned to the land and sea by precipitation (*24*). The additional nitrogen in precipitation over volatilization loss is provided by combustion, lightning and other oxidations. It has recently been demonstrated that plants may effectively take up and utilize ammonia from the atmosphere and also that they may give up lesser amounts of ammonia to the atmosphere (*125*, *144*). Agricultural areas adjacent to sites of high ammonia volatilization such as feed lots may obtain a large portion of their nitrogen by this physical transfer.

GLOBAL NITROGEN CYCLE

The five pools of global nitrogen (Figure 1) and the various transformations and transfers of nitrogen (Figure 2) are synthesized into a global nitrogen cycle in Figure 8 (*24*). On the left hand side of the chart are listed the various transfers and transformations contributing nitrogen to the atmosphere. These include outgassing of primary rocks, abiological denitrification, biological denitrification, combustion and volatilization. These inputs to the atmosphere are balanced by outputs due to industrial fixation, biological fixation, precipitation and reabsorption. Biological denitrification is a forced value used to balance the cycle and the value is substantially in excess of that which present measurements support. The interconversions of N_2, N_2O, NO and NO_2 in the atmosphere provide a catalytic

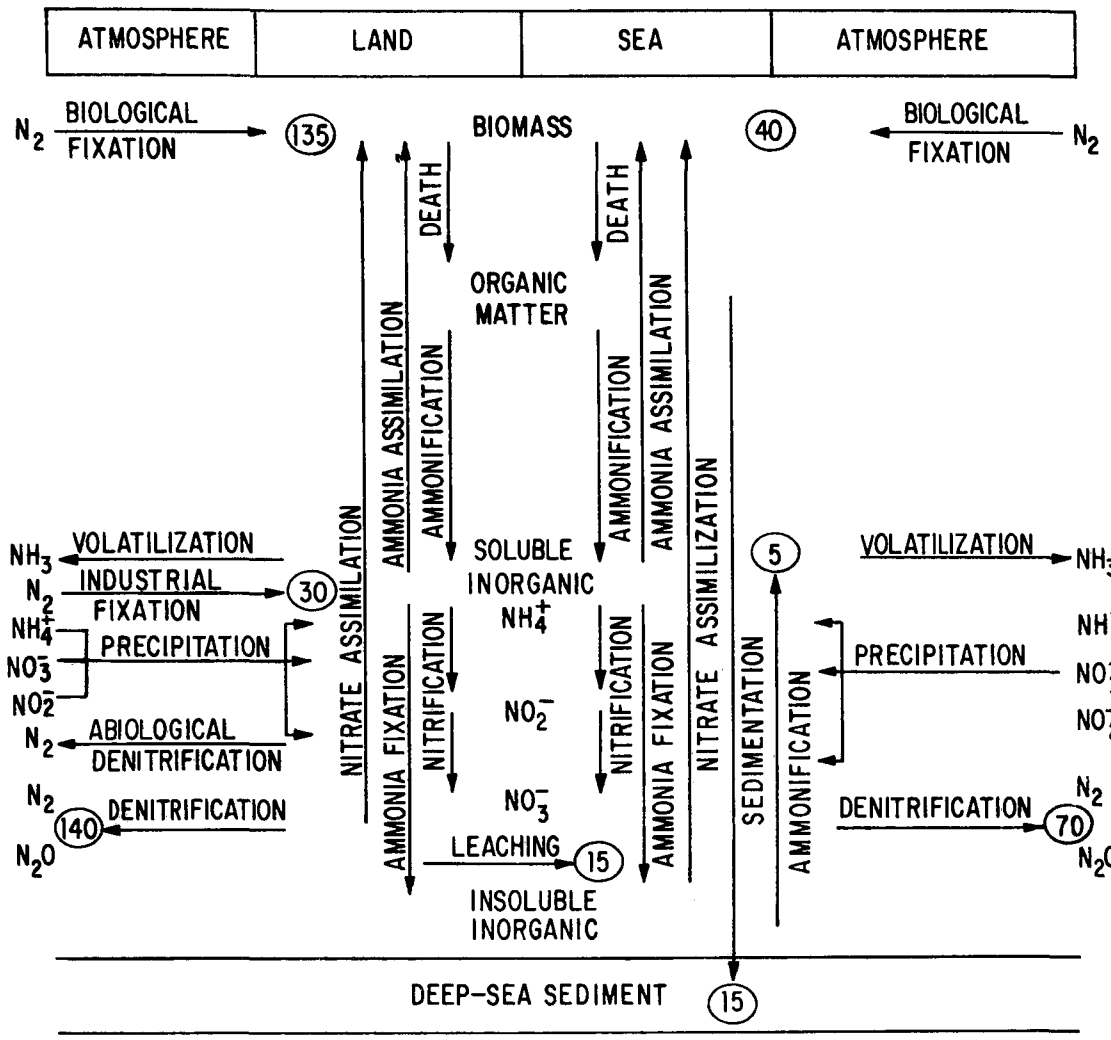


FIGURE 9.—Nitrogen Land-Sea Transformations and Transfers.

system for O_3 consumption. Concern has been expressed that increased nitrogen oxides in the upper atmosphere produced by combustion may lead to the depletion of O_3 which protects the earth's surface from UV rays (*95*).

In Figure 9 the land-sea core of the previous figure is divided between land and sea to show the inter- and intra-pool transfers and transformations. Both land and sea display most of the nitrogen transformations and transfers with a few exceptions *e.g.*, industrial fixation is restricted to land. An approximate allocation has been made in some cases. For example, 135×10^6 tons of biological N_2 fixation is assigned to land and 40×10^6 tons to sea. It is emphasized that the quantitation of the nitrogen cycle presented is only a best estimate from the limited data available.

TABLE 15.—Comparison of N budgets of two lakes

	Lake Mendota (19)	Lake George (91)
	Kg/ha.yr	
Nitrogen fixation	9.3	44
Precipitation on lake surface	11.3	9.5
Ground water		
Streams	9.2	11.1
Seepage	19.7 or 7.3 (103)	?
Urban runoff	3.5	
Rural runoff	6 0	?
Municipal and industrial waste	5.4	
Hippopotamus excreta		3.5
Denitrification	19.5 or 7.1 (103)	?
Outlet loss	10.5	109 (Plankton)
Weed removal	0.8	—
Fish catch	2.9	2.5
Ground water recharge	?	?
Other Losses—sediments	42.7	22.5

A comparison of the nitrogen transformation and transfer budgets of a temperate and a tropical lake are tabulated in Table 15. One comes from Lake Mendota adjacent to the University of Wisconsin campus and possibly the most intensively investigated lake in the world. The other is Lake George in Uganda. Even the data on intensively investigated Mendota is undergoing substantial modification. For example, note changes in denitrification rate and contrast the fixation rate recorded in this table *vs.* that for the same lake in Table 13.

ADDITIONAL NITROGEN MOVEMENTS

Additional natural movements of nitrogen are only now being recognized and additional synthetic or man-made movements are being introduced which will contribute to or modify the nitrogen cycle. Selected examples in Table 16 include N_2 fixation in rhizosphere associations, phylloplane associations, mammalian digestive tracts, white fir decay and *Escherichia coli.* Quantitatively, the digestive tracts are not significant contributors of fixed nitrogen. A recent report suggests that

TABLE 16.—Newly recognized or developed sites of nitrogen transformation

N_2 fixation
Rhizosphere associations—
Azotobacter—Paspalum (43)
Rhizomes of marine angiosperms (65, 138)
Phyl'oplane associations
Leaf algae, lichens, liverworts, bacteria in for example tropical rain forests (51, 96, 166)
Mammalian digestive tracts (11, 52, 67, 77, 147)
White fir tree decay (164)
Escherichia coli (42, 179)
Denitrification
Methanol to remove nitrogen from sewage (5)
NH_3 Assimilation
Foliar uptake and release of NH_3 (125, 144)

nitrogen-fixing bacteria may provide nitrogen for the fungi that decay white fir, a disease that destroys about a million board feet of lumber per year in the U.S. Northwest. The transfer of information for N_2-fixing activity to *E. coli*, a bacteria heretofore unable to fix nitrogen (*42*), has recently been accomplished by genetic manipulation. What other man-made diazotrophs may be developed and what will be their contribution to the nitrogen cycle? In the denitrification area, methanol additions are being planned to stimulate denitrification in sewage treatment plants (*5*). Foliar uptake of ammonia by plants is a recently recognized route for direct assimilation of nitrogen from the atmosphere by plants (*125, 144*).

LIMITATIONS AND NEEDS FOR NITROGEN CYCLE RESEARCH

The scarcity of reliable data is the major limitation of nitrogen cycle research and this limitation has been clearly recognized by many investigators. For example Robinson and Robbins (*156*) wrote that "The weak point in the nitrogen circulation model is primarily the small amount of available data that is applicable to the task. Differential data for land and ocean areas would be especially valuable in making more realistic estimates of the masses of material that enter into various portions of the cycle."

In conclusion, we would like to reemphasize the need to develop novel methods for measurement of nitrogen transfor-

mations and transfers. Alternate detection systems are needed. We propose that an example of such an approach may be the utilization of proton-reaction analysis to determine $^{15}N/^{14}N$ ratios in place of mass spectrometry (*153*). Advantages for proton-reaction analysis include high sensitivity for ^{15}N *vs.* ^{14}N possibly permitting more definitive measurement using samples with natural abundance. Furthermore, chemical treatments of a sample to isolate nitrogen and convert it to N_2 may not be needed. Systems capable of continuous monitoring are desirable. Seasonal or annual profiles of ecosystems are needed and extrapolation from limited measurements of an ecosystem should be avoided. Sufficient data and a suitable experimental design should be used to allow statistical evaluation.

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Questions and Comments Following Dr. Hardy's Talk

QUESTION FROM THE FLOOR: You showed on your charts that in ocean sediments there appeared to be more nitrogen coming out than going in. Do you have any idea of the natural processes in the sediments?

DR. HARDY: The figures presented were obtained from numbers that were available. Any of the numbers on that sheet except industrial input are to be considered as current best estimates. Little, if any information on the specific natural processes in ocean sediment are available.

QUESTION FROM THE FLOOR: I wonder why in your evolutionary scheme of nitrogen transformations that you put nitrogen fixation last or most recent. It's an aerobic system and it occurs so frequently in so-called primitive bacteria.

DR. HARDY: There are plausible arguments that you can make for either an early or late appearances for N_2-fixers. Our proposal is a logical sequence based on the stresses on the organisms. The random distribution of nitrogenase, is not explained in our proposal of late development. Our way of looking at the nitrogen metabolism evolutionary scheme makes it easy to understand.

Environmental Impact of Nitrate, Nitrite, and Nitrosamines

M. ALEXANDER

Laboratory of Soil Microbiology,
Department of Agronomy,
Cornell University,
Ithaca, New York 14850

Environmental microbiologists have been concerned with transformations of nitrogen for decades, and both soil and aquatic microbiologists have actively investigated the behavior of this element in the ecosystems of interest to them because of a variety of practical problems in agriculture and water pollution control. Moreover, a great deal of basic microbiological research has been done on the organisms and the biochemical steps involved in the decomposition, oxidation, reduction, volatilization, and fixation of nitrogen compounds.

Recent developments have again focused attention on the microbiology of nitrogen. A few of the issues of current interest are not really new. High nitrate levels in wells have been known for many years, and the danger of methemoglobinemia to infants and livestock consuming nitrate-rich waters has been a long-time concern of public health and agricultural specialists. Fertilizers have frequently been cited as poisons of biological cycles in the past also, and the recent claims that they are hazardous to critical biogeochemical processes taking place in natural ecosystems are, to a great degree, a repetition of statements made one or two generations ago.

On the other hand, certain of the concerns with environmental hazards arising from nitrogen compounds are quite new. Farmers in some regions of the United States are applying quantities of

fertilizer nitrogen heretofore unknown. Groundwaters which previously were reasonably free of nitrate are now occasionally showing levels in excess of the U. S. Public Health Service guidelines for potable water supplies. Feedlots with large numbers of animals are appearing in many areas, and the vast concentrations of manure would not likely have showed up even in the worst nightmares of farmers of 20 years ago. Eutrophic lakes are being recognized more frequently, and their increasing fertility may sometimes be attributed to an increased rate of release of nitrogen from agricultural, urban, or industrial sources. Furthermore, the possible significance to man's health of nitrosamines, a new class of toxicants, was not appreciated until the last few years.

Unfortunately, few microbiologists have been participating in investigations of these newer aspects of nitrogen pollution, and most of the research is being conducted by scientists in other disciplines. Owing to the absence of a sizable body of microbiological literature relevant to current problems, my comments will be based upon a few recent investigations, an assessment of the older literature, and a recent report of a committee of the National Academy of Sciences, which I was privileged to chair (*4*).

Microorganisms are the chief agents responsible for the cycling of nitrogen in the biosphere. For example, the organic nitrogen in soils, sediments, and terrestrial and aquatic plants is converted to ammonium by a large assortment of ubiquitous heterotrophic bacteria, fungi, and actinomycetes. Members of these microfloras are capable of tolerating essentially all natural conditions which allow for growth of living organisms, so that ammonium is produced in a wide array of circumstances. Ammonium in soil is retained by the clay particles, and thus it moves very slowly through the profile and into the groundwater. However, if O_2 is present and the pH is not too low, the ammonium is oxidized to nitrate in a process known as nitrification. Nitrate is not retained by clay, and hence it moves rapidly through soils to enter the subterranean water. Nitrate, in turn, serves as an electron acceptor for bacteria normally using O_2, and the nitrate is reduced and escapes in the form of N_2 or occasionally N_2O. The fate of the oxide in the biosphere is

presently unknown, but N_2 serves as a nitrogen source for blue-green algae, nodulated leguminous plants, and several bacterial genera and is thereby returned to the water and soil in a form suitable for subsequent plant growth and animal development.

Microorganisms are thus responsible for synthesizing most of the nitrate in soils, groundwaters, inland bodies of surface water, and presumably the oceans. Although any estimate must still be considered as only a first approximation, probably in the vicinity of 21.0 million metric tons of nitrogen are returned annually or are released in the soils of the United States to become potentially available for plant growth. Of this total, about 3.6 million tons is acquired by symbiotic nitrogen fixation resulting from the activities of *Rhizobium* and its hosts, 1.2 million tons is estimated to come from nonsymbiotic fixation largely by free-living bacteria, and 3.1 million tons arises from the microbial decomposition of organic nitrogen in soil and its release in the form of ammonium, which subsequently is converted to nitrate. In addition, 7.5 million tons is added to the soil each year in the form of nitrogenous fertilizers, and most of this too is acted upon by microorganisms, converting the reduced nitrogen to the nitrate form. Thus, probably about 75% of the nitrogen which is available for plant use is transformed by microorganisms in soil, and a large part of this 15.4 million tons is oxidized ultimately to nitrate (*4*).

Many other sources of nitrogen are acted on microbiologically in waters and soils. These sources include urban sewage, effluents from septic tanks, industrial wastes, and substances released from refuse dumps, food processing industries, and feedlots. The quantity of nitrogen provided in these various forms and the amount acted upon microbiologically to yield nitrate are still largely unknown at the present time, but available evidence suggests that a large part of this nitrogen will be nitrified if the environment is aerobic and not overly acid.

The remarkable rise in the number of cattle feedlots shown in Table 1 illustrates the growing problem of point and diffuse sources giving rise to high local concentrations of nitrate. Such large feedlots are frequently situated in regions having little or no adjacent cropland on which to apply the manure, and part

TABLE 1.—Numbers and sizes of feedlots in three representative years (4)

Animals per feedlot	Number of feedlots		
	1962	1966	1970
1,000–2,000	752	938	991
2,000–4,000	373	486	543
4,000–8,000	179	298	331
8,000–16,000	105	136	210
16,000–32,000	26	55	105
..32,000	5	8	41

of the nitrogen in the fecal matter is volatilized as ammonia and part is converted to nitrate by soil bacteria. The anion may leach through the soil and pollute the underlying waters. On occasion, this pollution is marked.

No one can doubt that fertilizers are also contributing to the increasing nitrate levels in certain groundwaters and, after lateral movement of the anion, in surface waters, too. Investigations of soil scientists have clearly shown that high fertilization rates do indeed give subterranean water concentrations of nitrate occasionally in excess of the Public Health Service guidelines for potable water supplies (7). The only real points of contention are the percentages of the fertilizer nitrogen added in any particular watershed that are leached as nitrate after it is nitrified and the percentages returned to the atmosphere as a result of denitrification. The heated debate that we are witnessing involves interested parties who have, sad to say, either inadequate or no data. Inasmuch as the nitrogen level in soil that has been cultivated for some time does not change appreciably, although there is a massive initial loss when virgin land is first tilled, all the fertilizer nitrogen added to soil is lost. Taking the 1970 figures for fertilizer use, 7.5 million tons thus disappear from surface soils.

Of course, only part of the nitrogen is lost by denitrification or leaching, following the nitrification phase. A considerable part of the nitrogen is assimilated by growing plants and that which is not allowed to decay in place is removed from the farm land. If the efficiency of nitrogen uptake by the plant were to be increased, then the loss through denitrification or by leaching

would be diminished. Agricultural scientists have long sought to increase nitrogen efficiency, but the justification has traditionally been to improve crop yield per unit of fertilizer applied. Now the interest is prompted by a desire to minimize environmental pollution. A number of means are being explored to increase the proportion of added nitrogen that is assimilated by the crop. Of particular interest to the microbiologist is the search for selective inhibitors of nitrifying microorganisms, inhibitors which would keep the element in the soil in the ammonium form. Another means of diminishing the nitrogen flow into waters is to enhance denitrification, that is, to carry out some operation in the field to increase the magnitude of the microbial reduction of nitrate before it enters the groundwater. At the moment, however, essentially no research is being done on this approach.

Indeed, surprisingly little data are available on the percentage of nitrogen actually denitrified under field conditions and the percentage that is leached. It was commonly believed not too long ago that denitrification was inconsequential and that most of the added nitrogen or that released from humus entered groundwaters. However, now that it is considered to be unwise to have much nitrate in water, the same groups of individuals who proposed that leaching was the major mechanism of nitrogen loss from soil maintain that denitrification is extremely important. Conversely, a second group states vociferously that the old view is completely correct. In point of fact, neither school of thought has presented convincing data, and except for occasional areas or in greenhouse trials, the significance of microbial denitrification to nitrogen losses from soil is totally unclear.

The type of watershed or ecosystem study which ought to be conducted is illustrated by the work of Biggar and Corey (*2*). They estimated the contribution of various sources to the input of nitrogen into Lake Mendota, Wisconsin. Their data suggest that a significant amount, namely 45%, of the nitrogen entering the lake is derived from the groundwater, an input quite likely resulting from the intensive dairy farming in the vicinity of the lake. A high proportion of the nitrogen entering the lake appears to come from manure washed off the land, and microorganisms

are here once again the culprits as they decompose the animal wastes washed into the lake during the spring thaw and heavy rains. In addition, the lake acquires 14% of its new supply by nitrogen fixation through the activities of microorganisms in the water, presumably the blue-green algae blooming so frequently in that body of water.

Some individuals have argued that the problem of increasing nitrate levels in certain water supplies is simply overcome by reducing the use of chemical fertilizers. Admittedly, in many areas this can and should be done because of injudicious use by farmers. However, high crop yields require much nitrogen, and it makes no difference to microorganisms whether the nitrogen is supplied as ammonium, sewage sludge, plant residues, or manure. The nitrogen is converted to ammonium, if it is not already in that form, and the ammonium is subsequently oxidized to nitrate. After analyzing the changes in fertilizer use and dietary patterns in the United States during the last several decades, the National Academy of Science committee came to the surprising conclusion that the recent marked increase in consumer demand for animal protein, at the expense of plant protein, has contributed materially to the greater use of fertilizer. An inordinate amount of nitrogen must be used to grow the plants which are then fed to livestock and poultry so that we can eat animal rather than plant protein. This is illustrated by the figures in Table 2.

With reference to making animal protein from fertilizers, the efficiency of nitrogen utilization has two components: (a) the efficiency of plants in taking up the nitrogen that is provided in the soil; and (b) the remarkable inefficiency of animals in converting plant protein to animal protein. In fact, of the 29.4 kg of nitrogen in plant protein used or consumed per person per year, only 1.7 kg comes directly from plants that man consumes. The remaining 27.7 kg, or 94%, is used to feed the animals which provide us with the meat, poultry, dairy products, and eggs we consume. Consequently, if Americans are to have an adequate amount of high quality food and continue to consume enormous quantities of animal proteins, agriculture is obliged to use vast tonnages of synthetic fertilizers, much of which will be lost to waterways.

TABLE 2.—Protein and nitrogen consumption in U.S.: 1968 (4)

Protein source	Protein consumed (g/person/day)	N in plant protein (kg/person/yr)
Meats	36.6	19.4[a]
Poultry	8.8	3.0[a]
Fish	2.5	—
Eggs	6.7	1.6[a]
Dairy	15.8	3.7[a]
All plants	28.7	1.7

[a] N in plants required to feed the animals.

A number of scientists and even a few technically trained individuals have again raised the alarm that fertilizers are poisons of microorganisms and inhibit microbial processes. This restatement of an old and largely forgotten issue is rather surprising in view of the enormous literature that has developed since the time that the question was first posed. Certain fertilizers do indeed cause a change in the abundance and activity of soil microorganisms, but this inhibition is both temporary and reversible. However, plants are obtaining the nutrients they need from the fertilizers, nutrients they would have gotten in only small quantities from natural microbial activities, so that the temporary suppression is of no practical consequence. Should fertilizer use terminate, the activities of microorganisms in releasing nitrogen, phosphorus, and other elements would resume at probably the same rate as before.

Why is there the concern with nitrate as a health hazard? If the human infant or the ruminant animal consumes reasonably high concentrations of nitrate, the baby or the animal may develop methemoglobinemia, a disease in which O_2 transport through the blood is impaired by the decreased supply of hemoglobin. The 10 ppm nitrate-nitrogen recommended standard was established on the basis of a number of studies in which it was demonstrated that methemoglobinemia is very infrequent in infants drinking water containing less than this level and frequent in infants consuming water with higher concentrations.

Despite the fact that many surface wells contain nitrate in excess of these recommended limits, the number of reported cases of methemoglobinemia in infants in the United States is decreasing (*4*). The reason for this decline in disease incidence, despite the apparent increase in some areas of high nitrate waters, probably results from the greater vigilance of public health officials, the awareness by pediatricians of the problem, and the information provided to mothers of infants. However, society cannot be complacent about its present good fortune because it is clear that high levels of fertilizers will continue to be used and that present practices will continue to lead to nitrification of the added nutrients with the consequent appearance of nitrate in water supplies. Inasmuch as the nitrate problem will get worse and not better in the absence of new technologies, a real need exists for imaginative microbiological research.

Nitrate may also be hazardous to man and animals because of its presence in food or feed. Certain plants accumulate enormous quantities of nitrate, and consumption of such vegetables or feeds may induce symptoms of methemoglobinemia in infants and are known to have caused deaths in animals. It must be emphasized, however, that commercially prepared baby foods have been implicated in only one instance of methemoglobinemia in the United States in recent years. This remarkable safety record attests to the lack of acute toxicity resulting from the nitrate per se. This point must be emphasized because irresponsible individuals have blown out of proportion the problem of this disease in infants due to baby food. However, high nitrate levels in vegetables fed to infants are of no value, and it should not be too difficult for plant breeders to develop varieties and for farmers to employ practices to minimize the nitrate levels in food and feed.

To a microbiologist, it is astounding that essentially no attention has been given to what happens to the nitrate in foods stored in the home, either at room temperature or in the refrigerator. Recent evidence suggests that nitrate in certain foods may be only partially reduced during storage, and potentially toxic levels of nitrite may appear in these stored commodities. No data actually demonstrate that a hazard exists,

but complacency is again foolhardy, particularly in view of the indications that pathological effects other than those of methemoglobinemia may be associated with the nitrite formed from nitrate. These include effects on thyroid function and vitamin A utilization.

Much has been said and written about eutrophication, and inasmuch as the problem species in many eutrophic lakes are algae, the issue is one belonging to microbiology as well as other sciences. In addition to algal growth being a problem of relevancy to microbiologists, one recognized by few of my colleagues, however, means for removing nitrogen from domestic wastes so it does not enter bodies of water should be of interest to microbiologists. Unfortunately, few of them have been involved in studies of denitrification and algal harvesting, both of which appear to be particularly promising ways of removing nitrogen from such wastes (*3*). Although these studies require microbiological techniques and knowledge, environmental engineers investigating these methods of nutrient removal have had essentially no assistance from professional biologists.

It would also seem worthwhile for a microbiologist to seek an organism, or to develop one by suitable genetic techniques, which is able to oxidize ammonium autotrophically to molecular nitrogen. This process is exergonic and probably thermodynamically feasible. Such an organism would bring about the destruction of a widespread cation and nitrate precursor to yield a volatile product of no concern to man. The organism should grow by using the energy released in the oxidation.

After nitrification was first described in the 19th century and shown to be important in natural environments, autotrophic bacteria able to catalyze the process were obtained in axenic culture. In the subsequent ninety years, essentially all environmental researchers have assumed that nitrification is always an autotrophic process and that nitrite, the only product known to appear outside of the cell, is released only in trace quantities. However, nitrite may occasionally accumulate in enormous amounts (*5*), and it could then constitute a significant albeit local problem. Investigations of models of poultry waste disposals systems with high organic matter loading rates suggest that nitrite accumulates here too. The accumulation probably

results from an inhibition of *Nitrobacter* by ammonia at the high pH resulting from the decomposition of nitrogen compounds in the waste (*1*).

Nevertheless, it is premature to assume that all products of nitrification in natural environments are known. In a recent study, for example, Verstraete and Alexander (unpublished observations) demonstrated that hydroxylamine and 1-nitrosoethanol are formed in sewage treated with simple organic materials. Hydroxylamine is a potent mutagen, and its presence in at least laboratory samples of treated sewage suggests the need for careful testing under natural conditions. A process identical to that observed in samples from nature has also been noted in cultures of an *Arthrobacter* (*6*). Of particular interest is the finding of the nitroso compound, the first report of such a microbial metabolite in natural waters.

C-Nitroso compounds are not known to be health hazards, but N-nitroso compounds, which are more commonly termed nitrosamines, are carcinogenic, mutagenic, and teratogenic. The nitrosation to yield such potent products can occur chemically, but it usually requires the presence of nitrite and a suitable secondary amine. On the other hand, nitrite is formed microbiologically from both nitrate and ammonium. Secondary amines are present in foods, plant materials, and other natural products, and they may be generated microbiologically in the decomposition of a variety of tertiary amines. Thus, one can postulate that microorganisms may contribute to nitrosamine formation in four ways: (a) by synthesizing secondary amines; (b) by forming nitrite; (c) in the nitrosation reaction itself; and (d) by modifying the environment, such as by acidification, in order that chemical nitrosation may take place.

Recent work in the author's laboratory has demonstrated that microorganisms residing in sewage or lake water can produce secondary amines from tertiary amines and from one fungicide. They are then able to nitrosate the secondary amine with the formation of dimethylnitrosamine, an extremely toxic compound. Analogous processes have been shown to occur in soil. These preliminary studies have been performed with concentrations of nitrate, nitrite, secondary and tertiary amines, or fungicide higher than those which would be formed or added

in nature. However, if one extrapolates from the concentrations used in these studies to the low levels that are present in nature and also assumes that the yield of the nitrosamine is linear with precursor concentration, then the quantities may indeed be of public health concern.

To verify that the processes observed in lake water, sewage, and soil actually result from microbial activities rather than being nonbiological reactions, axenic cultures of representative organisms have been isolated and their activity in nitrosamine formation demonstrated. Furthermore, enzymes obtained from these microorganisms were found to catalyze nitrosation of representative compounds (Ayanaba and Alexander, unpublished observations).

Microorganisms are thus extremely important in the formation of nitrate, nitrite, and possibly nitrosamines in natural habitats. The activities of particular applied interest are the mineralization of organic nitrogen, the formation of nitrate by autotrophic bacteria, the possible heterotrophic oxidation of nitrogen compounds to yield products containing oxidized nitrogen, the reduction of nitrate to nitrogen gases, the formation of nitrite by either oxidative or reductive pathways, and the possible biogenesis of nitrosamines or their precursors in water or soil. Despite the critical importance of these microbial activities for man's health and for environmental quality, the body of information remains sparse. Hopefully, microbiologists will soon be attracted to such environmental studies to help bridge the information gap.

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Questions and Comments Following Dr. Alexander's Talk

QUESTION FROM THE FLOOR: It is my understanding that applications of fertilizers on crops have leveled off the past few years, and I was wondering if you'd like to predict what the next crop would be that they're going to hit with nitrogen fertilizers. Do you think it will be wheat?

DR. ALEXANDER: Do you mean where our agriculturalists will be putting the nitrogen? I really wouldn't want to predict this. My own personal concern in this area, and I'm counting on some values which were calculated by members of our committee, is in the developing areas where many crops are going to get nitrogen. But I wouldn't try to guess what crops are going to be getting more nitrogen.

QUESTION FROM THE FLOOR: Would you say that we're going to level off the input?

DR. ALEXANDER: You're asking a microbiologist to give you an answer. If I were to give you an answer, it would be a microbiologists answer, and I wouldn't trust that.

QUESTION FROM THE FLOOR: Is there hope of utilizing the nitrogen build up in water by stripping the algae from waste treatment ponds and feeding it back to the cattle?

DR. ALEXANDER: There is very little hope if the nitrogen comes from diffuse sources, such as agricultural nitrogen where is released into waterways over the country-side. This differs

from an urban effluent, where one has a discrete point source which can possibly be used. But the problem here, too, is that you're not changing the nitrogen. You're recycling the nitrogen and the question is where it goes; down into the groundwater or up into the atmosphere. As far as I know, there are no data indicating that such a recycling will lead to less nitrate pollution of the groundwaters.

QUESTION FROM THE FLOOR: I'd like you to comment on a recent finding that indicates, a good deal of nitrogen as nitrate entering a lake was being denitrified. The nitrate in groundwater was being denitrified before it reached the lake by traveling through the sediments. I'd like to know what your comments would be on that kind of mechanism.

DR. ALEXANDER: I would be surprised that there would be significant denitrification in the groundwater because of the absence of appreciable amounts of electron donors for denitrification. I would not be surprised in the sediments themselves. It really is a matter of having an available energy source for the denitrifying bacteria.

Microbial Influences on Phosphorus Cycling

G. W. FUHS

Division of Laboratories and Research, New York State Department of Health, Albany

ABSTRACT

Measurements of phosphorus uptake kinetics and rates of phosphorus-limited growth show that the maximum uptake rates for orthophosphate exceed the rates of growth-related assimilation of phosphorus into biomass, even at maximum rates of growth. Half-saturation concentrations for phosphorus uptake, on the other hand, exceed those for growth, often by one or two orders of magnitude. Known and apparent half-saturation concentrations of growth for microorganisms are below 5 μg of phosphorus per liter, and uptake of phosphorus may exceed the need for this element whenever growth is restricted in the presence of an energy source and certain required ions (K^+, Mg^{++}).

In aquatic ecosystems the basic relationships are modified by the presence of multiple substrates (organically bound phosphorus) and physical factors which act differently on different species. While certain simple generalizations can be made for the effects of phosphorus on the growth of attached plants and microorganisms in flowing waters, an adequate description of lake ecosystems requires the measurement *in situ* of important rates (growth, sedimentation, decay, grazing) for both total biomass and individual species. These measurements provide a framework for exploratory and predictive modeling in two

opposite directions: the dependence of the total system's behavior on the limiting nutrient input, and the interdependence of species properties and system behavior.

Heterotrophic mixed bacterial systems, thriving on naturally grown organic matter in water or on waste from other heterotrophic systems, tend to be controlled by the availability of carbon and energy rather than of phosphorus. Conditions required for the known mechanisms of metabolic imbalance with respect to phosphorus ("luxury uptake") rarely if ever exist. Indications of aerobic uptake and anaerobic release of phosphorus by bacteria are strong, but the underlying mechanism is insufficiently understood for control and optimization of the process on a technical scale.

INTRODUCTION

The role of phosphorus in biochemical energy conversion and growth has received much attention during the past half-century of research. Its role as a minimum factor for microbial growth was recognized early in the fermentation and yeast-growing industry, and yeast for a long time was the object of choice in studies on its metabolic role. Concern for the effect of phosphorus on water quality stems from observations during the past 25 years that quite often, particularly in fresh-water ecosystems, phosphorus regulates the size of algal and possibly also bacterial populations (*21*, *25*, *34*). Not until very recently, however, have there been investigations into the kinetics of phosphate utilization and the growth rate of microorganisms during phosphate limitation.

In this paper I shall attempt to review the present state of knowledge of the phosphorus nutrition of microorganisms in relation to water-quality management, including the modeling of aquatic ecosystems. Following Mortimer (*24*), I shall emphasize our understanding of mechanisms and not restrict myself to the communication of numbers. If many mathematical models of entire ecosystems have little predictive value, this is due, not to lack of numbers, but rather to lack of the right kind of numbers and inadequate understanding of mechanisms. Unfortunately the traditional set of measurements of water quality

is almost useless for successful ecosystems modeling. Accurate determinations of the appropriate kind are just now becoming possible as a result of new developments in methodology.

Kinetics of Phosphorus Utilization by Microorganisms

Phosphorus occurs in microorganisms in a number of chemical compounds which can be grouped by function into three major categories:

Structural components. These are cell constituents which are indispensable for the viability and integrity of the cell. They include both the hereditary material, deoxyribonucleic acid (DNA), and phospholipids to the extent that they are essential components of a minimum complement of peripheral and internal membrane systems. During each cell cycle these constituents must be synthesized in the amount already present in order to produce two viable cells from one. During slow growth the synthesis of DNA, a major phosphorus-consuming reaction in phosphorus-starved microorganisms, can be discontinuous, possibly resulting in a cyclic demand for phosphorus. This may affect to some extent the diurnal phosphorus cycle in lakes and the competition among species.

Functional components. This category includes compounds which determine the rate of growth, i.e., ribonucleic acid (RNA) and low-molecular, phosphorylated metabolic intermediates, including nucleotides. To the extent that additional intracellular membrane systems and chloroplasts are developed during rapid growth, their phosphorus would also be included in this category.

Storage components. In microorganisms this category is represented by two fractions, which are often incorrectly identified. The first consists of high-molecular, linear polyphosphates in the potassium form, which aggregate as solid concretions of high refractive index and extreme basophilia. These are known as metachromatic or polyphosphate granules (7). In some bacteria and algae these granules appear during unrestricted growth in a rich, balanced medium. In others, the granules do not appear until cells are no longer growing but are still exposed to abundant phosphorus, potassium, and a source of energy. As

can be predicted from their consistency, the granules are built up slowly (several to 24 hours are needed for growth into a microscopically visible particle) and are degraded slowly. This explains the slow turnover of phosphorus in this fraction. The granules are soluble in cold 10 percent trichloroacetic acid, and the fraction therefore is identical with the *acid-soluble polyphosphate* of the biochemical literature (*16*, *35*).

The other fraction is an acid-insoluble component, found in bacteria and other microorganisms, which turns over very rapidly and which resembles polyphosphates biochemically but not cytochemically. Its exact chemical nature and its chemical or structural association are not clear. This fraction has been referred to as *acid-insoluble polyphosphate* (*16*, *35*) or polyphosphate-ribonucleic-acid complex (*2*) and recently has been characterized by Correll (*1*) as a polymer of phosphoric acid which contains imido groups. The structure proposed by Correll is interesting, since it combines high packing of PO_3 residues with a low basophilia (only one negative charge for three PO_3 residues), which would explain why this fraction cannot be stained with basic dyes and requires many fewer K^+ ions for synthesis than the granular form $(KPO_3)_n$. The K/P ratio during synthesis of this fraction is closer to 1:4 (*15*). Other authors also report a low K^+ requirement during the first stages of rapid uptake of phosphorus (*38*). In any case, the acid-insoluble storage fraction tends to appear within minutes after exposure of the cells to phosphorus, and it rapidly exchanges its phosphorus with that of the nucleic acids, so that this fraction and the nucleic acids often show complementary behavior during the growth cycle (*29*, *33*). This fraction is determined by treating trichloroacetic-acid-extracted cells with hot 1 N HCl for seven minutes and correcting for the nucleotide-P which is released simultaneously ("7-min phosphate").

Microorganisms starved for phosphorus or any other element are "programmed" to form as many cells as possible by cell division. All phosphorus then becomes concentrated in the structural components, while in the functional components it is restricted to a bare minimum. In this state the highest cell yield is obtained (expressed as the number of cells or the dry weight per unit of phosphorus consumed). Growing cells contain

phosphorus in the form of functional components as well as 7-min phosphate. A plot of growth rate (μ in Equation 1) vs. cell phosphorus has the form of a saturation curve which intercepts the abscissa ($\mu = 0$) at the minimum phosphorus content per cell (a_0) (*8*). This latter parameter appears to be a species constant. Half-maximum growth is obtained at two times the minimum phosphorus content; 95 percent of maximum rate of growth, at five to six times the minimum content. During unrestricted growth, and even more during growth restriction by nitrogen or certain other elements, phosphorus content per cell (a) increases further but without a concomitant increase in the rate of growth. Maximum rate of growth (μ_{max}) is a function of several factors, including light and temperature.

$$\mu/\mu_{max} = 1 - 2^{-(a-a_0)/a} \qquad (1)^*$$

A complicating factor in the evaluation of Equation 1 is that it holds only on a per-cell, not on a cell-mass basis. During phosphorus starvation and in the presence of an energy source, microorganisms accumulate carbonaceous reserve materials, with a resultant increase in cell mass. Also, as is evident from the preceding paragraphs, the popular concept of approximately constant yield per unit of nutrient spent—a concept derived from Monod's (*23*) experiments on heterotrophic bacteria limited in carbon and energy source—does not apply to phosphorus-limited microorganisms.

Because of shifts in cell composition during different phases of phosphorus limitation and shifts of lesser extent occurring with nitrogen limitation (*4*), it is not possible to predict the effects of simultaneous limitation of the growth rate by both elements from experiments where only one element is limiting. Both phosphorus and nitrogen limitations have certain effects in common, such as restriction of nucleic acid synthesis and excessive (luxury) uptake of carbon.

Although cell phosphorus determines the growth rate directly, external phosphorus determines the rate of replenishment of the internal phosphorus pool and therefore is the ultimate regulator of growth rate. Uptake of phosphorus follows Michaelis-Menten

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* Growth rate is in doublings per day (base 2 logarithms). Time basis is one day.

kinetics (Equation 3):

$$V = a(2^{\mu} - 1) \tag{2}*$$

$$V = V_m \frac{S}{K_s + S} \tag{3}$$

Equations 1–3 can then be combined to give a fourth relationship in a form which, for practical purposes, is indistinguishable from either Monod's (*23*) hyperbolic model or Teissier's (*32*) exponential model linking growth rate and substrate concentration. The hyperbolic equation has the form:

$$\frac{\mu}{\mu_{max}} = \frac{S}{K_m + S} \tag{4}$$

Arriving at Equation 4 via Equations 1–3 appears circuitous; but it is practical, since growth-limiting phosphorus concentrations are often too low to be measured directly in chemostat experiments where the use of the isotope ^{32}P is impractical (*8*). The isotope, however, is easily handled in short-term uptake experiments used in the evaluation of Equation 3.

The set of Equations 1–3 is not complete, however, because it does not make provision for a limitation of uptake of phosphorus from the environment, i.e., control of V by cell phosphorus, or by an active fraction thereof. An effect of cell phosphorus on V_m alone was postulated by Jeanjean (*14*), but our experiments suggest an effect on both V_m and K_s (*9*). More recent experimentation in our laboratory suggests that Haldane's (*12*) modification of the Michaelis-Menten formula can be used, provided that the velocity of the back reaction (V_r) is assumed to be zero. This assumption is in agreement with results by other authors who describe phosphorus uptake as a virtually unidirectional process (*11*, *17*, *31*). The modified Equation 3 reads:

$$V = \frac{V_m \cdot S/K_s - V_r \cdot Z/K_r}{1 + S/K_m + Z/K_r} \tag{3a}$$

Assuming that $V_r = 0$ and substituting cell phosphorus in excess of the starvation level ($a - a_0$) for the intracellular product, Z,

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* Equation 2 unfortunately was misprinted in an earlier article (9). Growth rate and time basis are as in Equation 1.

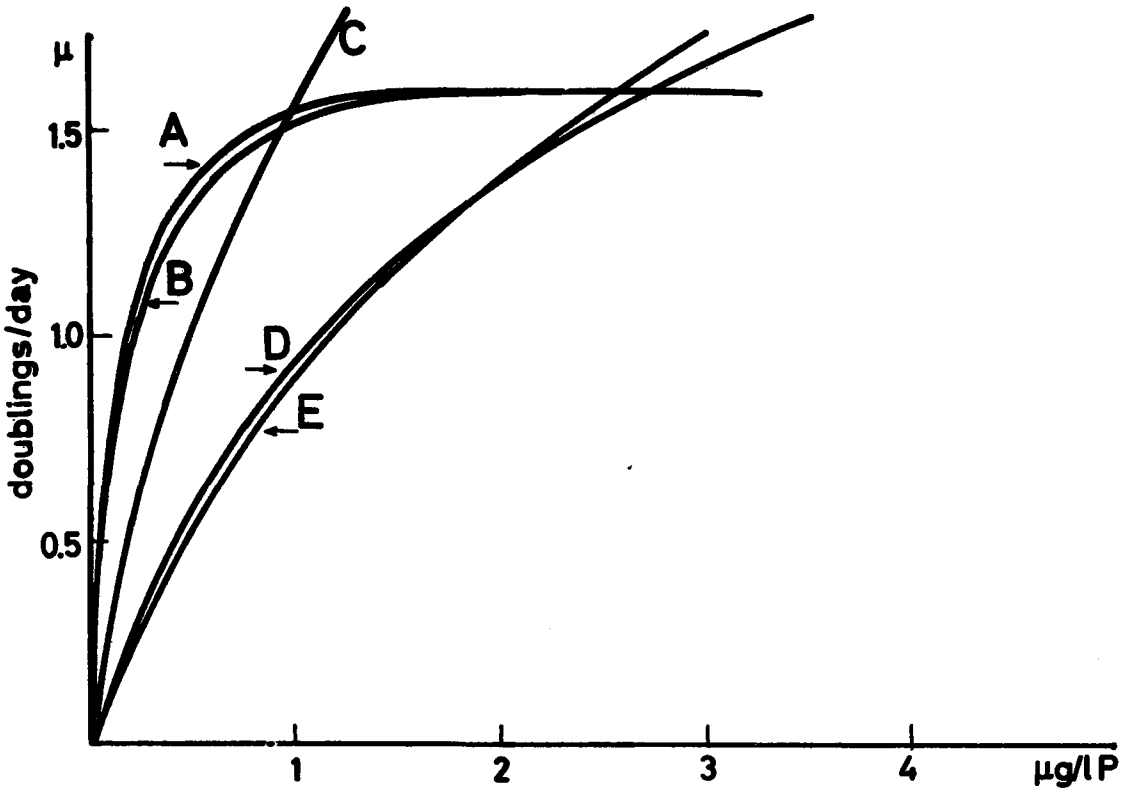

FIGURE 1.—Growth rate of two diatoms and three bacterial species as a function of P concentrations with orthophosphate as a source of P. A = *Cyclotella nana*, B = *Thalassiosira fluviatilis*, C = *Pseudomonas aeruginosa*, D = *Corynebacterium bovis*, E = *Bacillus subtilis*. From (*9*).

we obtain:

$$V = \frac{V_m \cdot S/K_s}{1 + S/K_m + (a - a_0)/K_r} \tag{3b}$$

where K_r is a constant which expresses the degree of end-product inhibition. In our experience, $(a - a_0)$ can also be replaced by "7-min phosphate," which suggests that this fraction is involved in the regulation of phosphorus uptake.

Two principal results have been obtained from studies of growth and uptake kinetics using orthophosphate as a source for phosphorus: The half-saturation constant for phosphate-limited growth (K_m) for all algae and bacteria examined so far is in the range below 5 μg P/l, sometimes below 1 μg P/l (*9*), as shown in Figure 1. Literature data giving a much higher range of limiting concentrations, particularly with *Scenedesmus* (*3*, *11*), have not been confirmed in chemostat experiments in our laboratory (Rhee, personal communication)

By contrast, the half-saturation constant for phosphorus uptake can be much higher; for various bacteria and algae examined so far, it is in the range of 5 to 400 μg P/l. Short-term uptake experiments alone, therefore, are not sufficient to predict the growth rate of an organism from a given phosphorus concentration. This is illustrated in Figures 2 and 3, which are based on earlier experimental data (*9*) for a planktonic diatom. It is evident that variations in V_m and K_s in the order of magnitude observed do not materially change K_m, although, all other factors being equal, the organism with a higher V_m or a lower K_s would realize a certain competitive advantage.

In nitrogen-limited algae K_s and K_m appear to be of similar magnitude (*4*). One possible explanation is the smaller storage

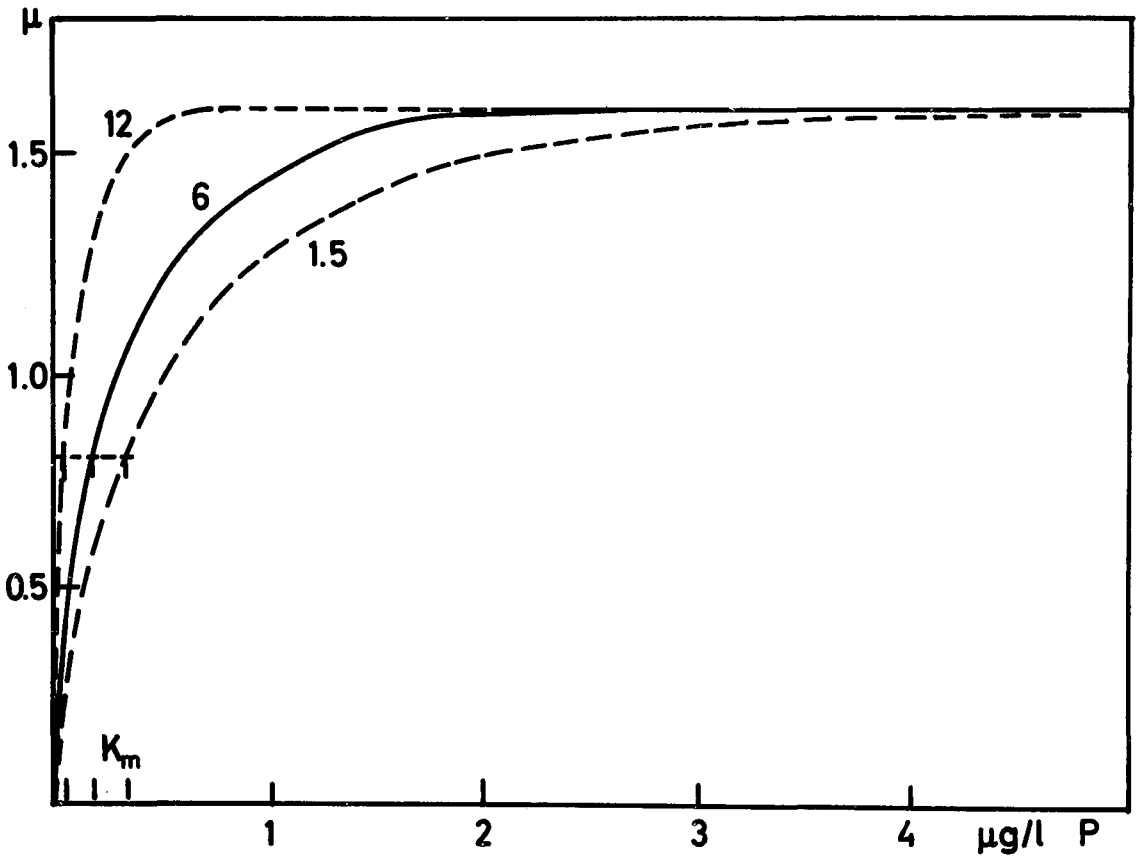

FIGURE 2.—The effect of V_m (maximum uptake rate) on substrate-dependent rate of growth. Solid curve based on experimental data for *Thalassiosira fluviatilis*, a planktonic diatom (*9*). With $\mu_m = 1.6$ doublings·day^{-1}, $a_o = 12.5 \times 10^{-15}$ g-atoms P·cell^{-1}, and $K_s = 50$ μg P·liter^{-1}, V_m varied as indicated, in units of 10^{-12} g-atoms P·min^{-1}·cell^{-1}. Wide variation of V_m causes K_m to vary only between 0.1 and 0.4 μg P·liter^{-1} (lower left of graph).

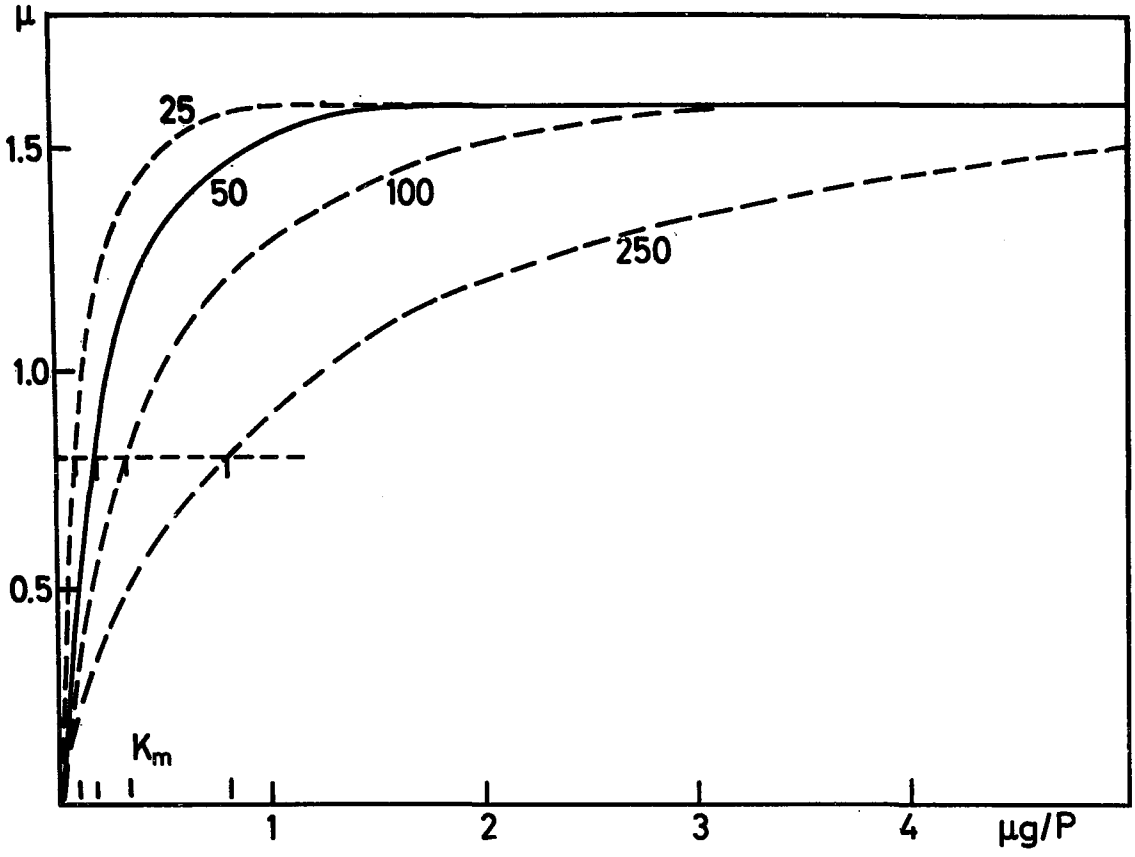

FIGURE 3.—The effect of K_s (Michaelis constant for uptake) on P-dependent rate of growth. Solid curve based on experiments with *Thalassiosira fluviatilis* (*9*). Constants as above, except for constancy of V_m (6×10^{-12} g-atoms P·min^{-1}·cell^{-1}) and variation of K_s as indicated, in units of μg P·liter^{-1}. As a result of variaiton in K_s, K_m varies between 0.1 and 0.8 μg P·liter^{-1} (lower left of graph).

capacity of microorganisms for nitrogen as compared with that for phosphorus.

Other Environmental Factors Affecting Phosphorus Uptake

Rapid uptake of phosphorus from very dilute solutions can result in the rapid depletion of microlayers around the microorganisms. Their repletion with nutrient by diffusion or water movement therefore can become an important regulating factor. Canelli and Fuhs (ms. in preparation) found that phosphorus uptake by diatoms was reduced when medium flow along the cells was slow or absent. At medium flows of 1 mm/min, uptake at any concentration between 1 and 100 μg/l was reduced to one-half of the maximum value (observed at velocities above

10 to 20 mm/min). Reduction of the surface-to-volume ratio and impediment of flow at the cell surface by gelatinous sheaths and in colony-forming microorganisms may also slow the uptake from very dilute solutions and extend the range of limiting concentrations.

When orthophosphate supplies are near exhaustion—a condition commonly found in freshwater lakes during summer—organic phosphates become a major source of phosphorus for microorganisms. These constitute a complex substrate for which no single half-saturation constant can be given. Uptake may vary from species to species and with the nature of the substrate (27), and the involvement of phosphatases excreted by phytoplankters is another complicating factor.

Application of Kinetic Data: Qualitative Considerations

From the facts presented in the preceding section we can safely conclude that in any standing body of water, phosphorus limitation of the microbial growth *rate* exists when *orthophosphate* falls to a concentration of 3 μg of phosphorus per liter (0.1 μg-at/l) or lower, regardless of the presence of *organic* phosphates. At higher concentrations, the rate may still be limited for some forms, depending on the replenishment of phosphorus in microlayers close to the cell surface.

For attached algae in flowing waters, or for suspended algae in turbulent streams, orthophosphate-phosphorus concentrations of 5 μg/l and above indicate with some probability an absence of phosphorus limitation. Concentrations below 5 μg/l are found in many lake effluents and in certain mountain streams (e.g. in the Adirondack Mountains in New York). If sewage effluents reach these waters, luxurious growths of attached algae may occur as a result of the increase in phosphate concentration. It is equally obvious that in such environments a massive increase in growth rate can result from a minuscule increase in phosphorus concentration—say, from 0.2 to 1.0 μg/l—on the steep part of the growth curve (Equation 4). A strong response may occur on periphyton slides immersed in such waters, although the development of massive weedbeds

which exhibit a marked phosphorus-stripping activity, or the development of an algal bloom in a receiving lake, would require a more plentiful supply.

Whenever massive weed growths occur below sewage discharges into streams with naturally high levels of phosphate, it may be suspected that the principal cause for growth stimulation is not phosphate but free carbon dioxide derived from sewage.

Application of Kinetic Data: Quantitative Considerations

The critical influence on algal growth of concentrations of orthophosphate too small to be measured with a high degree of accuracy, together with the effects of the other interfering factors mentioned above, poses a serious obstacle to the construction of conventional ecosystem models, because a substantial uncertainty is inevitable in any prediction of growth rate of a planktonic microorganism, alga, or bacterium, if that prediction is based on chemical measurement of substrate concentration and if the limiting substrate is phosphorus. Since a management model requires accuracy within ± 25 percent, a two- or threefold uncertainty in the estimate of the most important parameter, algal growth rate, obviously cannot be tolerated.

There is no viable substitute for the measurement of microbial growth rates *in situ*. Such measurement offers two advantages: a major difficulty in determining the principal process rates is solved, and by relating growth rate to chemical concentrations and kinetic constants, the interfering factors and lesser known properties of the species can be explored systematically. The results of these studies provide valuable feedback for the understanding of species dominance and are a necessary step toward more specific predictions of the response of the ecosystem to variations in phosphorus loading.

Going a step further, we can make this approach part of a larger plan for the characterization of a lake ecosystem. This master plan is being used with success in our studies of New York State lakes, which are designed both to improve water quality management and to generate data needed for basic research.

Our basic plan is illustrated in Figure 4, which shows a rigorously simplified model of a phosphorus-limited lake ecosystem. Criteria of phosphorus limitation have been described elsewhere (*9*). Only two pools are distinguished, both expressed as phosphorus. All other elements are rate terms: I (input), O (output via effluent, or overflow), dB/dt (change in biomass), and S (sedimentation). The term $(dB/dt)+S$ can also be approximately written $\mu-(G+X)$, that is, primary productivity less grazing by zooplankton and other effects, as explained below. In the equation

$$I+R=\mu-(G+X)+O \tag{5}$$

then, the nutrient regeneration rate (R) is the only unknown, and it can be calculated for each time interval studied. The comparison of I and R as forces driving μ reveals an important characteristic of the lake. The role of R with respect to I increases with the degree of eutrophy and with decreasing mean depth and is affected by wind mixing. Although sedimentation measurements can also provide us with data on M (mineralization), this parameter does not enter our simplified model.

G (grazing) is not measured at present; consequently the term $G+X$ stands for all interconversion of biomass and organic soluble phosphorus by grazing, excretion of organic phosphates by algae (*18*), and lysis. This complex remains to be resolved for a better characterization of population growth, and one might start by measuring its strongest component, which is probably grazing. Grazing in this context means the excretion of soluble phosphorus by all zooplankton and the solubilization of zooplankton fecal pellets by bacteria.

The growth of bacteria on detritus, as well as the feeding of protozoa and rotifers on bacteria, constitutes cycling of phosphorus within the particulate fraction and is not measured. One may go so far as to express some doubt whether the separation of bacterial processes in the epilimnetic cycle is essential except in rare circumstances. Soluble organic phosphorus compounds excreted by zooplankton may be taken up by algae as well as by bacteria or may be hydrolyzed by enzymes produced by either group of organisms. The effect of bacteria here is less readily identified than their role in the cycling of carbon and

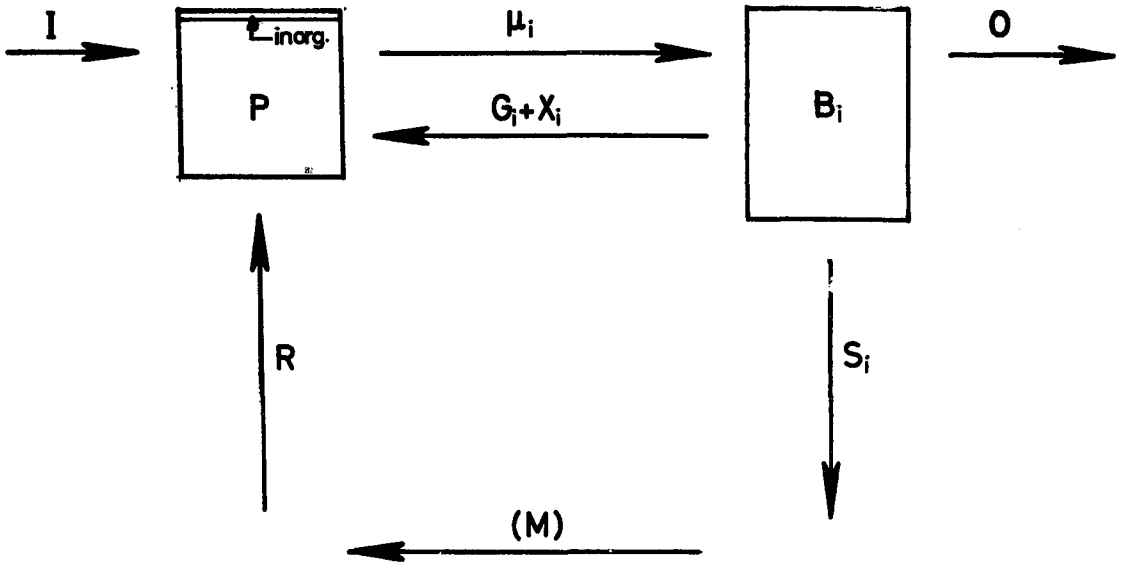

FIGURE 4.—Simplified conceptual model of P cycle in lakes. *I*, input; *P*, substrate (dissolved P); *B*, biomass; μ, rate of incorporation of *P* into *B*; *O*, output (overflow); *S*, sedimentation; *M*, mineralization; *R*, recycling of P. Subscripts indicate that rate and concentration terms can be determined for individual species as well as for total mass and rates of transformation.

nitrogen in the epilimnion and their role in the mineralization of organic matter at the sediment surface.

Subscripted variables in Fig. 4 are those that are determined not only for total standing crop but also for all major species of phyto- and zooplankton, using a conversion of basis from total phosphorus to volume. From dB/dt and S for each species, the "net" growth rate $(\mu - G - X)$ is determined. This approach was used with biweekly surveys in our studies of Lake Canadarago (Otsego County, New York). A few major conclusions deserve mention:

1. The maximum net growth rate of any alga observed so far was 0.6 doublings per day. A rate of 0.2 doublings was about average for many forms during active growth.

2. The development of blue-green algal populations can be accompanied by increases in the pool of soluble organic phosphorus, possibly by excretion or light-induced lysis. Algal production at the expense of dissolved organic phosphorus demonstrates indirectly the activity of phosphatase-producing species. If an algal population contributes to the soluble pool when disappearing from the water column, this is indirect evidence of grazing by zooplankton or lysis.

3. Our contention that grazing and sedimentation are by far the most important removal mechanisms for plankton algae is based on the relatively low mineralization rates in our sediment sampling devices (1 percent or less of cell phosphorus released into supernatant during a biweekly sampling period). This rate is in line with observations by Foree et al. (*5*).

4. During the growing season, total biomass production in terms of phosphorus is about 10 times the total phosphorus input, less overflow. This shows that incoming phosphorus is utilized 10 times before being precipitated irreversibly or flushed out, a characteristic of eutrophic lakes of this type. Our measurements in this lake also show the importance of what E. A. Thomas (*34*) has called the phosphorus "macrocycle" (involving the hypolimnion and the sediments), as compared with the "microcycle" (cycling within the epilimnion).

The important point is that the descriptive scheme of Fig. 4 does not yet represent a mathematical model. It lacks predictive value because it operates with a set of factors whose values vary continually (as determined at biweekly intervals, in our study). The factors are properties of the planktonic species and of rapidly changing meteorological conditions. As an example, growth rate (μ) not only depends on nutrient concentrations, solar radiation and temperature but also varies from species to species. Sedimentation (S) is affected by buoyancy, which in turn depends on the nutritional state and other properties of the species. Grazing (G) depends on the size and other properties of the several species, those grazing and those serving as food. Nutrient recirculation (R) varies with sediment exposure and wind action, which may cause upwelling of bottom waters or partial overturn.

By relating these factors to available information on species and weather, in addition to nutrient input, concentration data, kinetic constants, and other laboratory data on nutrient requirements, we can effectively explore the lesser known properties of the different species or groups of physiologically similar species, guided by observations rather than assumptions. In this manner we can come to understand the causes of species dominance and develop models as predictive tools. In the meantime, the simplified descriptive model can be used for qualitative

estimates and semiquantitative predictions, assuming no change in species succession and dominance.

This approach to the modeling of a lake ecosystem has the advantage that it relies on quantities and rate terms which can be determined with some degree of accuracy in phosphate-limited, confined systems. By contrast, in nitrogen-limited marine systems the current approach is one of reliance on nutrient concentrations and kinetic data for the estimation of growth rates (*4*). This appears justified because kinetic data on nitrogen uptake by microorganisms can be applied more easily to practical situations than kinetic data on phosphorus utilization and because sedimentation rates are much more difficult to measure in vast, open and complex oceanic systems.

Phosphorus and Microorganisms in Sewage Treatment

All organisms, when given ample nutrients and a supply of energy, are programmed to synthesize preferentially the essential components of their cell such as nucleic acids, protein, and membrane and cell wall material. Intracellular carbohydrates are formed for temporary storage of energy only. This principle is a useful one, since it permits the production of a maximum number of progeny containing a minimum complement of essential constituents.

If a heterotrophic organism utilizes such cell material, part of it is consumed for energy, which is derived from the degradation and oxidation of the carbonaceous skeletons of the various cell constituents. Unless the material happens to be almost pure carbohydrate, nitrogen and phosphorus are released from the part of the substrate utilized for energy and are excreted as waste materials. An excess of nitrogen and phosphorus relative to energy content is therefore found in most organic food for heterotrophic organisms—and even more so in waste from heterotrophic organisms (humans, animals), relative to the need of other heterotrophic organisms (bacteria) which feed on it. This is the situation in biological sewage treatment, where bacteria are degrading carbonaceous material in the presence of an excess of both nitrogen and phosphorus.

The ability of microorganisms to take up phosphorus in

excess of immediate need has received much attention and is summarily referred to as "luxury uptake." In reality this term subsumes three or possibly four very distinct mechanisms, which are activated under very specific sets of environmental conditions. The first, which was mentioned in the beginning of this paper, is uptake to sustain the growth rate. During unrestricted growth, a microorganism contains five or six times as much phosphorus as during phosphorus starvation. This additional amount is indispensable for rapid growth. Although it exists partly in the form of storage materials (e.g. 7-min phosphate), reduction of this amount would reduce the rate of growth. If phosphorus supply were interrupted, the organism would grow and divide at a steadily decreasing rate until all progeny contained only the minimum amount of phosphorus required for survival.

Luxury uptake in the stricter sense of the microbiological literature is dead storage of phosphorus, typically in the form of solid polyphosphate granules. This phenomenon normally occurs when bacterial growth is limited by lack of some nutrient which is not required for phosphorus uptake and storage. Phosphorus uptake requires, besides phosphorus, a source of energy, magnesium as an enzyme activator, and potassium as a neutralizing cation. Growth limitation by lack of any of these factors will not normally induce luxury uptake of phosphorus. Nitrogen limitation, however, is a good condition for phosphorus storage, and so are some less likely conditions, such as sulfur starvation.

The third condition, known as the "phosphate overplus" phenomenon, was first demonstrated in yeast and was subsequently observed in algae and certain bacteria (*13*, *19*). It occurs when phosphorus-starved organisms are exposed to phosphorus-rich media or solutions. In this case, phosphorus accumulation proceeds at a far higher rate than the synthesis of other cell constituents, but it is still dependent on the availability of an external or internal energy source.

If we compare these conditions for luxury uptake in its different forms with the conditions prevailing in biological sewage treatment, it is clear that luxury uptake in the strict sense is unlikely. In domestic and most other wastes, bacterial growth

is limited by the supply of carbon and energy; the exceptions are certain carbohydrate-rich industrial wastewaters. Occurrence of the phosphate-overplus phenomenon is equally unlikely, as it requires a phase of phosphorus starvation. Such starvation does not occur at any time during the biological treatment of domestic sewage.

A new biological mechanism has been described by Levin and Shapiro (*19*) and Shapiro (*30*). It occurs when activated sludge cycles between aerobic and anaerobic phases, and its kinetics resembles those of the phosphate-overplus phenomenon and the storage of granular (acid-soluble) polyphosphates (with maximum phosphorus content reached in four hours). Phosphorus accumulation is faster than during "metabolic upshift" (increase in growth rate) and much slower than chemical precipitation. Selection of suitable species may be involved, since the bacterial mass must apparently be exposed to the aerobiosis-anaerobiosis cycle over an extended period of time before the process is operational. More experimentation is also needed with regard to the exact nature of the storage material and its localization, since according to Shapiro phosphorus is stored in an acid-soluble form (*30*), whereas Yall *et al.* (*36*) find increases mainly in the acid-insoluble fraction. At present, this mechanism poses more new questions than have been answered so far, partly because of the general scarcity of data on the release of phosphorus from viable microbial cells.

Not to be confused with these mechanisms is the chemical precipitation of sparingly soluble phosphates on microbial cells and colonies, a process that occasionally has been confused with uptake and release of phosphorus by the organisms. A case in point is the alleged diurnal cycle of uptake and release of phosphorus by algae in phosphorus-rich natural waters or laboratory media (*26*). Reinvestigation of the phenomenon has shown that together with phosphorus, a stoichiometric amount of calcium was bound; and autoradiography with ^{45}Ca showed that calcium phosphate had been precipitated on the cell surface during daylight hours, when the pH was high due to photosynthetic removal of CO_2 from the medium. At night, release of respiratory CO_2 lowered the pH to such an extent that the precipitate dissolved (*6*). The same phenomenon is easily demon-

strated with activated sludge, particularly in hard water. During periods of aeration, respiratory CO_2 is expelled and the pH rises; in the settling tank, the pH is lowered by CO_2 accumulation (*22*).

CONCLUSIONS

Bacteria and algae, sharing the basic properties of all organisms and a plantlike mechanism of nutrition (uptake of soluble or solubilized phosphorus), show similar absolute requirements for phosphorus and similar uptake and growth kinetics, exemplified in the existence of very effective uptake of orthophosphate from very dilute solutions.

Interspecies variation which already exists with regard to orthophosphate utilization is so greatly intensified in the presence of mixed organic phosphates and under the modifying action of physical factors that serious errors are introduced in the prediction of growth rates from simple chemical measurements. Predictions of standing crop are even more dangerous, as they depend on additional variables which are affected by the characteristic properties of species. Only the recognition of species differences and the introduction of more meaningful measuring techniques, particularly the direct measurement of certain transfer rates *in situ*, can prevent ecosystems models from becoming detached from reality and largely worthless as predictive tools.

The investigation of the role of phosphorus in heterotrophic systems reveals the need for research into the operation of known biochemical mechanisms under environmental conditions and for the development of a basic explanation of phenomena which cannot be predicted from existing knowledge of cell physiology. Capabilities for control and optimization of environmental processes cannot be expected to develop without such research.

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Questions and Comments following Dr. Fuhs's Talk

QUESTION FROM THE FLOOR: I wonder if you could comment on aquatic macrophytes as a sink for phosphate wastes.

DR. FUHS: I have been looking for data to show that the source of phosphorus for aquatic weeds is entirely in the surrounding water. Certainly you see things growing on old mud banks of streams where sewage treatment has newly been instituted. But I have no data on the kinetic uptake of phosphorus from the aqueous phase.

STATEMENT FROM THE FLOOR: There have been radioactive uptake studies which have shown that aquatic plants do take up phosphorus.

Microbial Recycling of Naturally Occurring Refractory Material

DR. R. R. COLWELL

Department of Microbiology
University of Maryland
College Park, Maryland 20742

ABSTRACT

Sampling devices specifically designed for microbiological work in the field are needed. The methods presently available for enumeration and characterization of *in situ* microbial activities are also limited in scope and application.

Detailed information on the cycling of matter in nature is lacking and would be more easily obtained, if knowledge of indigenous microbial communities, i.e., the natural flora, were available. The carbon cycle is, at the present time, described in the literature in simplistic terms, with much of the present understanding of the breakdown of carbon polymers derived from studies of pure substrates, such as, cellulose, chitin, lignin and pectin. Recycling of carbon compounds in nature can be shown to involve interrelationships with the annual cycles of organisms, as exemplified by the association of chitin-digesting vibrios with zooplankton. These relationships, as well as the biochemistry of breakdown and assimilation of carbon compounds, should be incorporated into carbon cycle models.

The assigned task of this presentation was to discuss the role of microorganisms in the carbon cycle of the aquatic habitat, insofar as the available knowledge of the biochemistry and ecology of carbon compounds permits. This is at once extra-

ordinarily difficult and yet not burdensome, i.e., difficult because of the enormous complexities involved in the mobilization of carbon by microorganisms in the aquatic habitat, yet deceptively simple because of the paucity of data. A simplistic over-view thus can be presented without undue embarrassment or apologies.

Before moving into the main theme of the discussion, some comment on the problems of obtaining samples for aquatic microbial ecology is appropriate. Aquatic microbiology, herein defined as encompassing fresh water, estuarine and marine microbiology, inevitably involves field work at some point in any serious investigation. The methods used in the field, in the main, are primitive, despite the fact that we are in the computer age. A fair statement of the art would be, I believe, to say that no new method has been developed by aquatic microbiologists specifically for use in aquatic microbiology field studies since the turn of the century. A variety of modifications of the sterile glass bottle sampler and the adaptation of some potentially sophisticated practices, such as radiorespirometry for *in situ* work, have been done, but expenditure of intellectual creativity in the design and fabrication of equipment to overcome the multitude of problems besetting any microbiologist working in the field has not been made to any significant degree. The only possible exception to this sweeping statement may be the ATP assay system devised by Holm-Hansen and his colleagues (1966). The methods for studying aquatic microorganisms are not unique and are, in general, taken directly from soil, food and medical microbiology. In our own work we have employed data processing by computer to identify bacteria isolated by use of selective, elective and enrichment culture, grouping bacteria by enzymatic capabilities, nutritional requirements and level of complexity of nutritional requirements, with relative incidence of these groups used to characterize water masses, as was suggested originally for comparison of rhizosphere versus non-rhizosphere soils (Lochhead and Burton, 1957).

The usual method of quantitating the microflora in a sample of water or sediment is by dilution plate, membrane filter and most probable number count methods. These are all indirect means of enumerating microorganisms in such samples and

possess inherent sources of error due to limitations imposed by the nutrient medium, temperature, pH, pressure, salinity, etc. employed. Direct counts, via direct examination of sample aliquots suspended on slides, the use of fluorescent antibody microscopy, submerged slide methodology or the more recently developed infrared microscopy method for visualization of unstained microorganisms in natural habitats (Casida, 1968) provide somewhat greater detail concerning distribution of particular organisms in water and sediment and in the Aufwuchs community (Seligo, 1905; Cooke, 1956; Potter, 1964), i.e., on the surfaces of animals, plants and submerged inanimate objects. Transmission electron microscopy has also been used by Bae, Cota-Robles and Casida (1972) to separate and concentrate indigenous microorganisms from soil and ocean sediment without the occurrence of growth. "Dwarf" cells, less than 0.3 μm in diameter were thus discovered in untreated soil and sediment samples. As high as 72 percent of the cells observed in a natural sample were dwarf cells. Scanning electron microscopy has been used by Sieburth (1972) to view the alignment of bacteria, yeasts and fungi on the surfaces of seaweeds, wood and sand grains. Fåhraeus (1947) observed that the cellulases of certain *Cytophaga* spp. were not extracellular and cellulose digestion required physical contact between the cells and the substrate, whereas in several fungi the cellulases are freely diffusible exoenzymes. Scanning electron microscopy thus may assist in elucidation of cellulytic activity by *Cytophaga* spp.

In summary, a major problem faced by the aquatic microbiologist is that he must attempt to ferret out details of the carbon, or any other cycle, while lacking an appropriate sampling technology. Only two, perhaps three, samplers that can be considered to be reasonably reliable are available for taking water samples at desired depths. These include the J-Z sampler (ZoBell, 1946), employing sterile rubber bulbs and restricted to neritic ocean or coastal region sample-taking, the Niskin sampler (Niskin, 1962), which consists of a sterile plastic bag, messenger-triggered for opening at coastal or deep-ocean depths, and the sterile glass bottle for streams and estuaries (ZoBell, 1946).

Sediment samplers designed strictly for microbiological work are not yet available. Coring devices with autoclavable plastic

liners are the best compromise of corers available on the market (Colwell and Zambruski, 1972). However, these must be sent down open so that contamination with surface water cannot be avoided. A variety of samplers have been constructed for chemical and physical oceanography applications. Some cannot be adapted; for example, the Nansen bottle, and others have been used because nothing else was available, but the oligodynamic effect of the metal sampler and its non-sterile state must be contended with. The gravity corer, bucket sampler for sandy sediments, dredge, oyster dredge and plankton net are all apparatus not suitable for microbiological work in the strictest sense. Thus, instrumentation is an immediate need, and it is in this area that research and development would provide substantial benefit, since the necessary technology is sufficiently advanced to meet the requirements of instrument development. Unquestionably, without the proper tools, aquatic microbiology is seriously impeded.

Naturally occurring refractory compounds are generally considered to include the less readily degraded components of plant and animal residues, such as, cellulose, chitin, pectin, lignin and humus. In most textbooks, the carbon cycle is very simplistically presented showing carbon moving from the atmosphere, as carbon dioxide, to organic carbon via green plant photosynthesis, also via algae and some bacterial species. The plant and animal tissues deposited in soil and streams thus become part of the organic complex of the sediments. Organic carbon degradation is viewed chiefly as microbial attack on cellulose, lignin, pectin and humus materials to complete oxidation, producing carbon dioxide and water, thus completing the cycle. See Figure 1.

Higher plant tissues are essentially boxes or tubes made up of cellulose, pectin and minor polysaccharide components, such as, galactans, mannans, xylans and arabans. These cellular building blocks are cemented together by an interstitial mortar of pectic substances, which in ageing tissues may contain an increasing proportion of lignin (Codner, 1971). The pectin, cellulose and other components of the plant cell are complex and variable, as are the microbial enzymes degrading these systems. Pectic substances are a group of colloidal polymeric materials formed mainly from anhydrogalacturonic acids in

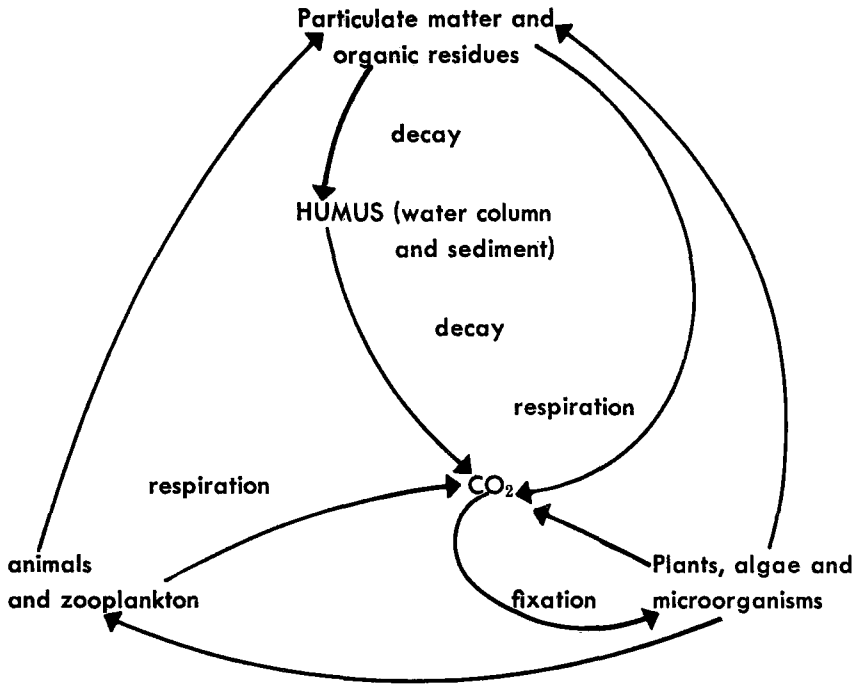

FIGURE 1.—The Carbon Cycle in the Aquatic Environment.

linear α1-4 glycosidic linkage. Compounds referred to as pectic acids or low methoxyl polygalacturonic acids are compounds which contain unesterified carboxyl groups of the polymer or only esterified to a very limited extent by methoxyl groups. Where greater than 5 percent of the carboxyl groups is esterified with methoxyl, the term pectinic acids is applied. A wide range of microorganisms, both terrestrial and aquatic, produce pectin-methyl esterase capable of hydrolyzing the ester groups with the production of methyl alcohol. A variety of microorganisms can also effect hydrolytic cleavage of pectic acid.

Cellulose, one of the world's most abundant polysaccharides, is composed of repeating 1-4β-linked glucose units. Microbial breakdown of cellulose is brought about by hydrolytic glycosidases specific for the β1-4 D linkage, i.e., cellobiase activity. Most of the cellulose decomposition in nature is catalyzed by fungal and bacterial enzymes derived from gram negative saprophytes, anaerobic rumen bacteria of several genera and anaerobic thermophilic sporeformers.

Cellulolytic, pectinolytic and ligninolytic microorganisms, including many species of bacteria and fungi, are common in soil and aquatic sediments and have been isolated from the

aquatic habitat without great difficulty. Generally speaking, the breakdown product of cellulose is glucose and other hexoses, which is readily metabolized by a great variety of microorganisms. Again speaking generally, the aerobic bacteria produce CO_2 and cell material, whereas anaerobes produce gases, fatty acids and alcohols and other neutral products, i.e., an "incomplete" utilization. Plant and animal residues in soil and sediment and comprising a portion of the particulate matter which in water, when in the last stages of decomposition, are referred to as humus: dark-colored, amorphous material containing compounds not readily decomposed by microorganisms. Humus, then, is used to denote the organic fraction of soil that is generally resistant to decomposition, distinguishing it from the organic matter of leaf litter, for example. Initial plant litter contains some water-soluble materials, such as sugars and amino acids, which are leached out and readily decomposed. The humus in soil contributes to the bulky nature of soil, acting as a buffer and water-holding agent. Krey (1967) reported on studies of detritus in water samples of the North Atlantic and suggested that detritus acts as an adsorber of dissolved organic matter and plays a role in the ocean similar to that of humus in arable soils.

Interest in organic particles in seawater was focussed by Nishizawa, Fukuda and Inoue (1954) who published pictures of "sea snow." Organic matter in the aquatic environment was the subject of a symposium, where much of the work done on the subject has been summarized (Hood, 1970). For the purposes of this discussion, the nomenclature proposed by Riley (1963) is used, i.e., the term organic aggregates refers to the flat, plate-like forms or flocculent, heterogeneous amorphous aggregations and detritus to the recognizable particles, which include zooplankton carapaces, diatom tests, faecal pellets and plant fibers. The assumption has been made by many investigators that large numbers of bacteria are attached to these particles, both organic aggregates and detritus. Documentation by direct observation has been made by only a few investigators (viz., Jannasch, 1958). Wiebe and Pomeroy (1972) undertook the examination of the phenomenon of microbial attachment to particles in seawater and concluded that in the open ocean,

regardless of the location, two types of aggregates were dominant: flat, plate-like flakes and flocculent forms. The chemical composition of both types was undertaken mainly by staining procedures. They observed that "mucus-like" material was more associated with flocs than with flakes. Carbohydrate was a more substantial part of the aggregates and aggregates were more chemically heterogeneous. Seldom were bacteria or other recognizable microorganisms found associated with flakes. Wiebe and Pomeroy (1972) concluded that relatively few bacteria are directly associated with particulate matter, although bacteria became extremely abundant and overgrew large flocs during incubation. This finding was true of estuarine as well as open ocean waters. Thus, it may well be that the flakes comprise the humus of the aquatic environment, either settling to concentrate in the sediments or remaining suspended in the water column. Size distribution of particles in sea water has been studied by Sheldon *et al.* (1972).

Thus, organic matter is found abundantly in water and bottom sediments, in solution in the water or as fractions of planktonic and organic debris, i.e., the seston, and suspended in the water. Organic material is clearly abundant in sediments. Organic matter in the aquatic environment derives from two main sources: (1) incoming waters and wave action at the shore, which introduces allochthonous matter into lakes, streams and estuaries. This accounts for part of the total organic matter, with the nature of the substances brought in being varied, ranging from leaf litter of shorelines to upland soil leachings and organic pollution; (2) autochthonous matter produced by living organisms and by decomposition of plant and animal bodies (Reid, 1961). The relative contribution of these sources is difficult to measure, since appropriate techniques are not perfected.

The nature of the soil that forms in given areas, hence the allochthonous material contributed to the aquatic regions, is greatly dependent on climatic conditions affecting microbial action, as well as the kinds of plants that develop.

Organically poor lakes contain 1–2 mg C/l, with the total organic content, *ca* 73 percent carbohydrate and 24 percent protein. Richer lakes contain up to 26 mg C/l, with about 90

percent of the organic matter carbohydrate and 10 percent protein. The total organic content of most "average" lakes is as much as three or four times that of the open seas. Seawater generally contains about 2 mg C/l and about 0.2 mg organic N/liter. Most estuaries receive considerable quantities of allochthonous materials from stream inflow and drainage from surrounding marshes or other land features.

Cycling of refractory materials in the aquatic habit can be considered to take place chiefly at the water-sediment interface. Aquatic ecosystem surface sediments act as a boundary between the circulating dynamic medium primarily dominated by the properties of water and its solutes and the structurally more stable medium, the sediment, with properties much like soil. This boundary area is the site of intense microbial activity in most natural waters. A variety of bacteria can be isolated from surface sediments in high numbers relative to the overlying water column. Harrison, Wright and Morita (1971) examined

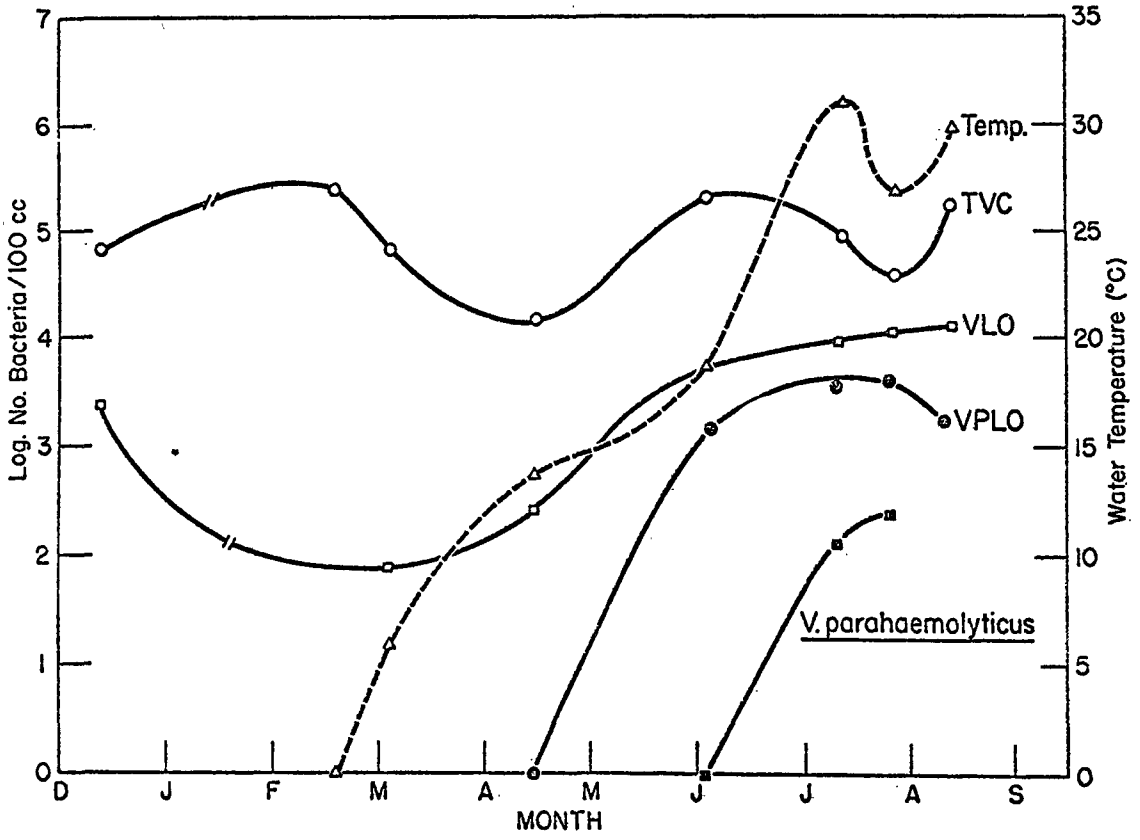

FIGURE 2.—Bacterial counts/100 cc water showing relationship of the incidence of *Vibrio* spp. (VLO), organisms closely related to *Vibrio parahaemolyticus* (VPLO) and *V. parahaemolyticus* strains with temperature. VPLO appeared in the water column at 14C and *V. parahaemolyticus* at 19C.

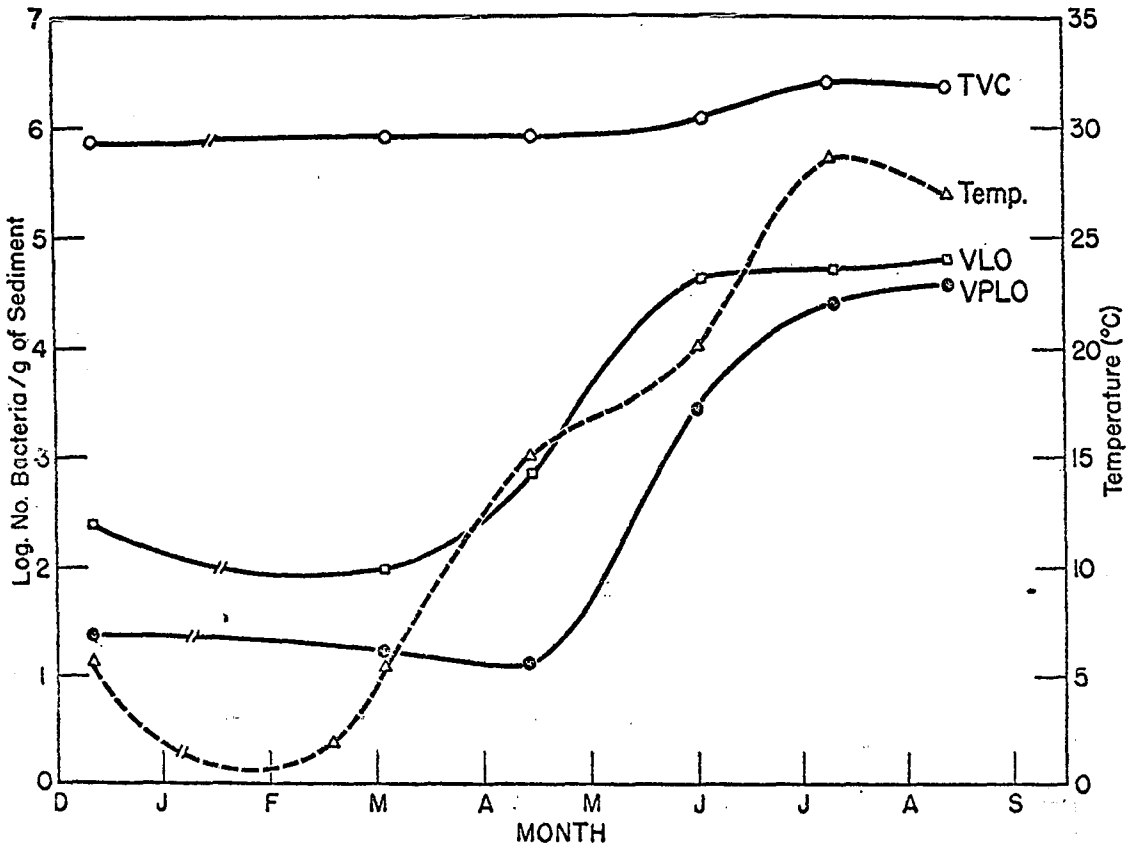

FIGURE 3.—Changes in total viable count (TVC), numbers of *Vibrio* spp. (VLO) and organisms closely related to *Vibrio parahaemolyticus* (VPLO) and temperature, measured during the period December 1970 through August 1971. Survival of VPLO in sediments during the winter months is shown.

the uptake of ^{14}C-labeled glucose and acetate by lake sediments and observed that the top sq. cm of sediment was capable of mineralizing 24 × the amount of glucose as the overlying sq. cm water column of Klamath Lake water, based on a mean depth of 2.7 m. The short turnover time indicated such biologically active substrates as glucose could not accumulate in lake sediments. This was in agreement with the findings of Vallentyne and Whittaker (1956) that extremely low glucose concentrations of the order of 5 μg/liter were present in interstitial water in lake sediments.

The mud-water interface contains most of the organic matter sedimented from the surface water. In the case of particulate matter in the great ocean depths, observations of Barber (1968) and others indicate that particulate matter in deep waters below the photic zone is comprised of material only very slowly degraded by bacteria, i.e., refractory or humus-like material.

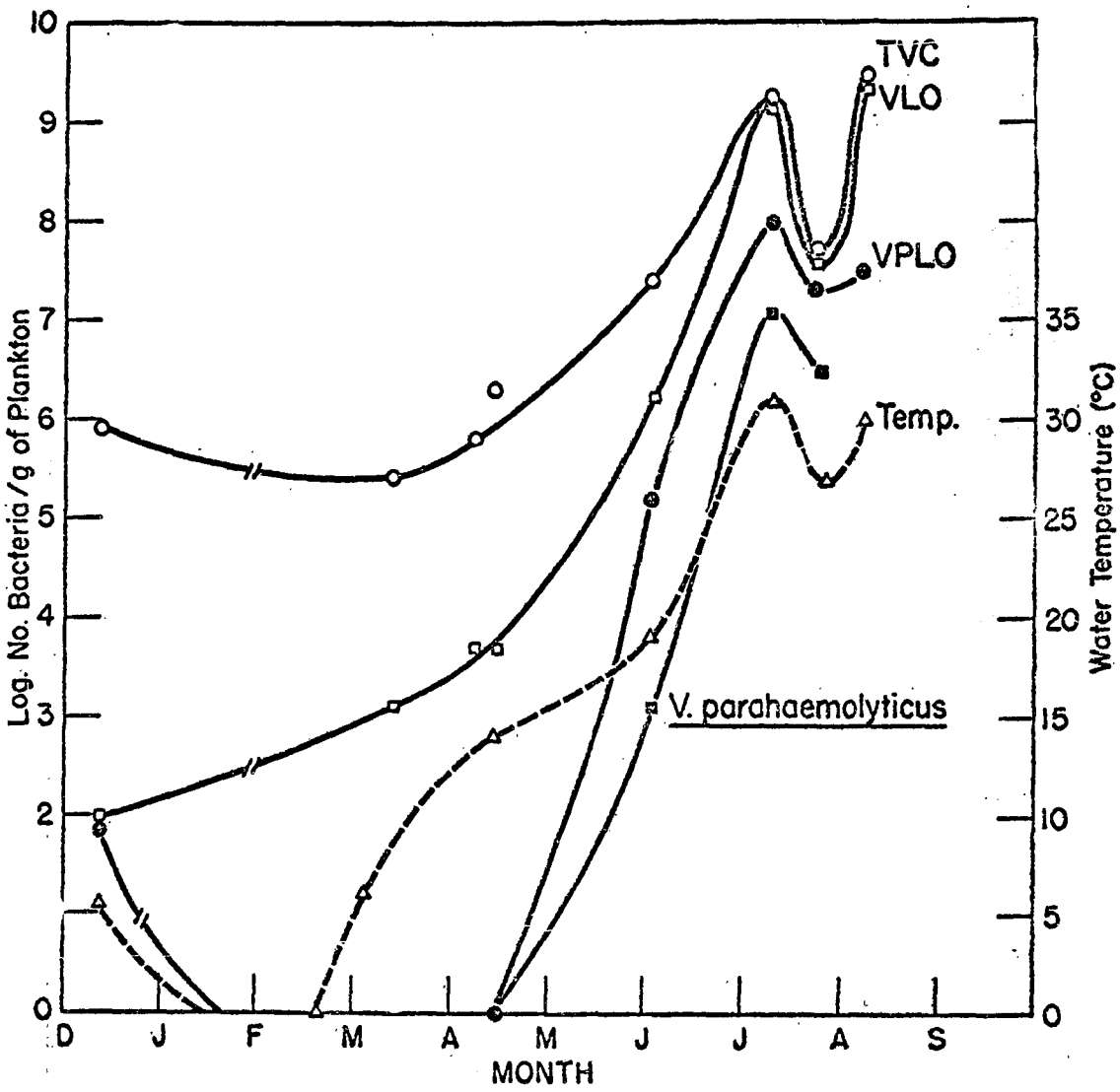

FIGURE 4.—Bacterial counts/gram plankton, showing seasonal changes in the bacterial flora of plankton. Organisms closely related to *V. parahaemolyticus* (VPLO) and *V. parahaemolyticus* strains appeared simultaneously in and on plankton when the water temperature reached 14C.

Thus, organic materials not mineralized while floating in the sea, deposits and forms part of the sediments where the residual organic matter would be further utilized by the bacteria, fungi, protozoa and benthos. Residual refractory material would thereby be permanently trapped in the sediment. In most of the open ocean, the rate of deposition is slow, in the order of <1 cm/100 years for chitin (Seki, 1965(a)).

Chitin is a naturally-occurring refractory material that has been best studied in ocean and estuarine waters. Decomposition of chitin has been shown to occur mainly in the sediments,

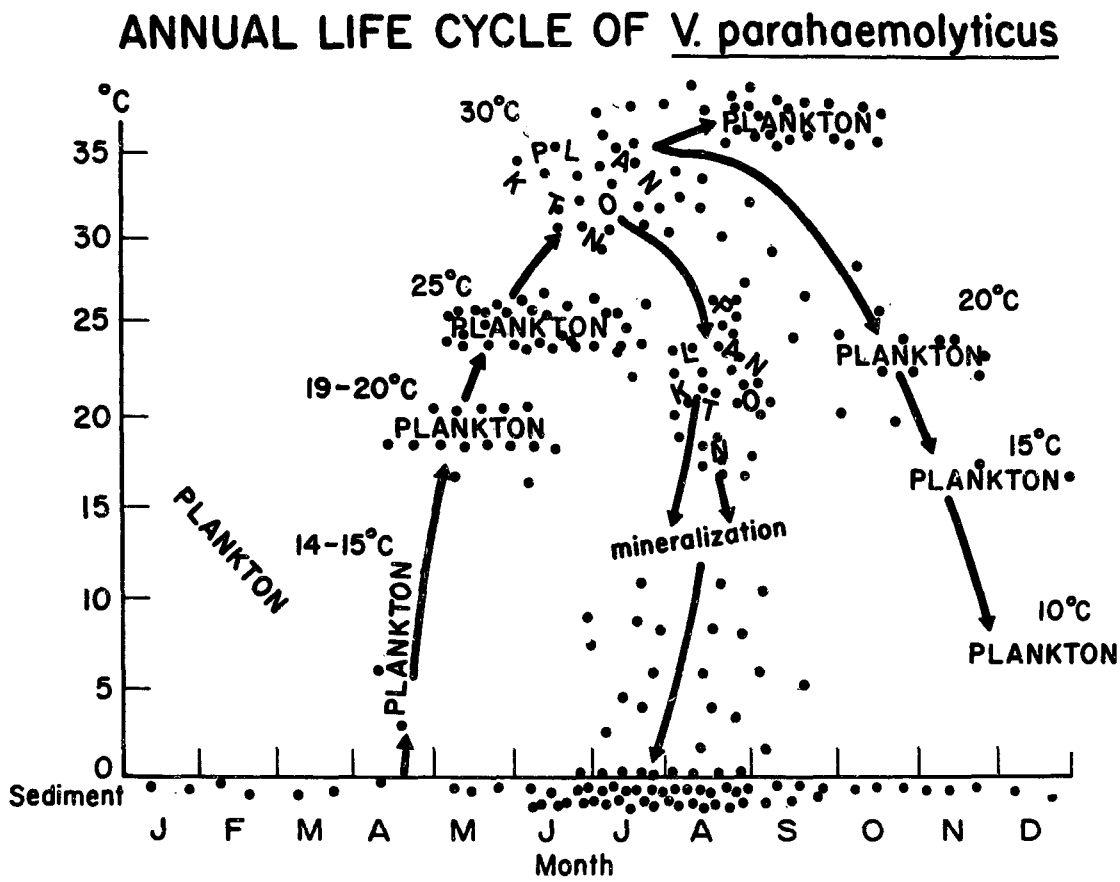

FIGURE 5.—Annual Life Cycle of *V. parahaemolyticus*.

with very little remains of crustaceans being found in sediments of shallow marine bays and inlets where planktonic and benthic production of crustaceans is high. Chitinoclastic bacteria, generally speaking, are aerobes or facultative anaerobes. Thus, the greater part of precipitated chitin is mineralized aerobically in the topmost layer of the sediment. Aleschina (1938) showed that sulfate-reducing bacteria also may have the ability to oxidize chitin. Thus, in bays or estuaries, where significant amounts of hydrogen sulfide are present in the sediment, sulfate-reducing bacteria may be largely responsible for the decomposition of chitin. Seki (1965(b)) estimated that for planktonic crustaceans in natural seawater, a theoretical time for decomposition of chitin could be calculated as 40–70 days in surface waters at 15°C, within 370 days in intermediate waters at 5°C and within 500 days in deep waters at 1–2°C. Seki and Taga (1963) found marine chitinoclastic bacteria to be widely distributed in seawater, on plankton and on suspended matter (detritus). Chitinoclastic bacteria comprised less than 0.4 per-

cent of the total bacterial number in the neritic zone. Many chitinoclasts were found on copepods.

These results are interesting in view of our own studies on a chitinoclastic bacterium, *V. parahaemolyticus*, in Chesapeake Bay. The distribution of this organism in water (Fig. 2), sediment (Fig. 3) and on plankton (Fig. 4) led us to hypothesize a relationship between this organism and the copepod, *Calanus* sp., in Chesapeake Bay (Fig. 5), (Kaneko and Colwell, 1973).

In conclusion, the bits and pieces of information available on refractory materials and their synthesis and catalysis in the aquatic environment suggest that the cycling of these materials does occur in lakes, estuaries and the oceans. However, the data are fragmentary, and the patterns of true "cycles" of these materials remain to be painted in full detail.

ACKNOWLEDGEMENT

The support of the National Science Foundation (Grant No. GB-35261X) and of the National Oceanic and Atmospheric Administration (Sea Grant Project Grant No. GH-91) for studies of *Vibrio parahaemolyticus* quoted in this paper is gratefully acknowledged.

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Questions and Comments following Dr. Colwell's Talk

QUESTION FROM THE FLOOR: Does *Vibrio parahaemolyticus* occur in fresh water?

DR. COLWELL: This organism in general demonstrates a preference for salinites above 12 parts per thousand, so it is restricted to coastal and estuarine areas.

QUESTION FROM THE FLOOR: Have you looked at the contamination in a Zobell sampler due to the messenger going down the slide wire?

DR. COLWELL: I have looked at this problem with a Niskin sampler but not with the Zobell sampler.

STATEMENT FROM THE FLOOR: I would bring an endorsement for the record on Dr. Colwell's request for modern equipment and the development of equipment as a research priority for deep ocean and ocean sampling and work. Some of the problems are the need to miniaturize ocean sampling equipment for inland water, for continuous monitoring of the ocean environment, and in particular for engaging in rate measurements.

Microbial Interactions with Hydrocarbons: Physiological and Structural Correlates

W. R. FINNERTY
and R. S. KENNEDY

Department of Microbiology
University of Georgia
Athens, Georgia 30601

INTRODUCTION

At present, little detailed knowledge exists at the molecular level or the organismic level as to the means by which diverse microorganisms grow at the expense of various hydrocarbons. Advances relevant to hydrocarbon microbiology have appeared at frequent intervals (*1*, *6*, *11*, *21*, *28*). These reviews serve to emphasize our paucity of knowledge concerning detailed mechanisms in hydrocarbon oxidation processes by microorganisms. A large number of microorganisms have been shown to utilize hydrocarbons quite efficiently as sole carbon sources. Also, general classes of hydrocarbons (paraffins, olefins, aromatic, and non-aromatic polynuclears) have supported growth of bacteria, yeasts, and fungi. Summation of these studies have revealed that products of hydrocarbon utilization are predominantly acids, alcohols, ketones, and esters. Oxidative mechanisms have been proposed following a consideration for structural formulae involved with comparisons being made between starting compounds and products obtained following growth. Such chemical analogy has allowed meaningful analysis of microbial hydrocarbon degradation patterns.

While a great number of microbiological studies have been directed towards a compilation of hydrocarbon utilizing micro-

organisms, very few workers have undertaken serious studies concerning the detailed mechanisms of hydrocarbon assimilation and utilization. The exact nature of the biochemical mechanisms which regulate hydrocarbon oxidations by microorganisms are generally poorly understood at best.

Micrococcus cerificans represents a microorganism capable of prolific growth on a large variety of paraffinic and olefinic hydrocarbons greater than ten carbons in length. Published work has delineated the nature of compounds arising from growth of *M. cerificans* on many of these hydrocarbons (*4*, *9*, *25*, *26*, *27*). Further studies have provided additional insight into the biosynthesis of characteristic esters resulting from growth of *M. cerificans* on specific hydrocarbons (*5*, *9*). Detailed biochemical analyses have appeared with this microorganism defining the comparative aspects of hydrocarbon assimilation (*14*, *15*, *16*, *17*, *18*, *19*).

This report extends these studies with observations concerning the effect and fate of hydrocarbon on a microorganism in pure culture. The results of these studies emphasize our lack of detailed knowledge concerning the impact of petroleum on biological entities in the environment and the consequences which arise from this relationship.

MATERIALS AND METHODS

Organism and Growth Conditions. All studies were carried out with the HOl-N strain of *Micrococcus cerificans* as described by Finnerty et al. (*3*). The organism was grown on a mineral medium consisting of (in grams per liter): $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; Na_2HPO_4, 6; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; pH 7.5.

n-Alkanes and n-1-alkenes (Humphrey Chemical Co., New Haven, Conn.) were added to a final concentration of 1 percent.

Organisms were also grown on nutrient broth (0.8%)-yeast extract (0.5%), acetate (2%), and ribose (2%) for comparative studies. All hydrocarbon and non-hydrocarbon growth substrates were added to the defined mineral medium.

Cultures were grown on a gyratory shaker at room temperature (25 C) to the late exponential growth phase and harvested

by centrifugation at room temperature. Cell pellets were washed two times with the mineral medium to remove the external non-metabolized hydrocarbon.

Fixation: Two fixation procedures were used. Method 1 followed the techniques described by Kellenberger et al. (*10*). In method 2, the procedure of Glauert and Thornley was used (*8*). A cell suspension was mixed with equal parts of 5 percent glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) containing 2 mg of $CaCl_2$ per ml.

Dehydration and Embedding. The fixed cells were dehydrated by one of two procedures. Method 1 involved processing the samples through a graded series of water-ethanol mixtures followed by propylene oxide. These samples were infiltrated with Epon according to Luft (*13*) or with Maraglas as described by Freeman et al. (*7*). In method 2, the samples were dehydrated by processing through a graded series of Durcopan as described by Staubli (*24*) and embedded in Araldite. Ultrathin sections were cut on a Reichart OMU-2 ultramicrotome and mounted on uncoated 300 mesh copper grids. The sections were stained with lead citrate (*23*) followed by uranyl acetate and examined in a Philips-200 electron microscope operating at 80 Kv.

Extraction of Cells. Cell pellets obtained from the growth of *M. cerificans* on specified substrates were washed 5 times with distilled water by repeated centrifugation. Each cell pellet was processed through a graded series of water-ethanol mixtures as used in the dehydration for EM (50:50, 30:70, 5:95, 5:95, 0:100, 0:100, v/v). The alcohol extracts were pooled and reduced to dryness. All alcohol extracts reduced to dryness except those obtained from hexadecane and hexadec-1-ene grown cultures. The alcohol extracts were dissolved in chloroform, dried with anhydrous sodium sulfate, and dissolved in hexane for analysis by gas chromatography.

Gas-Liquid Chromatography (GLC). A Packard gas chromatograph, series 7500, consisting of a dual-column oven with coiled-glass columns (4mm inside diameter, 1.83 m long) was used for the analysis of alcohol extracts. The detection system was an argon ionization detector with column support systems consisting of liquid phases of 10 percent Apiezon L and 20 percent diethylene glycol succinate (DEGS) on a support of 70–80

Anakrom A. Operating conditions were: column temperature, 120 C; detector temperature, 190 C; injection temperature, 180 C; outlet temperature, 205 C; argon flow rate, 60 ml/min; chart speed, 2.5 min/inch.

X-Ray Diffraction Analysis. Cultures of *M. cerificans* grown on specified hydrocarbon and non-hydrocarbon substrates were washed extensively and lyophilized. These cell preparations were encapsulated in a Mylar film and cooled to −10 C with a cold air stream. The cooled specimens were exposed to a 1 mm diameter beam of Ni-filtered X-rays from a Cu anode and a flat

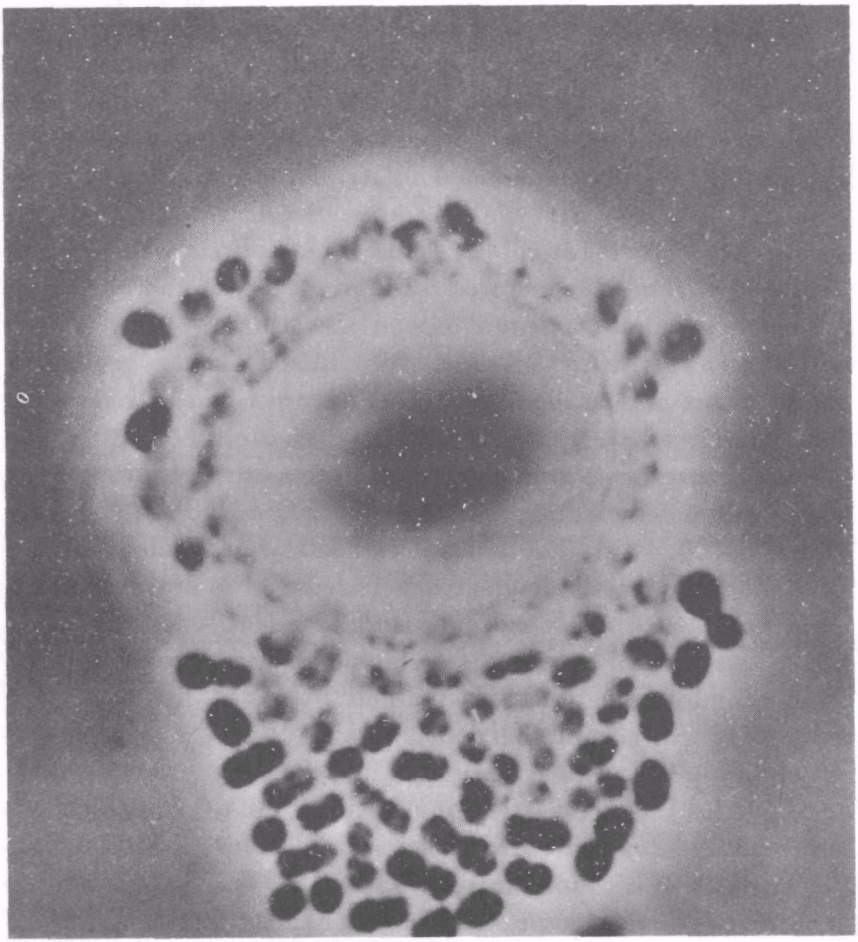

FIGURE 1.—Photomicrograph of *M. cerificans* associated with hexadecane during the early exponential growth phase. 5000 ×.

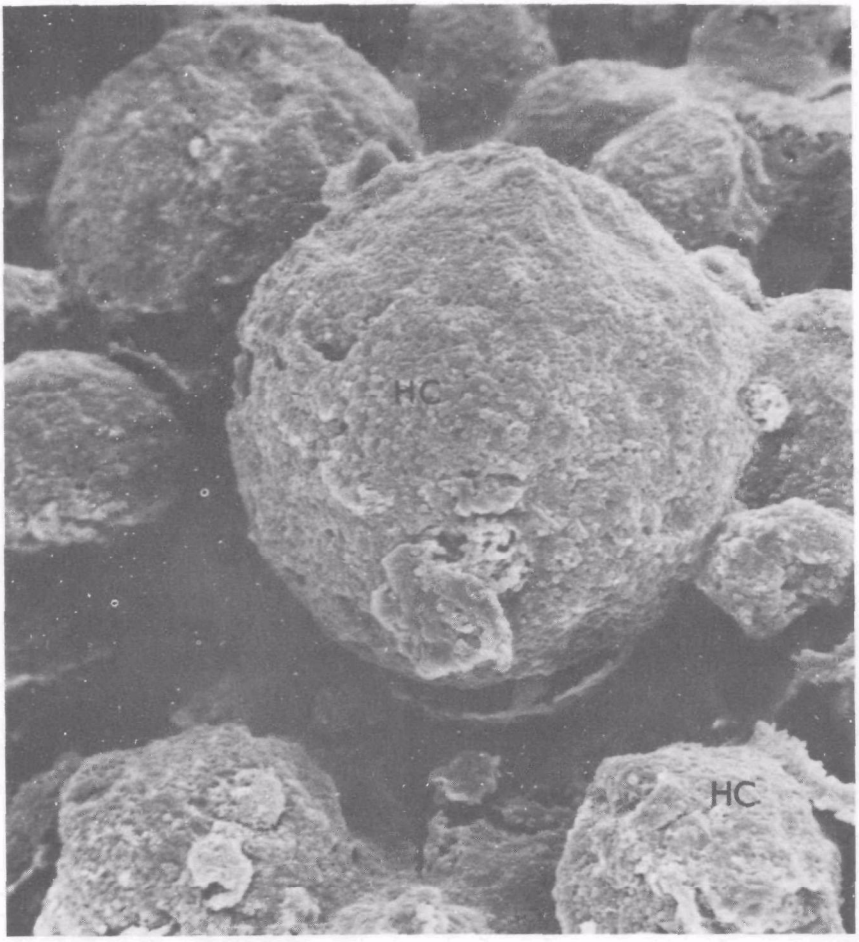

FIGURE 2.—Scanning electron micrograph of *M. cerificans* associated with hexadec-1-ene (HC). The hydrocarbon appears as large ovoid bodies. 1750 ×.

film was set perpendicular to the beam approximately 5.3 cm behind the specimen (flat-plate forward-reflection technique). The exposed film was quantitatively analyzed by microphotometry along a diametric line.

Scanning Electron Microscopy. Cell preparations for scanning electron microscopy were either fixed in glutaraldehyde or dried unfixed onto specimen stubs, coated with gold-palladium (40:60) in a vacuum evaporator and viewed in a Cambridge Stereoscan Scanning Electron Microscope.

RESULTS

A preliminary phase microscopy study of *M. cerificans* actively growing at the expense of hexadecane revealed that the hydrocarbon was dispersed as small microdroplets in the culture. Bacteria were observed to adhere to the surface of these hydrocarbon microdroplets as shown in Figure 1. Interestingly, hexadecane did not disperse into stable microdroplet emulsions when shaken with uninoculated growth medium. The presence of bacteria preinduced to growth on hydrocarbons was essential and necessary for the formation of stable hydrocarbon-water emulsions.

This phenomenon was studied in further detail with the aid of the scanning electron microscope to better determine the relationship of the bacteria to the hydrocarbon microdroplets. Figures 2, 3, and 4 are scanning micrographs obtained by

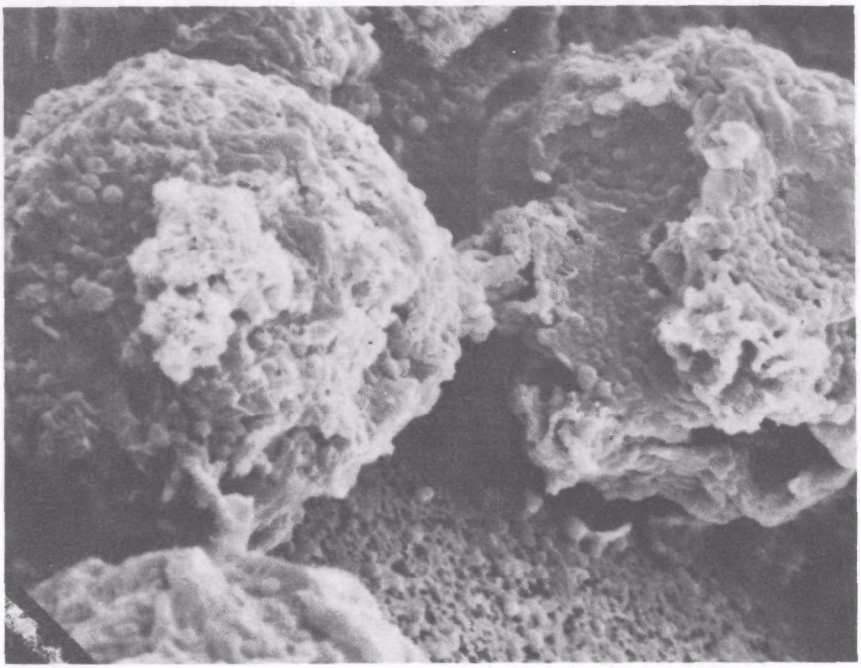

FIGURE 3.—Scanning electron micrograph of *M. cerificans* cells coating the surface of the hydrocarbon-eicosene. The surface of the hydrocarbon is irregular with the bacteria randomly positioned on all surfaces. 3400 ×.

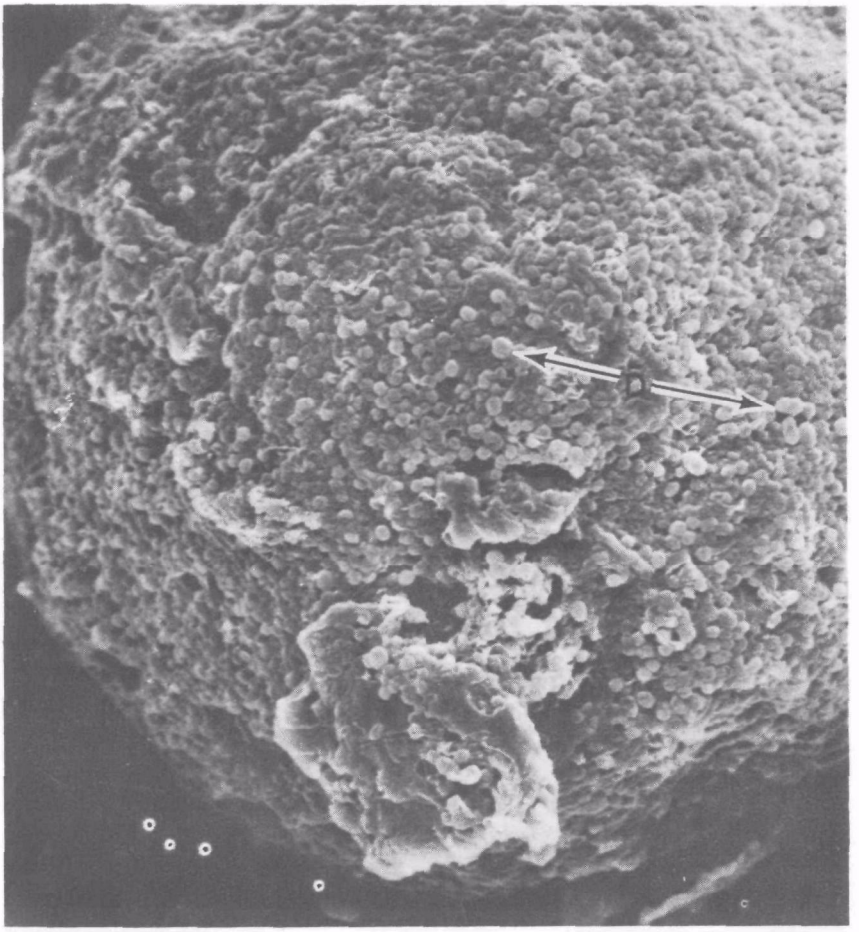

FIGURE 4.—A micrograph of higher magnification showing bacterial cells on the surface of the hydrocarbon-hexadec-1-ene. The sample was taken from the mid-exponential growth phase and cells in the process of division (D) can be seen. 3650 ×.

sampling a culture of *M. cerificans* during exponential growth on hexadecane. Figure 2 is of low magnification (1750 ×) and includes many hydrocarbon microdroplets. A larger magnification (Fig. 3) reveals the surface of the microdroplet to be uniformly covered with bacteria. Further magnification shows quite clearly the bacteria densely packed over the surface of the microdroplet (Fig. 4).

A whole cell preparation negatively stained with phosphotungstic acid reveals topographically the presence of intra-

cellular inclusion bodies (Figure 5). This topographical pattern is characteristic only of bacteria grown at the expense of hydrocarbons.

A detailed study was initiated into the ultrastructure of *M. cerificans* grown on various hydrocarbon and non-hydrocarbon growth substrates. Cell specimens were prepared for examination by transmission electron microscopy as specified in Materials and Methods. Ultra-thin sections obtained from cells grown on acetate, ribose, or nutrient broth-yeast extract (NBYE) were identical with respect to their fine-structure detail (Figure 6). In contrast, cells obtained from growth on hydrocarbons exhibited unique characteristics that served to dis-

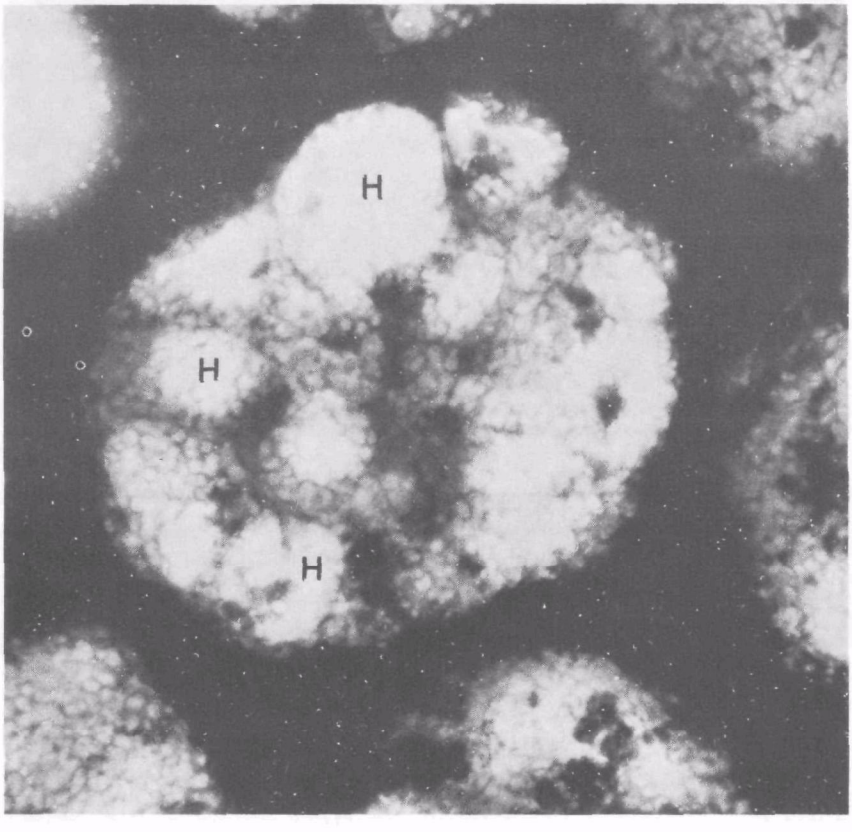

FIGURE 5.—*M. cerificans* grown on hexadecane and negatively stained with phosphotungstic acid. Large electron translucent granules of the hydrocarbon (H) substrate are observed in the whole cell preparation. 92,000 ×.

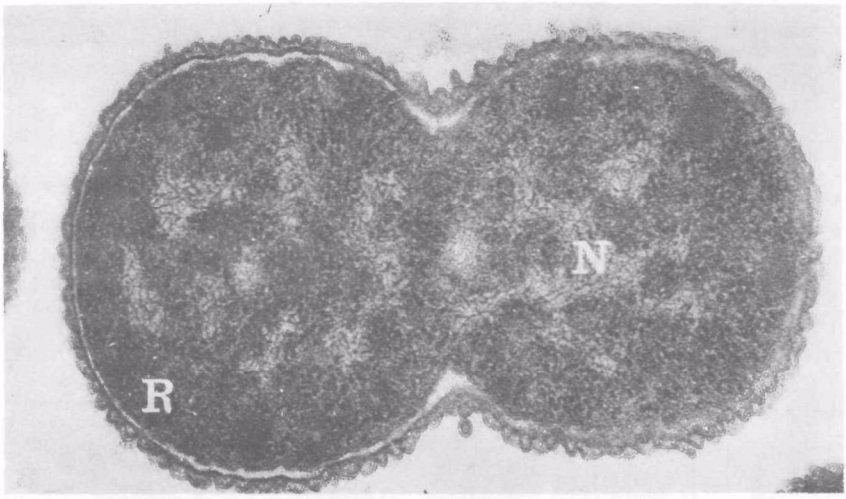

FIGURE 6.—Electron micrograph of *M. cerificans* grown on nutrient broth-yeast extract. Ribosomes (R) and nuclear material (N). 71,500 ×.

tinguish them from nonhydrocarbon grown cells (Figure 7). Multiple lipid inclusion bodies were characteristic features of only hydrocarbon grown cells. This fine-structure lipid inclusion was a characteristic feature associated with the growth of *M. cerificans* on a homologous set of alkanes varying in chain length from 12–20 carbon atoms. Alk-1-enes as sole carbon and energy source exhibited a characteristic fine-structure pattern (Figure 8) in that 1–2 lipid inclusion bodies were observed per cell. These lipid inclusion bodies have been observed to be present in yeast and fungal species capable of growth on hydrocarbons. Under no circumstances have these patterns of inclusion bodies been observed in bacteria unable to grow at the expense of hydrocarbons.

The chemical identification of these inclusion bodies was established by gas-liquid chromatography and X-ray diffraction. Positive identifications of pentadecane, hexadecane, hexadec-1-ene, heptadecane, and octadecane were confirmed in alcohol extracts of cells grown on these respective hydrocarbons by gas chromatography. Table 1 shows the X-ray diffraction data obtained from analysis of lyophilized cells grown on the specified hydrocarbons. The cells from each group were compared with

TABLE 1.—X-ray diffraction analyses of *M. cerificans*

Hexadecane				Hexadec-1-ene				Heptadecane				Control Cells			
Standard		Cells		Standard		Cells		Standard		Cells		NBYE		Acetate	
d	I	d	I	d	I	d	I	d	I	d	I	d	I	d	I
20.40	10	20.41	6												
10.40	10	10.40	8	21.20	50	21.21	10	22.40	30	22.4	15	.....	.....	.....	.....
6.96	5	.....	.....	11.00	50	11.00	8	11.90	50	11.9	25	.....	.....	.....	.....
5.20	1	.....	.....	7.43	20	.....	.....	7.88	40	.....	.....	.....	.....	.....	.....
4.63	80	4.62	45	5.59	5	.....	.....	5.94	30	5.94	20	.....	.....	.....	.....
4.50	70	4.50	30	.....	.....	.....	.....	4.76	5	.....	.....	.....	.....	.....	.....
4.20	5	.....	.....	4.67	20	4.65	5	4.14	100	4.14	100	.....	.....	.....	.....
4.03	20	.....	.....	4.30	5	.....	.....	4.03	40	4.03	.....	.....	.....	.....	.....
3.82	100	3.82	100	4.22	100	4.22	100	3.93	5	.....	.....	.....	.....	.....	.....
3.60	100	3.60	100	4.07	30	4.07	5	3.81	10	3.81	6	.....	.....	.....	.....
3.45	20	.....	.....	3.88	80	3.87	30	3.71	90	3.72	50	.....	.....	.....	.....
3.24	5	.....	.....	.....	.....	.....	.....	3.54	5	.....	.....	.....	.....	.....	.....
2.60	16	2.60	7	3.82	5	.....	.....	3.26	2	.....	.....	.....	.....	.....	.....
2.31	2	.....	.....	3.65	5	.....	.....	2.50	40	2.49	20	.....	.....	.....	.....
2.28	30	2.28	18	2.52	20	2.53	6	2.32	30	2.30	15	.....	.....	.....	.....
2.14	5	.....	.....	2.29	10	2.29	5	2.22	40	2.22	15	.....	.....	.....	.....
2.04	5	.....	.....	.....	.....	.....	.....	2.14	30	2.14	15	.....	.....	.....	.....
2.01	5	2.01	3	.....	.....	.....	.....	2.07	20	.....	.....	.....	.....	.....	.....

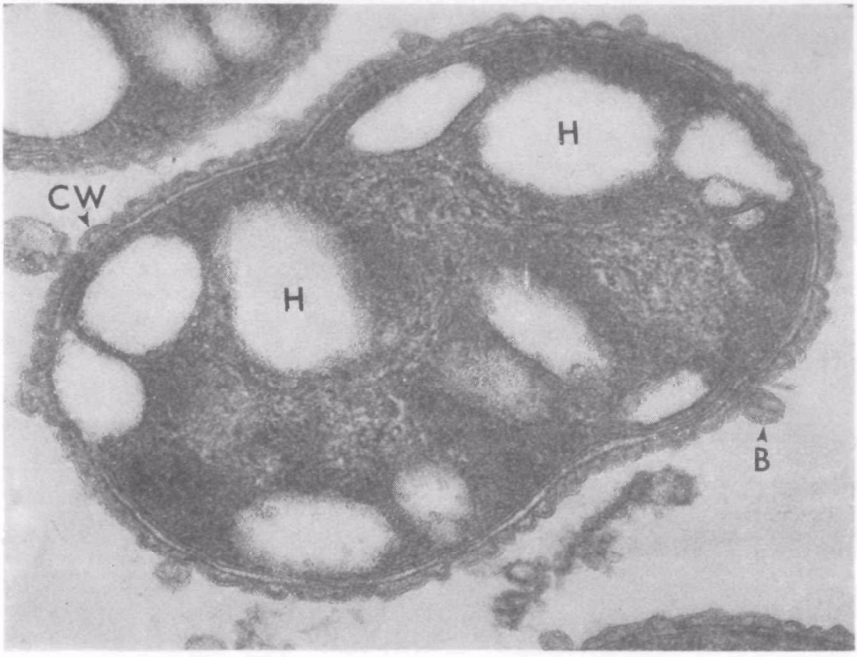

FIGURE 7.—Ultrathin sections of *M. cerificans* grown on hexadecane medium showing the large translucent hydrocarbon (H) inclusion granules. Hexadecane grown cells contain numerous granules in each cell. The cell envelope is similar to that of cells grown on other substrates with an occasional bleb (B) appearing on the cell wall (CW). 117,000 ×.

samples of chemically pure hydrocarbon and the D values and relative intensity (I) determined. Only bacteria obtained from hydrocarbon cultures exhibited a X-ray diffraction pattern. The D values obtained from hydrocarbon grown cells enabled positive identification of the intracellular hydrocarbon pools with the ability to discriminate the identity of each hydrocarbon. These results serve to confirm the presence of intracellular pools of hydrocarbon in *M. cerificans*.

Ultrastructure Modifications Associated with Hydrocarbon Oxidation

An unexpected ultrastructure feature revealed a new dimension to the complexities associated with the biochemistry and enzymology of hydrocarbon oxidations by biological systems.

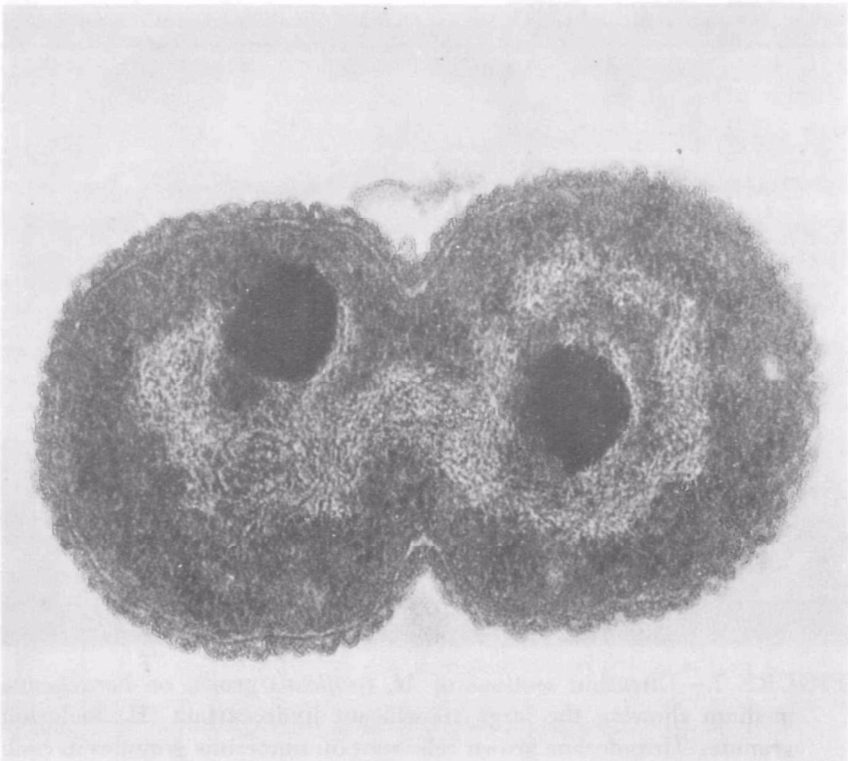

FIGURE 8.—Electron micrograph of *M. cerificans* grown on hexadec-1-ene. Cells normally contain one or two large granules in each cell. These cells were processed with Durcupan in order to retain the unsaturated, osmiophilic, hydrocarbon granules as electron dense inclusions. 124,000 ×.

These ultrastructure differences became apparent in a comparative study of the fine-structure of *M. cerificans* grown with hydrocarbon and non-hydrocarbon substrates. Hydrocarbon grown cells exhibited the synthesis of complex intracytoplasmic membranes. These membranous structures do not appear as a result of bacterial growth on non-hydrocarbon substrates. Figures 9 and 10 show the variation and structure of these membranes in *M. cerificans* when grown on hexadecane. Figure 11 shows the type of membrane structure observed with hexadec-1-ene grown bacteria. These structures appear as highly ordered bilayer lamellar membranes extending throughout the cell. An

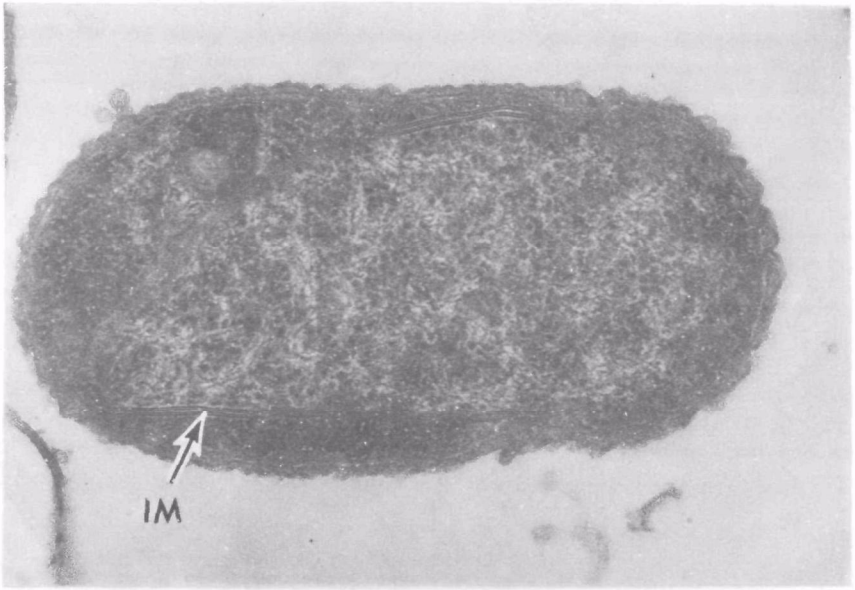

FIGURE 9.—Intracytoplasmic membrane (IM) development in *M. cerificans* grown on hexadecane. 89,000 ×.

occasional physical relationship between the intracytoplasmic membrane and a hydrocarbon inclusion body can be observed.

A growth phenomenon currently inexplicable concerns a gross cellular transformation in hydrocarbon grown bacteria. At irregular intervals cultures of *M. cerificans* growing on alkane or alk-1-ene transform into giant cells. Individual bacteria will become 4–10 times larger, greatly extended and elongated, and exhibit extensive intracytoplasmic membrane development. Figure 12 shows a thin-section of one of these giant cells obtained from a culture growing on hexadecane. Figure 13 is a micrograph of a cell obtained from a hexadec-1-ene culture. This induced transformation occurs only when bacteria are grown on hydrocarbons. Subculturing these transformed cells to new hydrocarbon containing media maintains giant cell populations, with approximately 30 percent of the total cell population undergoing this transformation. Reversions to normal cell size and shape occurs by subculturing to non-hydrocarbon nutritive media, e.g., nutrient broth-yeast extract or acetate. A physical property associated with these transformed cultures is their

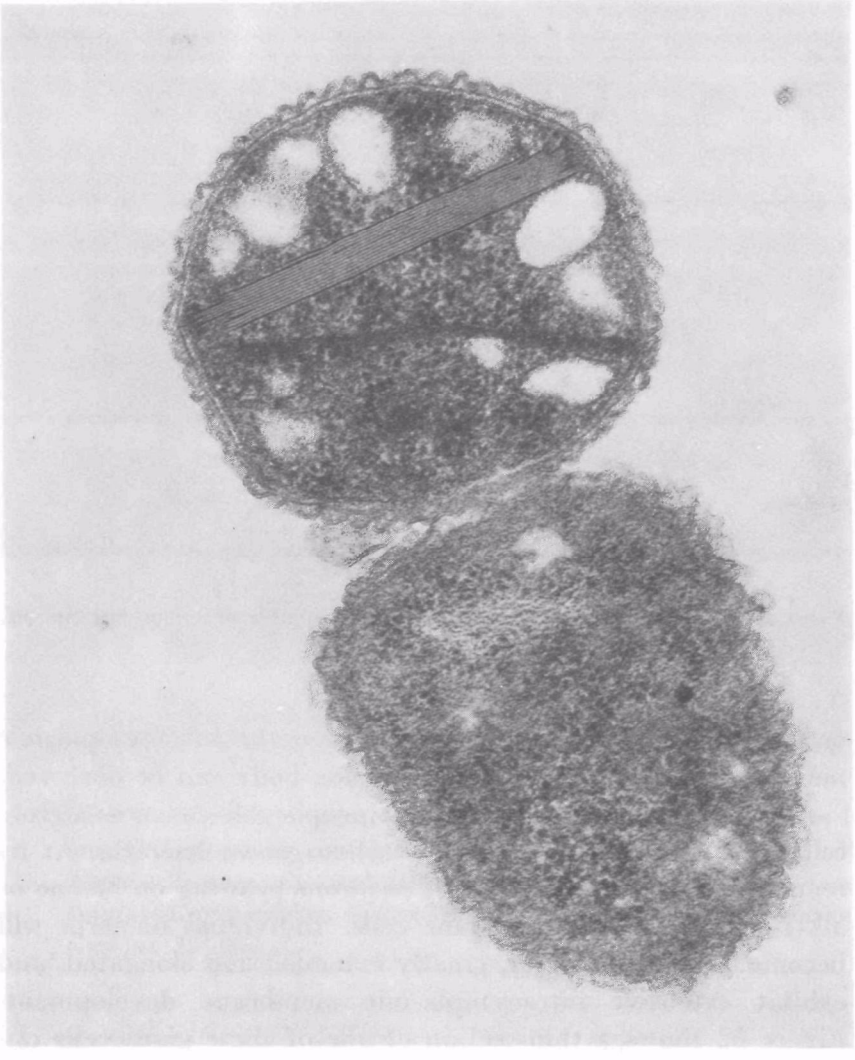

FIGURE 10.—A complex intracytoplasmic lamellar membrane system observed in *M. cerificans* grown on hexadecane and dehydrated with Durcopan prior to embedment in Araldite. 94,500 ×.

buoyant density. It is impossible to sediment these cells in centrifugal fields as high as 40,000 ×g. The transformed cells remain as a floating granular pellicle at the top of the centrifuge tube.

Quantitative Relationships of Lipids in Relation to Hydrocarbon Oxidation. A comparative study of lipids in relationship to

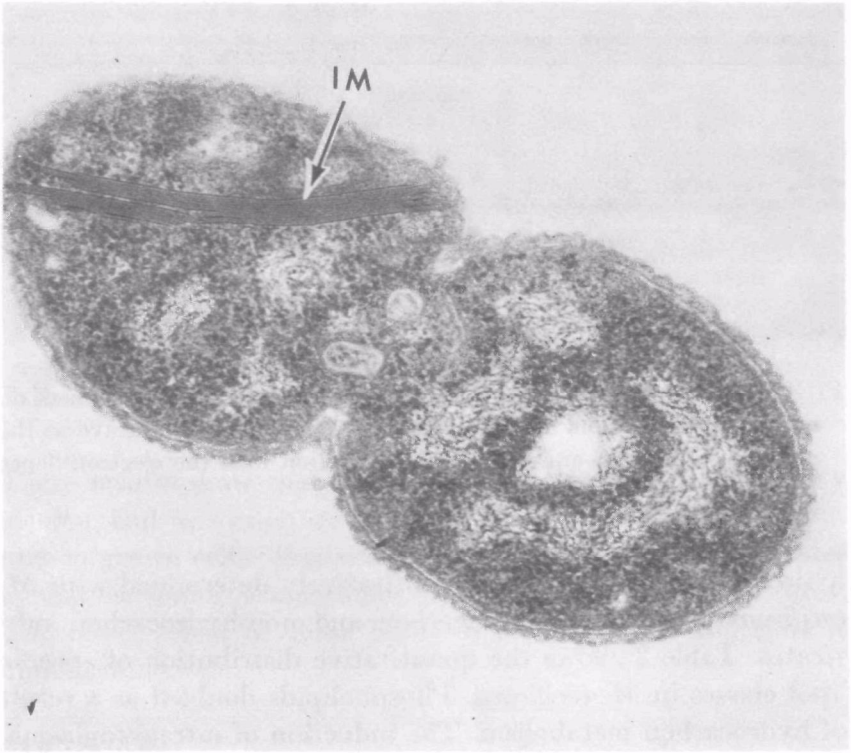

FIGURE 11.—*M. cerificans* grown on hexadec-1-ene and dehydrated in a graded series of ethanol exhibits a complex intracytoplasmic membrane system (IM). 96,000 ×.

FIGURE 12.—Electron micrograph of *M. cerificans* transformed to large cells by repetitive growth on hexadecane. Intracytoplasmic membrane (IM) and hydrocarbon (H). 50,000 ×.

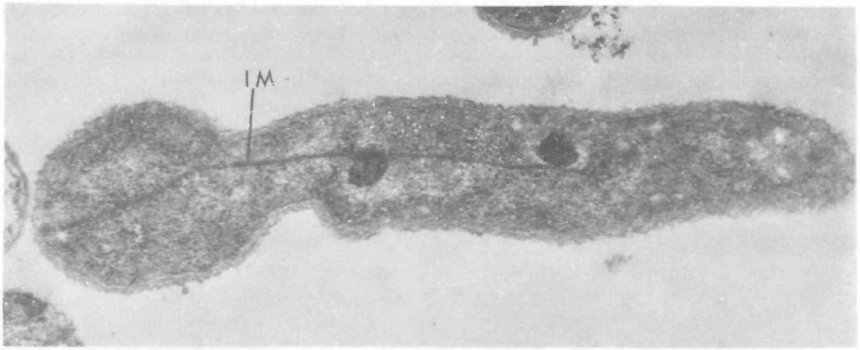

FIGURE 13.—A transformed cell of *M. cerificans* obtained by growth on hexadec-1-ene. The membrane system (IM) appears to traverse the length of the cell and is in close association with the electron dense hydrocarbon inclusions. 49,000 ×.

hydrocarbon oxidation was quantitatively determined with *M. cerificans* growing on hydrocarbon and non-hydrocarbon substrates. Table 2 shows the quantitative distribution of specific lipid classes in *M. cerificans*. Phospholipids doubled as a result of hydrocarbon metabolism. The induction of intracytoplasmic membrane in hydrocarbon grown cells accounts for a doubling of phospholipid. Free fatty acid pools remain constant under the two growth conditions while significant differential concentrations of free fatty alcohol and wax ester (cetyl palmitate) were measured only in hydrocarbon grown cells.

The extracellular accumulation of lipids is shown in Table 3.

TABLE 2.—Quantitative distribution of lipids in *Micrococcus cerificans*

Lipid components	Micromoles/g dry cell weight	
	Non-hydrocarbon[a]	Hydrocarbon[b]
Phospholipids	60	120
Triglyceride	4.8	2.5
Free Fatty Acid	7.5	8.2
Free Fatty Alcohol	0	2.7
Wax Ester	0	7.3

[a] Nutrient broth-yeast extract grown bacteria
[b] Hexadecane grown bacteria

TABLE 3.—Quantitative distribution of extracellular lipids

Lipid components	Micromoles/liter of culture medium	
	Non-hydrocarbon[a]	Hydrocarbon[b]
Phospholipids	0	0
Triglyceride	2.4	25.6
Free fatty acid	4	60
Free fatty alcohol	0	0.5
Wax ester	0	280

[a] Nutrient broth-yeast extract grown cells
[b] Hexadecane grown cells

These results show that triglyceride, free fatty acid, free fatty alcohol, and wax ester are present in the culture broth of hydrocarbon grown cells. Triglyceride, free fatty acid, and wax ester appear as major extracellular lipids in hydrocarbon cultures. The direct effect of these lipids in solubilizing hexadecane is undetermined.

DISCUSSION

These studies have served to direct attention to problems associated with petroleum which have been either totally ignored or unrecognized. The recent impact of petroleum in the environment tends to emphasize the cogency of providing concrete answers to these problems. Specific implications which stem from this study are:

(a) cellular transformations induced by hydrocarbons;
(b) the sequestering of hydrocarbons by microorganisms with potential impact on food chain interrelationships; and
(c) the microbial production of specific solubilizing agents which aid or promote hydrocarbon metabolism.

The fate and effect of petroleum or petrolic byproducts on the environment has received cursory attention at best. A summary discussion of the effects of oil pollution on birds, mammals, fish, molluscs, plankton, plants, as well as marine communities has defined a few of the overall aspects of pe-

troleum pollution (*22*). The examination of shell fish and marine sediments demonstrated the persistence of oil in sediments and shell fish several months following the pollution incident. Of significant interest was the retention of aromatic hydrocarbons by these shell fish (*2*). Recent studies have shown the rapid uptake of heptadecane, 1,2,3,4-tetrahydronaphthalene, toluene, naphthalene, and 3,4-benzopyrene by marine mussels. The data indicated that mussels did not metabolize these hydrocarbons but significant amounts were retained by the tissues (*12*).

We have observed that a microorganism undergoes a morphological transformation as a result of growth on chemically pure hydrocarbon. Whether this transformation is the result of synergism between hydrocarbon and a metabolic byproduct or a singular effect of hydrocarbon is unknown. Further evidence has demonstrated fine-structure modifications in the form of induced membrane synthesis. Quantitative biochemical analyses of the simple and complex lipids of *M. cerificans* have demonstrated an increase in the cellular and extracellular lipids as a result of hydrocarbon metabolism. The pharmacological and toxicological properties of petroleum and petroleum byproducts on the aquatic environment are undetermined. It is well known that many polycyclic aromatic hydrocarbons are carcinogenic. A hydrocarbon oxidizing microorganism has responded physiologically and morphologically in a rather drastic manner to a hydrocarbon molecule which is tacitly assumed to be innocuous. The inescapable question of "why?" remains.

Sequestering of hydrocarbons by the microbial flora through active transport becomes of practical concern. Hydrocarbons which are pooled by the indigenous microbial flora could relate to the sequential transfer of hydrocarbons through the food chain. The phenomenon becomes of fundamental concern as a mechanism for passage of potentially dangerous chemicals through the food chain webs to higher life forms. Compounds which can undergo such processes are: (a) paraffinic and olefinic hydrocarbons; (b) aromatic and polycyclic aromatic hydrocarbons; and (c) halogenated hydrocarbons. We have investigated hydrocarbon utilizing bacteria, yeasts, and fungi and found all to accumulate paraffinic and olefinic hydrocarbons in the cytoplasm. The critical question becomes just how far does

hydrocarbon move through the food chain progression before it disappears, is converted into a potentially harmful intermediate, or is metabolized to non-toxic intermediates?

The conclusion appears warranted that *M. cerificans* undergoes a loss or alteration of biochemical control mechanisms for cell wall synthesis, membrane synthesis, and morphological shape when grown repetitively on hydrocarbons. The analogy to neoplastic transformations in higher cellular systems is evident. The "how" and "why" of what triggers a microbial cell to "pool" a hydrophobic hydrocarbon against a concentration gradient, to form complex intracellular membranes, and to undergo gross cellular transformations are undetermined.

It is a well recognized fact that all microorganisms are not capable of hydrocarbon assimilation presumably lacking the requisite enzymatic complement to effect such conversions. A study of hydrocarbon structure in relationship to microbial oxidizability has established a set of biological guidelines for determining the biodegradability of specific substituted hydrocarbons (*20*). Hydrocarbons as hydrophobic, water-insoluble compounds have circumstantially been considered as refractory to active transport processes. These considerations necessitated an extracellular modification of the hydrocarbon by the microbial agents with assimilation processes being effected on the byproducts of this activity. Our findings that hydrocarbons are pooled suggests a mechanism for active transport of hydrocarbons. The component parts and requirements for active transport of a water-insoluble substrate are ill-defined. However, if hydrocarbon oxidizing microorganisms possess the ability to synthesize a biodetergent which promotes pseudo-solubilization of hydrocarbon, a mechanism for active transport against a concentration gradient can be affected. An analysis of cellular and extracellular lipids revealed that a number of specific lipids were present in a hydrocarbon utilizing system that could fulfill this purpose. The explicit role of these lipids as solubilization agents for hydrocarbons has not been determined within this system. Their known physical properties would suggest that an influential role could be served in maintaining a finite level of hydrocarbon in aqueous solution.

Future directions for analyzing the impact of petroleum on

the aquatic environment can be formulated as a series of experimental questions:

(a) what is the diversity of microorganisms in the aquatic environment capable of hydrocarbon metabolism?;
(b) do differential kinetic parameters exist with respect to uptake of specific hydrocarbons?;
(c) what is the magnitude of hydrocarbon pooling in the microbial flora and what are the rates of hydrocarbon elimination from such microbial populations?;
(d) can internal pools of more than one hydrocarbon be maintained or do selective uptake and oxidation rates prevail depending on the hydrocarbons available?;
(e) do hydrocarbons induce cellular transformations throughout all microbial species?;
(f) what pharmacological and toxicological properties do specific hydrocarbons exert on higher cellular systems?

Answers to these questions would provide basic information towards formulating specific policies and procedures in water quality management and control as well as providing needed insight and knowledge into the biological effects of petroleum in the environment.

ACKNOWLEDGMENTS

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Questions and Comments following Dr. Finnerty's Talk

QUESTION FROM THE FLOOR: Are the hydrocarbons true emulsions in the sense that they are clear suspensions?

DR. FINNERTY: No they are not. They are turbid suspensions.

QUESTION FROM THE FLOOR: Have you tried growing the bacteria in a medium in which both alkanes and alkenes are present?

DR. FINNERTY: No, I haven't. They have all been the single substrate type of experiments. Granules form even when there is a vapor phase over the liquid medium.

QUESTION FROM THE FLOOR: If there is to be recycling of hydrocarbons to the sediments, then there must be a lot of anaerobic activity for hydrocarbons to be degraded. Have you observed anaerobic degradation of hydrocarbons?

DR. FINNERTY: I don't know that anyone has shown conclusively that there is such a thing as anaerobic degradation of hydrocarbons.

COMMENT FROM THE FLOOR: I might add that there is evidence for the anaerobic degradation of the benzene ring with nitrate as a terminal electron acceptor. This is a special case of the hydrocarbon degradation and not the degradation of a linear hydrocarbon but there is no explanation as to how the ring is cleaved.

COMMENT FROM FLOOR: You also mentioned that you had looked at yeasts and fungi. I wonder what particular species of yeasts you looked at.

DR. FINNERTY: Mainly *Candida* species.

QUESTION FROM THE FLOOR: Have you sampled and looked at any bacteria in natural oil slicks to see if they have the characteristics of the organisms you grow in culture?

DR. FINNERTY: I have not. All the studies we have done have been with pure cultures, under carefully defined conditions.

QUESTION FROM THE FLOOR: Have you established any rates of degradation for the specific hydrocarbons?

DR. FINNERTY: No definitive rates have been defined, but we have done some preliminary experiments. We cannot furnish any numbers right now.

QUESTION FROM THE FLOOR: When you remove the cells from these certain types of media do you find that there is a time lag before the cells begin to lose the inclusion bodies which you showed?

DR. FINNERTY: If you put the cells back in fresh medium without hydrocarbon intracellular hydrocarbon inclusions disappear in approximately 60 minutes.

QUESTION FROM THE FLOOR: Did you find nitrogen to be a limiting nutrient in the utilization of the hydrocarbon?

DR. FINNERTY: We have never quantitated the exact utilization of nitrogen in the growth of this organism. We always grow the organisms in an excess of nitrogen, for the organism will not grow in the absence of nitrogen.

QUESTION FROM THE FLOOR: Do you think any of the inclusion bodies could have been polyhydroxybuterate?

DR. FINNERTY: No. We specifically looked for PHB, but our organism does not make PHB. We grew many microorganisms on crude oil and we found that many of them had PHB as inclusion bodies.

QUESTION FROM THE FLOOR: What is the generation time of the organism grown in hydrocarbon when compared to the generation time of the organism grown on acetate medium?

DR. FINNERTY: The generation time on hydrocarbon is approximately 30 minutes and 50 minutes on acetate.

QUESTION FROM THE FLOOR: Does the structure shown on your slides appear to be correlated with growth phase?

DR. FINNERTY: The structures are seen throughout the growth cycle.

Microbial Conversions of Dissolved Organic Carbon Compounds In Sea Water

D. W. HOOD

Institute of Marine Science
University of Alaska

and T. C. LODER

University of New Hampshire

Interest in the organic components of sea water has a long history generated largely by biologists, whose thinking was first summarized by Lucas (1955), in emphasizing the significance of so-called *organic metabolites* on the distribution and growth of marine algae, bacteria, protozoa, and larvae. Most parameters in the early studies indicate little difference between "good" and "bad" ocean waters, a terminology based on the ability to support productivity. Good waters support heavy growth; bad waters are nearly sterile. Recent studies seem to indicate that complexation of trace metals by organic matter is at least one consideration in distinguishing possible "good" and "bad" waters (Barsdate et al. 1973; Ryther et al. 1971). Observations suggest other critical factors such as growth substances, toxins, and antimetabolites may hold the key to the productivity of the sea. Recent reviews (Hood 1963, 1970; Johnson 1955; Provasoli 1963; Vallentyne 1957; Saunders 1957) have pointed out the importance of dissolved organic matter in the biology of the sea from six viewpoints:

—Organic compounds, even in dilute solution, serve as energy sources for bacteria, yeast, fungi, larvae, and possibly algae;

—Vitamins serve as growth stimulators for algae, bacteria, yeast, fungi, and larvae;
—Organic toxins such as those derived from *Gymnodinium brevis* kill large populations of marine organisms.
—Organic compounds are important in migration, schooling, predator-prey relationships and sexual behavior of organisms.
—Certain organic compounds trigger feeding processes of organisms, even though these compounds may not be nutrients;
—Nutrient or toxic trace elements may be associated or complexed with organic compounds, thus controlling the level of these elements in the aquatic medium.

Organic matter also has importance in geological and geochemical processes in nature. The literature on this topic is voluminous and will not be covered in this discussion. It is clear, however, that the diagenic processes leading to fossil fuel formation involve microbial alteration of deposited organic matter. Part of this transition must involve a soluble or at at least colloidal state for the organic matter. Clay-organic association in this process is not yet fully understood, but evidence indicates it has a central role in sedimentary processes involving organic matter.

Physical processes in the sea that are affected by organic material include sea-foaming, surface tension, air-sea interaction, light absorbancy, sound transmission, viscosity, and the character of surface films.

The importance of organic matter to the chemistry of the sea is well established. There have been many problems in obtaining quantitative data even of the gross amounts present, and the presently used techniques are good only to about ± 0.1 mg C/liter. Group-type organic analysis has been used extensively and is useful in some considerations; however, many reactions, especially those involving organisms, are compound-specific. Thus, an understanding of the stimulus, depression, or activity of organisms influenced by organic matter depends upon a careful evaluation of the constituents present, their rates of production and degradation, and distribution in the ocean. At this time, no single water sample has been analyzed

for all the organic components present. In sea water only about 20 to 30 percent of the total present can be accounted for by the classes of compounds such as proteins, including amino acids; lipids, including hydrocarbons; and carbohydrates. The remaining organic constituents, best classified as "fulvinic acids" or marine humates, are extremely difficult to classify. They probably do not consist of specific molecular structures, but rather are conglomerates of different sized, highly complex polymeric chemicals with varying numbers of functional groups. Analysis of this fraction for the purpose of understanding reactivity in the environment might best be done on a functional group basis rather than by the conventional compound-specific means.

In this paper, an attempt is made to review what is known about dissolved organic carbon distribution in nature, particularly the ocean, as it reflects information on decomposition rates. Most attention is directed to those compounds that may affect the sea on a world ocean scale. Since those materials that are easily oxidized have already been considered by others in this meeting as they have dealt with nutrient regeneration, I will discuss the more refractory material.

Distribution of Dissolved Organic Matter in the Ocean and Evidence for its Role

Dissolved organic matter is defined in this paper as that fraction of the organic matter which passes through a filter of an average 0.4 micron pore diameter. This is an operational definition and has little to do with the physical-chemical state of the material. The filtered fraction will contain the crystalloidal and colloidal portions; cell contents of ruptured phytoplankton, especially nannoplankton and dinoflagellates; small aggregates; and some minute organisms.

In the case of the decomposition of dissolved organic matter, however, the ambiguity of the above definition is probably not critical. Organic material as utilized by most organisms must go through a single-molecule stage before absorption into the metabolic pool of the organism. Whether the material starts as an aggregate and is attacked in this form or is in true solution would probably affect only the *rate* of utilization. The concept

of dissolved organic carbon has limited scientific utility, since observed dissolved organic carbon concentration is the result of a number of diverse processes such as heterotrophic utilization, adsorption to particulate matter, transformation between dissolved and particulate organic carbon, extracellular excretion by living plants and animals, and decomposition of dead organisms. In order to study the dynamics of the carbon cycle, however, some separation is necessary between living and non-living carbon; large particles which settle quickly can be separated from smaller particles or colloids which remain in suspension. The size fractionation method currently in use by most investigators is a practical way to reach those goals and is probably the best technique now available.

Distribution. The largest quantity of organic carbon on this planet, with the exception of that in sediments and soils, is contained in the oceans. Nearly all of this is non-living and in the dissolved form. The concentration of organic carbon in sea water is less than 1 mg C/liter in the major portion of the ocean and higher at the surface and near land; yet it exceeds by an order of magnitude the particulate portion, which in turn exceeds the living matter present by a proportionate amount (Wangersky 1965).

There have been a number of studies on the distribution of both dissolved organic carbon and particulate organic carbon in the world ocean. Investigations in the Atlantic include work by Duursma (1961), Parsons (1963), Menzel and Ryther (1964), Riley et al. (1965), Skopintsev et al. (1968), and Gordon (1970a,b). Among Pacific studies are those conducted by Holm-Hansen et al. (1966), Hobson (1967), Barber (1967), Holm-Hansen (1969), and Gordon (1970c). Work in the Indian Ocean includes studies carried out by Menzel (1964), Starikova (1967), Szekielda (1967), Newell and Kerr (1968), and Newell (1969). Organic carbon in the Arctic has been studied recently by Loder (1971) and Kinney et al. (1971); work in the Bering Sea has been reported by Loder (1971), Nishizawa and Tsunogai (1973); and a glacial inlet has been investigated for organic carbon by Loder and Hood (1972).

There is general agreement among most of the authors cited as to the concentrations of DOC and POC both near the ocean

surface and at depth with two exceptions: (1) The DOC values reported by Skopintsev and colleagues from dry combustion of evaporated samples are several times higher than the concentrations found by other workers. (2) There has been a trend toward finding smaller amounts of POC in the deep sea than was earlier reported; this is probably due to more careful sample preparation, larger samples, and more precise methods.

Summary of available data shows that from surface to about 300 m the DOC concentration is about 1.0 mg C/liter (range 0.3–2.0) and the POC concentration is about 100 ug C/liter (range 30–300). In dense plankton blooms, however, DOC values may temporarily reach 3–5 mg C/liter and POC values 1000–2000 ug C/liter. Below this surface zone DOC values are relatively constant with an average of 0.5 mg C/liter (range 0.2–0.8 mg C/liter) and POC values averaging about 10 ug C/liter 2–30 ug C/liter). It should be made clear at this point that the methodology used for acquiring these numbers is generally that of Menzel and Vaccaro (1964), in which certain components of the dissolved organic fraction may become lost. The sparging process used to free the system of inorganic carbon may also remove low molecular weight volatile compounds. In addition, some of the high molecular weight hydrocarbon type compounds may not be oxidized to carbon dioxide by the procedure used.

The composition of the deep water particulate organic matter appears to be mainly carbohydrates and proteinaceous material (Parsons and Strickland 1962, Gordon 1970a and Handa 1970). There have been conflicting reports on the nutritional value of this material. Although Menzel and Goering (1966) observed that particulate organic matter is uniformly resistant to biological decomposition, Gordon (1968) found that 19–26 percent of the material (mostly protein) could be removed with a mixture of digestive enzymes. Lorenzen (1968) found that, although *Artema nauplii* did not survive feeding on concentrated deep water particulate organic matter, 43 percent of the POC nevertheless disappeared when the concentrated portion was allowed to sit for 90 days at 20 C in the dark. Deep water zooplankton do ingest organic aggregates, but whether or not they digest them is unknown. These organisms have an abundance of 0.1 to 1 mg/m^3 at 4000 m (Vinogradov 1962) and

require about 1 to 2 percent of their body weight per day for respiration. This energy must come from organic carbon.

In the surface layers, the particulate organic matter consists mainly of detritus from the death of organisms and organic aggregates described by Riley (1963, 1970). Living matter contributes only about 10 percent of the total. The ratio of DOC to POC ranges from about 3–10 in the surface layers to 200–300 for some deep water. The average deep-water ratio is about 50 (Williams 1969).

Dissolved organic matter appears to contain small portions (about 20 percent) of the following compounds: amino acids (free and combined) (Degens 1970, Pocklington 1970); sugars (free) (Handa 1970); fatty acids and hydrocarbons (Jeffrey 1970, Blumer 1970); and even smaller amounts of aromatics (substituted phenols) and vitamins (Natarajan 1968). The amount of these compounds present in the near-surface waters is quite variable because of changes in biological activity involving both uptake and liberation of extracellular products and decomposition (Parsons and Seki 1970). Both Williams (1969) and Degens (1970) feel that these compounds comprise only a small percent (10–25) of the total amount of DOC. Based on low C/N ratios reported by several workers, Degens (1970) proposed that most of the remaining dissolved organic material is in the form of *heteropolycondensates*, a clathrate-type molecular structure. Williams (1969), however, suggests that the remaining material is *humus* or *lignin*-type material and is refractory to microbial and chemical oxidation. Jannasch (1970) suggested that the low concentration may effect utilization but found that bacteria would not utilize DOC from deep water even in five-fold concentration.

Duursma (1961, 1965) and later Menzel and Ryther (1968, 1970) have suggested that DOC in deep waters appears to be a conservative property and can therefore be used to trace water masses. Menzel and Ryther (1968) concluded from their study of the Antarctic Intermediate Water that oxygen concentrations observed in the water as it moved north were a function of mixing and were not primarily due to oxidation of entrapped organic matter. They suggested that dissolved organic matter tends to be conservative in nature once water masses sink below

the surface and that the cycling of organic matter is restricted almost entirely to relatively shallow surface water.

Carbon-14 dating and stable isotope studies (carbon-13: carbon-12 ratios) have provided further insight into the status of organic carbon in sea water and its age. Williams and Gordon (1970) reported that the range of $^{13}C/^{12}C$ ratios for both POC and DOC was between -22.0 and -24.4 percent relative to the PDB_1 standard, meaning the carbon is relatively depleted in ^{13}C. They found that the ratios are constant with depth, sample location and time in the northeast Pacific. In addition, they reported that the ratios found for both DOC and POC most closely approximate the cellulose and lignin-like fractions of surface plankton and concluded that particulate organic matter is similar in composition to dissolved organic matter. Williams et al. (1969), using carbon-14 dating techniques, have reported an average age of 3400 years (b.p.) for the total organic matter in 2 samples taken at 2000 m off southern California. This value is almost twice that of inorganic carbon and is suggestive of different input and utilization pathways for the two fractions.

With the concept of the inert nature of sea water organics in mind, a review of other evidence infers that a portion of organic material at depth is in dynamic equilibrium rather than totally inert. There are a number of problems that remain unanswered by Menzel, Williams and their co-workers: Some of these include the seasonal and spatial variation of POC and DOC in the sea (Hobson 1967; Menzel 1964; Gordon 1970a,c), and the possible DOC-to-POC transformation and subsequent role as a food source for deep sea filter feeders.

The role of DOC-to-POC transformation is still not well understood, although it is generally accepted that under the proper conditions particulate organic matter does form from part of the dissolved fraction. Baylor et al. (1962), and Sutcliffe (1963) found that phosphate adsorbed to bubbles passing through sea water. The next year Baylor and Sutcliffe (1963) found that cultures of *Artemia* could survive on particles produced by the bubbling of sea water. Sutcliffe et al. (1963) suggested this process might be associated with Langmuir circulation and be a source of organic food particles in the sea.

Riley et al. (1965) found that particles could be produced by bubbling both surface and deep water from the Sargasso Sea, although deep water produced fewer particles.

In further studies Menzel (1966) found by using ignited, filtered air and trice-filtered sea water that bubbling is not a likely mechanism for the production of particulate carbon. Barber (1966) reported he was unable to produce significant particulate carbon by bubbling sea water "sterilized" by filtration through 0.22-μ filters and concluded that bacteria played a role in particle formation. Batoosingh et al. (1969) explained these apparent contradictions with a set of very carefully controlled experiments. They found that small particles (0.22–1.2 μ in size) are important as nuclei in formation of larger particles during the bubbling process and that the concentration of particles inhibits further formation until particles are removed. Thus it appears that entrapment of bubbles near the sea surface may play an important role in production or organic particles suitable as food for the small grazing organisms. This process does not provide particles for the deep sea, however.

Riley et al. (1965) proposed that there is a dynamic balance between utilization of particles and further adsorption from the filterable fraction. Sheldon et al. (1967) and Parsons and Seki (1970) described the formation of particles and the possible role of bacteria in particle formation and enlargement by bacterial clumping. Khaylov and Finenko (1968) found that dissolved high-molecular compounds are sorbed into detritus, broken down by enzymatic hydrolysis and then utilized by bacteria populating the detritus. This would move dissolved organic matter into the particulate phase and make it available to filter-feeding organisms. The role of adsorption of organic molecules by inorganic particles has been shown by several workers (Bader et al. 1960; Chave 1965, 1970; Degens and Matheja 1967), but the extent to which this process contributes to particle formation is unknown. These, then, are some of the problems concerning the relationship of DOC to POC.

Several theories have been suggested concerning the source of food for deep-living zooplankton: (1) Non-living particulate organic material may settle slowly out of the euphotic zone and provide a continuous supply of food material. (2) Migrating

zooplankton populations may utilize food materials above them in the water column and in turn be eaten by organisms below them in a series of overlapping food chains (Riley 1951; Vinogradov 1962); (3) Detrital organic matter, some of which originates in the euphotic zone, settles in the water column in a state of dynamic equilibrium with the dissolved organic matter through a series of interactions perhaps mediated by heterotrophic microorganisms and physical adsorption (Riley et al. 1964; Sheldon et al. 1967; Riley 1963, 1970). (4) High concentrations of particulate matter may exist at various depths, possibly in discontinuous layers for short periods of time, providing a concentrated food source (Parsons and Seki 1970). Since data have been found to support all of these theories to some extent, it is suggested that the actual mechanisms are a combination of some or all of these factors, with the amount of food contributed by each mechanism still unknown.

Several recent studies on the role of POC and DOC in sea water may help our understanding of the very complex role played by organic matter. Organisms may release natural organic chelators which are necessary for increased phytoplankton growth (Barber and Ryther 1969). Surface-charge studies on organic particles may give insight into aggregate formation (Wangersky 1968; Neihof and Loeb 1972). Studies on the release of dissolved organic matter by phytoplankton (Anderson and Zeutschel 1970), zooplankton (Johannes and Webb 1970) and macrophytes (Sieburth and Jensen 1970) suggest additional sources of DOC.

Considerable advance has been made on the differentiation of living particulate matter from the non-living by the use of ATP analysis (Holm-Hansen 1970). This technique is now being widely used to determine the biomass of living organisms associated with detrital material.

Decomposition of Organic Matter in the Deep Sea

The distribution of organic matter with depth in the Atlantic Ocean is shown in Table 1 (Menzel 1970) and for the Arctic Ocean in Table 2 (Loder 1971). The locations of the samples designated in Table 1 are shown in the original paper of Menzel (1970). It may be seen from these data and those obtained by

many others that the concentration of dissolved organic matter ranges from 0.8 to 2 mg C/liter in the surface layer and decreases gradually with depth (Duursma 1961; Menzel 1964; Ogura and Hanya 1967; Barber 1968; Menzel and Ryther 1968; Ogura 1970; Loder 1971; Loder and Hood 1972; Ogura 1972). The concentration of DOC below 400 m is fairly uniform and

TABLE 1.—The distribution of dissolved organic carbon (mg C/liter) in selected areas of the Atlantic Ocean. Each value is the average of concentrations measured at the indicated number of stations

Area	A	B	F	G	E	H	D
number of stations	6	5	9	8	10	7	13
Depth (m)							
1	1.04	0.88	0.82	1.03	0.99	0.81	0.87
50			0.74	0.80	0.98	0.82	0.87
100	0.70	0.61	0.62	0.65	0.86	0.65	0.77
150		0.57	0.50	0.59	0.88	0.56	0.67
200	0.67	0.50	0.50	0.59	0.78	0.52	0.58
250			0.46	0.56	0.72	0.48	0.58
300	0.67	0.47	0.46	0.51	0.66	0.46	0.52
350			0.46	0.52	0.61	0.48	0.45
400	0.66	0.42	0.47	0.49	0.58	0.42	0.45
450			0.46	0.50	0.57	0.43	0.45
500	0.64	0.44	0.46	0.48	0.55	0.43	0.45
550			0.44	0.46	0.56	0.44	0.42
600	0.64	0.39	0.40	0.47	0.53	0.45	0.40
650			0.39	0.48	0.50	0.45	0.38
700	0.64	0.38	0.38	0.48	0.53	0.43	0.38
750			0.40	0.44	0.52	0.44	0.37
800	0.70	0.40	0.37	0.48	0.50	0.44	0.34
850			0.46	0.44	0.54	0.44	.....
900	0.66	0.40	0.39	0.48	0.53	0.40	0.36
950			0.42	0.48	0.49	0.44	.....
1000	0.66	0.46	0.44	0.45	0.51	0.44	0.34
1100			0.42	0.50	0.49	.....	
1200	0.63	0.43	0.40	0.53	0.51	0.46	.....
1300			0.43	0.52	0.53	.....	
1500	0.60	0.41	0.43	0.49	0.50	0.42	0.34
2000	0.60	0.40	0.49	0.42	0.51	0.45	0.37
2500	0.60	0.42	.....			0.50	0.36
3000	0.58	0.42	.....			0.44	0.36
3500	0.60	0.40	.....			0.44	0.36
4000	0.60	0.40	.....				0.34
4500	0.65	0.45	.....				0.38
5000			.....				0.38

Source: Menzel 1970.

TABLE 2.—Organic matter in the water of the Amerasian Basin of the Arctic Ocean at Station T-3-703, 82 N°, 157°W, from March 29 to April 6, 1968

Depth (m)	POC (µg/liter) wet combustion	DOC (mg/liter)	DOC/POC
5.....	11.0	1.31	119
30.....	11.0	1.14	106
60.....	7.1	1.31	185
200.....	14.0	1.20	86
250.....	8.8	1.26	143
500.....	5.5	0.77	140
1500.....	6.4	0.76	119
2500.....	14.0	0.80	57
3500.....	6.9	0.75	109

Source: Loder 1971.

ranges between 0.3 and 0.80 mg C/liter if the results of Skopintsev are eliminated (Table 3). The remarkable regularity of these data led Menzel (1970) to an analysis of the way in which DOC could affect non-conservative constituents in the sea and thereby reflect on its rate of decomposition. The variability in DOC in 400 m water is about ±0.2 mg C/liter if absolute values are disregarded and only variations within any set of observations are considered. This variability is equivalent to a utilization rate of less than 1.0 ml/liter (2.6:1 atomic ratio of oxygen to carbon, as derived by Redfield et al. 1963) in areas where the oxygen concentration is between 0.1 ml to 6.4 ml/liter.

TABLE 3.—The concentration of dissolved organic carbon at depths below 400 m in the Pacific and Atlantic Oceans

Range (mg C/liter)	Location	Authority
1.34–1.74 (500 m).....	10°S 51° N: Atlantic	Skopintsev et al. (1966)
0.34–0.70.....	35°S 35°N: Atlantic	Menzel and Ryther (1970)
0.24–0.82.....	40°N 66°N: Atlantic	Duursma (1961)
0.34–0.70.....	20°N 28°N: Gulf of Mexico	Fredricks (1968)
0.40–0.80.....	10°S 0°: E. Pacific	Barber (1967)
0.43–0.79.....	15°S 10°N: E. Pacific	Fredricks (1968)
0.36–0.77	27°N 35°N: N.W. Pacific	Ogura (1967)

Source: Menzel 1970.

These data indicate the maximum influence that the decomposition of DOC can exert on the oxygen present at depth may amount to 15 percent of the total change. Even this variation seems unrealistic, because DOC distribution is not consistent with other criteria used to differentiate deep water mass distribution, nor are gradients evident in the direction of decreasing O_2 values. Further evidence by Barber (1968) and Ogura (1972) show that DOC from deep water samples does not decrease during prolonged storage of water samples and that stable isotope ratios of organic matter are constant as a function of depth (Williams 1968; Williams and Gordon 1970). Also, Menzel (1967) and Newell and Kerr (1968) found that particle concentration below 200 m was independent of surface production and that density discontinuities do not lead to the accumulation and thus accelerated decomposition at depth. These observations led Menzel (1970) to conclude that the compounds included in the DOC which found its way into the deep ocean were biologically and chemically stable.

What may be considered to be a disagreement on the stability of the DOC fraction based on other data has recently arisen. Nishizawa and Tsunogai (1973), in extensive studies of particulate organic matter distribution in the Bering Sea, found that the minimum concentration of particulate carbon occurred close to the dichothermal layer and concentrations below this depth were 2 to 3 times higher. Most significant, however, they found that the *average* POC in deeper layers correlated closely with the concentration in the upper 50 m layer. These authors concluded that the major fraction of POC in the deeper layers is directly derived through rapid sinking from the immediate overlying layer, implying a much closer coupling between the surface and depth than determined by Menzel (1970). It must be clearly understood in comparing these data that in the Bering Sea, vigorous mixing in some areas may place the productivity under *physical* control—thus allowing excess production over consumption. The conditions studied by others that supported the conclusion of conservative-type organic matter at depth, were under *biological* control (probably by the zooplankton community), thus allowing only limited amounts of organic matter to remain available for sinking.

A second consideration concerns the sources of carbon dioxide in the water column. The major changes in the concentration of carbon dioxide in the oceans are effected through exchange with the atmosphere, production during respiration, consumption during photosynthesis, and in the formation and solution of particulate calcium carbonate. The oceans of the world contain about 2×10^{-3} moles/liter of total carbon dioxide. The deep waters of the Atlantic and Pacific contain about 10 and 20 percent, respectively, more total carbon dioxide than the surface water. The addition of CO_2 to the water by solution of $CaCO_3$ is accompanied by a concomitant increase in calcium and carbonate alkalinity. Each mole of CO_3^{2-} which enters solution will increase the alkalinity by two equivalents. Tsunogai (1972), computing differences in total-CO_2, Ca^{2+} and alkalinity between the surface and deep water, concluded that only about 20–30 percent of the increase in carbon dioxide could arise from $CaCO_3$ dissolution, the remainder being derived from decomposition of organic matter.

Kroopnick et al. (1971) has estimated the source of carbon dioxide in deep water based on two methods: the consumption rate of oxygen and the mean isotopic composition of carbon isotopes added to the water column. Both of these methods indicate that about 70 percent of the excess CO_2 is derived from oxidation of organic matter.

Some direct measurements of decomposition rates of DOC have been made by Barber (1967) and Ogura (1972). Ogura, during extensive cruises on the *Hakuho Maru* in the north equatorial Pacific collected samples from the surface layer and incubated them aboard ship for a period of up to 75 days while observing dissolved oxygen and DOC changes with time. His data were found to best fit a three-component decay system. The first part (F_I), consisting of 10–20 percent of the total, was utilized in the first 40 days of storage; disintegration of the second fraction (F_{II}), consisting of 20–25 percent of the total, required a much longer period; and the third portion (F_{III}), essentially refractory, constituted 50–60 percent of the total DOC. Table 4 summarizes these conclusions. From these data, he was also able to compute rate constants (Table 5).

The decomposition of phytoplankton has been studied both

TABLE 4.—Proportion and nature of three organic fractions in surface sea water.

Fraction	Percentage	Nature of DOC
F_I	10–20	Easily utilized by microorganisms
F_{II}	30–40	Utilizable by microorganisms
F_{III}	50–60	Refractory, not easily utilized by microorganisms

Source: Ogura 1972.

in mixed and single specie culture by several workers (Skopintsev et al. 1966; Khaylov and Finenko 1968; Grill and Richards 1964; Handa 1970; Ogura 1971). Their results show that decomposition occurs usually at two different rates: between 0.03–0.10 mg day^{-1} during the first few days and between 0.0004–0.02 mg day^{-1} for extended periods. The available data do not appear to permit a precise breakdown of decomposition rates for different organisms under various conditions; the general conclusion may be drawn, however, that there are rapidly decomposing fractions together with more refractory materials that decompose at slower rates. The decomposition of the easily decomposed organic matter occurs mainly in the surface water, leaving the more refractory materials as the bulk of dissolved organic matter found in the ocean.

There appears to be no real basis for the apparent disagreement on the stability of DOC in the deep ocean. Zooplankton in the Pacific Ocean have a biomass of 0.1–1 mg/m^3 at 4000 m (Vinogradov 1969). This biomass is about 10 percent carbon and will require about 1–2 percent of the body weight per day as food. Thus, the consumption of carbon would be about 0.1–1 μg C/m^3-*day* or 0.036–0.36 mg C/m^3-*year*. The distribution of particulate carbon alone is between 3–10 mg C/m^3 available for zooplankton consumption. If this were the source of zooplankton food, a turnover time of approximately 10 years is indicated.

Based on the above assumptions and the integrated abundance of zooplankton from 400–4000 m, the flux of carbon required is about 2g C/m^2-year. This estimate is similar to that of Packard et al. (1971), based on potential electron transport

TABLE 5.—Rate constant for decomposition and half-life of DOC in surface sea water

Fraction	Rate constant (mg × day^{-1})		Half-life (days)	
	25 C	12 C	25 C	12 C
F_I	0.033	0.016	20	40
$F_I + F_{II} + F_{III}$	0.0052	0.0030	130	230

Source: Ogura 1972.

measurements, which indicated carbon requirements of 2–3 g/m^2-year. This flux may be provided through the sinking of plants, animal feces, zooplankton migration, particulate carbon, or dissolved organic carbon.

The oxygen depletion rate resulting from the consumption of this much carbon is between 9–13 μliters/liter-yr, which would result in a depletion of about 0.1 ml O_2 in 25 years. This is within the presumed accuracy range of the presently used method for oxygen determination and, therefore, such a depletion would normally be undetectable in the standard deviations of oxygen data.

There appears to be little basis for the differences in opinion expressed above on utilization of DOC in deep ocean water to account for oxygen utilization, excess carbon dioxide or isotope ratio differences. Although it is clear that the deep water biomass requires approximately 2g C/m^2-yr to sustain its metabolic requirements, this amount may easily be derived from other sources. Alternately, a plausible model compatible with existing data may be advanced: The DOC in deep water is in dynamic equilibrium, which maintains its concentration at a remarkably constant value throughout the oceans. The particulate organic carbon, although low in concentration, is not only a source of usable carbon but might in fact function in a sorption-desorption equilibrium with DOC components. Through such a process POC would serve as a source of oxidizable dissolved organic matter that may escape surface oxidation. Both the carbon dioxide required to satisfy the increase in total carbon dioxide with depth and the metabolic energy required by zooplankton

and deep ocean microorganisms is derived by oxidation of organic matter on surfaces provided by sinking particulate matter. Such a mechanism would also justify the very old dates obtained for dissolved organic carbon and the correlation of surface productivity with particulate carbon found at depth. Some recyclable DOC will be added to the pool by metabolizing organisms; if such a contribution is equivalent to 10 percent of the metabolizable carbon taken in, about 0.4–4.0 mg C/m^3-yr would be released, an amount which would not cause concentration changes significantly detectable by present methods of measurement.

Man-Made Additions of Organic Matter to the Marine Environment

Easily oxidized compounds constitute the major portion of organic matter that man adds to the marine environment. Many of these compounds do not pose a serious pollution problem unless their concentrations are high enough to reduce the dissolved oxygen content. Other compounds or their degradation products may directly inhibit vital biological processes. Although easily oxidized compounds are eliminated relatively rapidly by oxidation and thus do not ordinarily constitute a serious pollutant threat, even their very brief residence time in the case of certain highly toxic compounds is sufficient to cause considerable harm through their specific individual properties. The more resistive organic compounds are of greater concern because of their potential role as major pollutants, depending upon the manner in which they interact biologically and chemically with environmental components.

The effect of contaminants on vital life processes can often be measured quantitatively, such as inhibition to photosynthesis or respiration, energy conversion, or even decrease in motility. Other deleterious effects are subtler, more difficult to detect, and if not monitored carefully can go unnoticed until a particular species is eliminated. These latter effects may be manifested as reproductive failure, thermotactic interference or alterations of predator-prey relationships. Inhibition of particularly sensitive life processes such as photosynthesis or respiration occurs often at contaminant concentration levels below those affecting

TABLE 6.—United States annual production rate of some synthetic organic chemicals in 1968

	Million metric tons	Boiling point (C)
Formaldehyde	1.70	−21
Acetaldehyde	0.59	21
Acetone	0.59	56
Dichloro-difluoro-methane (freon 12)	0.13	−29
Carbon disulfide	0.34	45
Methylene chloride	0.12	40–41
Diethyl ether	0.05	35
Ethanol	0.86	78.5
Methanol	1.50	65
Cyclohexane	0.86	81
DDT	0.74	—

Source: U.S. Tariff Commission 1968.

other vital functions. This condition enables use of a control technique based on a maximum tolerable concentration threshold, below which photosynthesis and respiration are unaffected, thus providing some degree of protection against the less conspicuous effects. Because of the rapid increase and diversity in production of synthetic organic chemicals over the past few decades and their release to the environment by multiple methods of transport, it is difficult to predict the future extent of contamination. The 1968 U.S. production rate of some commonly used organic chemicals is presented in Table 6; hundreds of other synthetic organic compounds are produced in varying quantities. Since the quantity produced of these compounds tends to follow the gross industrial production of the world, it is apparent that the environment will continue to be under ever-increasing stress from additions of man-made organic chemicals.

Biodegradation and geochemical removal are probably the two most active factors in depleting the oceans of added organic contaminants. All organic materials are eventually biodegraded. Although the rate of biodegradation has been measured for very few compounds, it is clear that this process proceeds very slowly in the case of chemicals such as DDT, polychlorobiphenyls, some plastics, and certain petroleum fractions.

A review of the general topic of critical problems in the coastal zone has recently appeared in a workshop report *The Water's Edge* (Ketchum, ed. 1972). One of the recommendations of this workshop reads in part:

The Coastal Zone Workshop recommends basic biological, chemical, and physical research directed toward the following types of problems in the coastal zone:

(a) Transport, dispersion, upwelling and cycling of nutrients and hazardous chemicals as they affect the functioning and stability of coastal zone ecosystems;
(b) Surveillance of input levels of contaminants, especially chlorinated hydrocarbons, petroleum and heavy metals.
(c) Effects of chronic, long-term, sublethal contaminants on organisms and ecosystems;
(d) Assimilative capacity of the coastal zone for all kinds of waste;
(e) Factors affecting stability, diversity, and productivity of coastal zone ecosystems.

Until a major portion of these problems are resolved, particularly concerning the natural capacity of the environment to degrade and assimilate, the question of organic contaminant additions will remain in question. Particular emphasis is needed on refractory compounds such as components of petroleum, polychlorobiphenyls and pesticides; at the same time a constant surveillance system is needed to detect any new additions that may be accumulating in the marine ecosystem. Since biodegradation may be the key factor in removing organic wastes from the environment, a thorough knowledge of specific reaction rates under varying environmental conditions is essential.

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Questions and Comments Following Dr. Hood's Talk

QUESTION FROM THE FLOOR: What about sinking of the oil and high sediment loading that may occur in Port Valdez?

DR. HOOD: There is a problem with sediments—oil interaction in glacial inlets. In Cook Inlet, however, where oil spills frequently occur, hydrocarbons have not been found in the sediments. I know this is not true in general. These are the results obtained on over a hundred samples in this inlet. We are watching the developments in Port Valdez. If the oils do sink there will be probably be active degradation in the surface sediment.

QUESTION FROM THE FLOOR: In your measurement of carbon, did you use a carbon analyzer?

DR. HOOD: No. The oceanographers are using a method which involves a 5 ml sample of ocean water taken in a vial. Persulfuric acid and phosphoric acid is then added and the vial sealed. The vials are autoclaved and the CO_2 evolved is measured by gas chromatography or infrared analysis.

Bacterial Bottom Sampler for Water Sediments

DALE J. VAN DONSEL*
and EDWIN E. GELDREICH**

An important aspect in assessing the water quality of a lake, river or estuarine area zoned for swimming may be the bacteriology of the bottom sediment. The sediment deposits may provide a stable index of the general quality of the overlying water, particularly where there is great variability in the bacterial quality of the water (*1*). Bottom sediments may also prove to be of critical significance in shellfish culturing waters. Attempts to collect sediment samples for bacteriological analysis have met with difficulty because of the limitations of available equipment. Bottom material is variable in composition, ranging from sand, gravel, marl, clay and hard rocky outcroppings to bottoms littered with refuse and sludge banks. Dredges are of limited value when attempting to obtain representative samples from a fixed area in some of the above sediments and are unsatisfactory for recovery of bottom sludges. Core sampling devices were found to be difficult to sterilize and the nature of the sediment limited the sample size that could be recovered. Field tests with the Emery bacterial bottom sampler were disappointing in that the spring loaded cut off plate frequently jammed in gritty substrates, preventing complete closure of the open end of the glass vial during recovery of the bottom sampler. In addition to this problem, the sample size was too small for multiple testing procedures. The need for a device that would

* Research Microbiologist, Arctic Health Research Center, College, Alaska.
** Chief Bacteriologist, Water Supply Research Laboratory, National Environmental Research Center, EPA, Cincinnati, Ohio

function in a wide variety of bottom materials, incorporate a disposable sterile container of adequate size, and permit hand operation in shallow water, or remote control operation from a boat, motivated the development of a new sediment sampler.

GENERAL DESCRIPTION

It is desirable that the sampler (Figure 1) be constructed of stainless steel, but brass material may be used except for the nose piece which requires stainless steel for durability. Samples are collected directly in sterile "Whirl-Pak" type plastic bags (6-ounce size) that are easily closed after use and serve as convenient containers during subsequent handling. A nylon cord

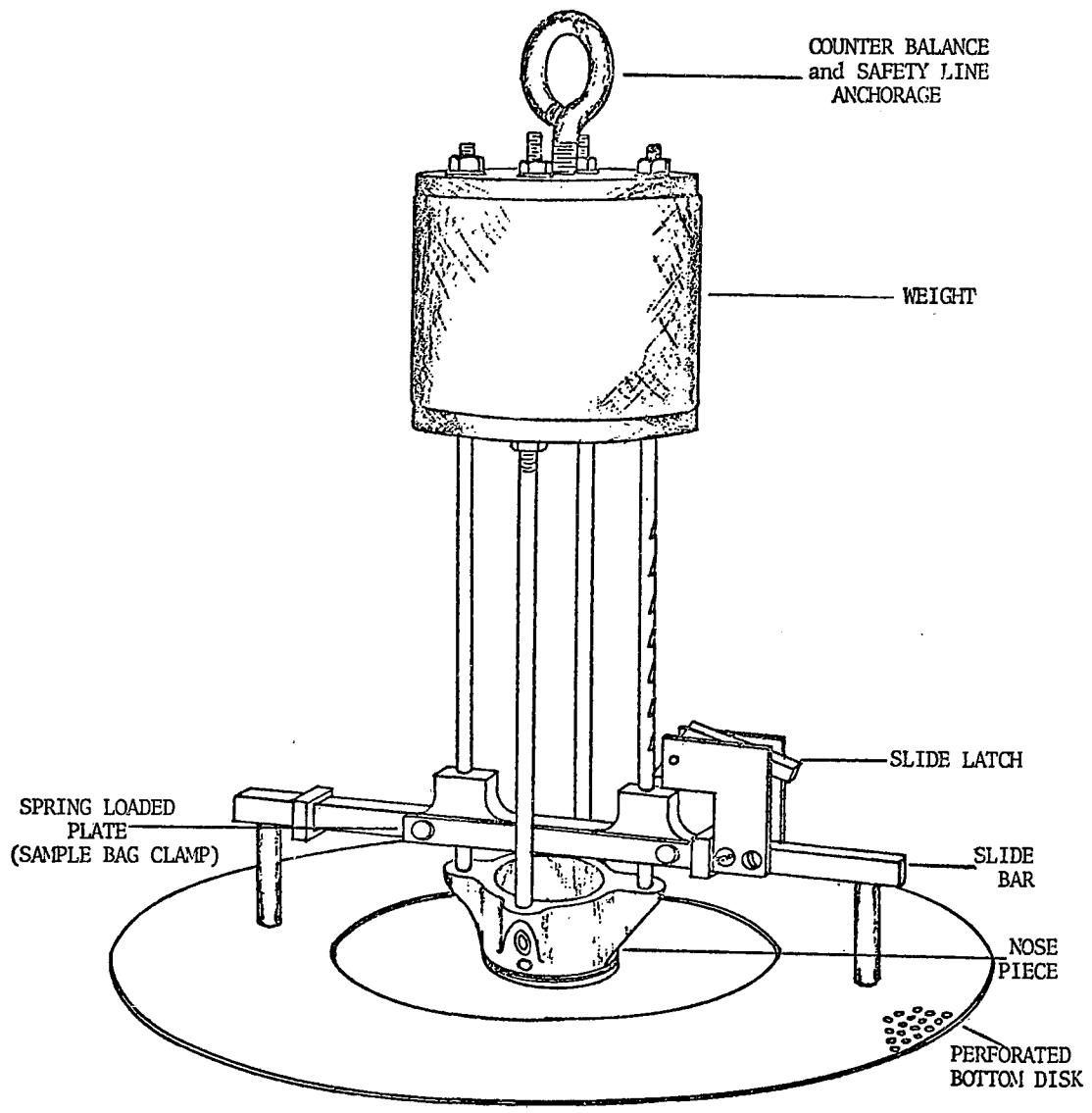

FIGURE 1.—Bacteriological Bottom Sampler.

loop arrangement closes the bag from the outside after the sampler penetrates the bottom sediment. A slide bar keeps the bag closed during descent and thus prevents air pressure build-up or entry of water into the bag. This slide bar permits the bag to open only during penetration of the sediment and then only as much as necessary to contain the in-flow of sample. Adding a heavy metal weight jacket to the assembly and using a counterbalances hook release makes it possible to use the bottom sampler in deep water.

Major Components

THE NOSEPIECE

This section holds the sampling bag and provides the cutting surface for penetration of the sediment. The nylon closing loops are inside the nosepiece but encircle the open end of the sample

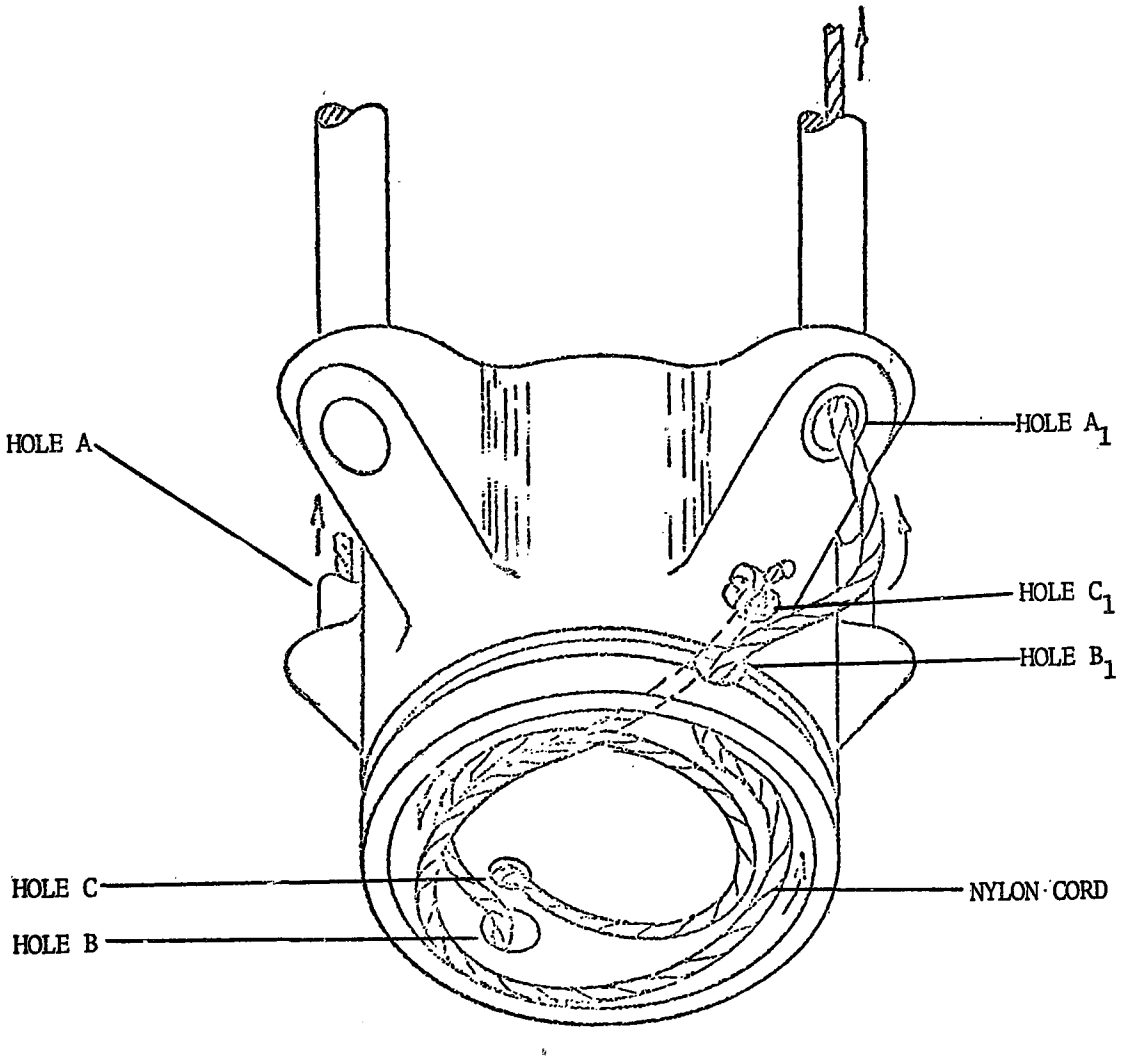

FIGURE 2.—Sampler nosepiece and closing loop.

bag, which is fitted over this nose piece (Figure 2). There are two holes on each side of the nosepiece; the upper set (B and B_1) serves only to anchor the free ends of the closing loops, which pass through the nosepiece and are knotted on the outside. The lower set of holes (C and C_1) allows the cord to pass through two hollow rods in the sampler to the operator's hand directly or indirectly by attachment to the counter-balance drop plate.

SLIDE BAR

The slide mechanism consists of a bar that rides on two solid rods attached to the nose. A plate is attached to one side of the slide and is held against the slide by slight tension from two small springs. Except for the open mouth, the sample bag is held shut by the spring-loaded plate. A short standoff mount on each side of the slide bar serves as an attachment point for a perforated bottom plate or disk. Thus, as the sampler nosepiece penetrates the bottom sediment, the perforated disk presses against the surface of the sediment, and the weight of the sampler causes the slide to traverse a distance equal to the penetration depth of the nosepiece. This procedure allows the sample bag to open and the bottom sediment core to enter the space provided. A latch holds the slide bar in the uppermost position during sample retrieval to prevent its dropping back to a lower position and cutting the bag.

CLOSING LOOPS

A #18 nylon cord, 160-lb. test strength, is used to make the closing loops that constrict the bag opening in a sphincter-like manner (Figure 2). The closing loops are made by: passing one end of the cord through the upper hold C of the nosepiece; knotting this end; and lightly flaming the free nylon cord tips to prevent fraying. The unknotted end of the cord is passed through the lower hole B and threaded through the hollow rod A to the counterbalance drop plate or extended long enough to be controlled by manual operation. This portion of the cord is then returned threaded, through the other hollow rod A; passed through the lower hole B; inter-looped through the companion closing loop and then through the upper hole C, where this end

is locked in a knot and the free tip is flamed. Spreading the interlooped cord in the nosepiece forms an opening through which the sample bag is inserted. The bag is opened and its mouth turned back over the nosepiece. When the cord at the top of the sampler is pulled, the bag is tightly closed.

COUNTERBALANCED DROPPING PLATE

In shallow water, a counterbalanced dropping plate is not needed. Instead, a rod is screwed into the top plate for handling the bottom sampler and the closing loops are operated by pulling the cord by hand. In deep water, bottom sediment collections will require attachment of a suitable jacketed weight and an eye bolt to the top plate of the sampler. In operation, the sampler is then lowered from a boat by a counterbalanced dropping plate (Figure 3), which also operates the closing loops. As soon as the sampler touches bottom, tension is removed from the dropping plate, permitting disengagement of the hook and eye. This release then permits the sampler to penetrate the bottom sediment by force of its own weight. Adjustment of the line length from the screw hook on the dropping plate to the sampler eye bolt will influence free fall force, depth of core collected, and speed of sample bag closure. The initial motion of retrieving the sampler via the line attachment to the eye bolt will simultaneously pull the closing cords to cut off the bottom sample and close the plastic bag. When the sampler is used in this manner, a safety line should be connected to the sampler eye bolt, passed through a hole in the dropping plate, and then joined to the lowering rope to ensure against loss of the sampler in the event the closing cords break.

SAMPLER OPERATION

The sampler is inverted and the closing loops are opened, keeping these cords within the nosepiece. Place a bag inside the loop opening, parallel with the slide. Open the bag, and fold it back over the rim of the nosepiece. The spring-loaded plate on the slide is opened and held open with the two stops. While feeding the free end of the bag through this opening, advance the slide bar until it comes to rest against the nosepiece and then

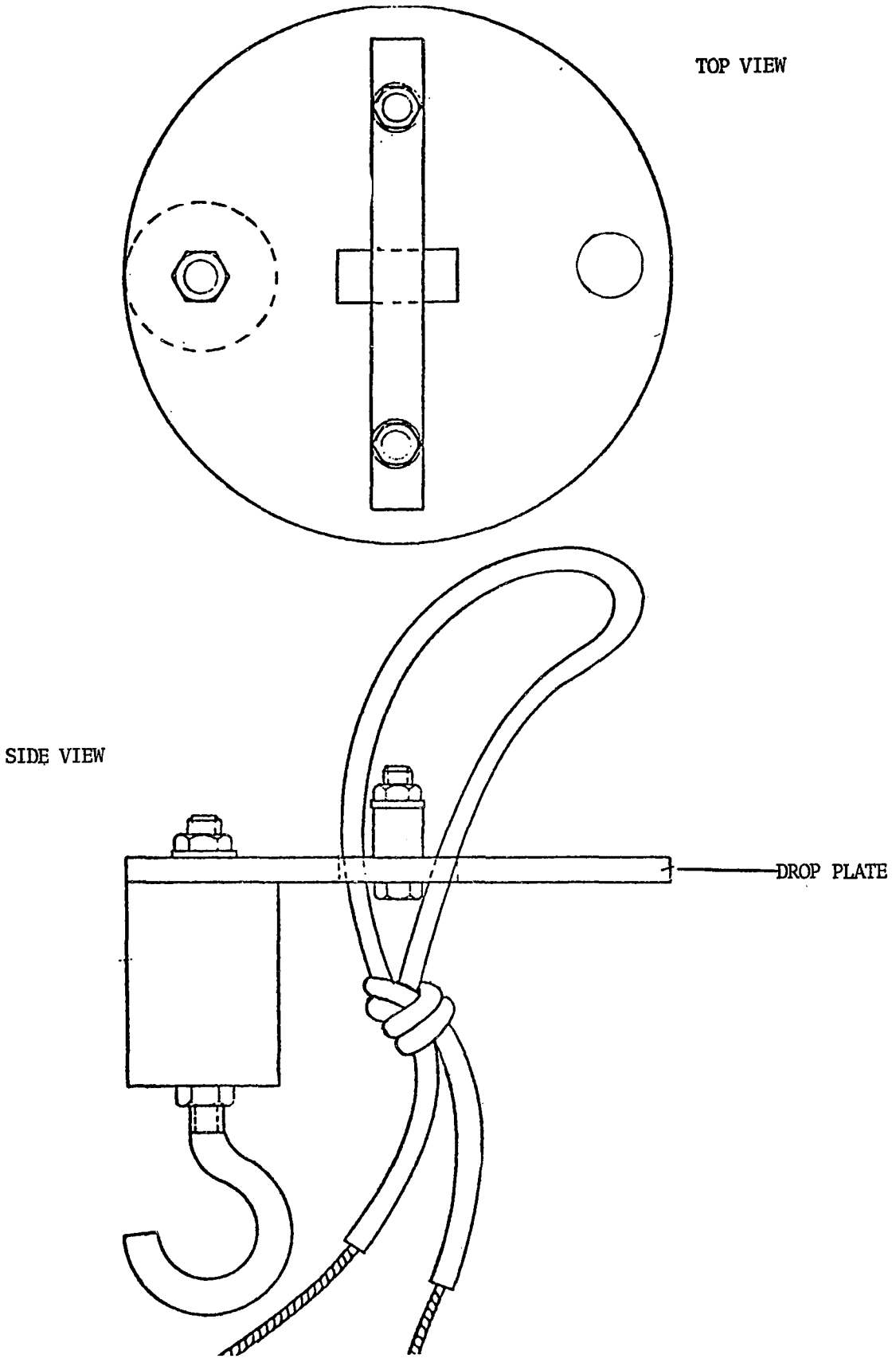

FIGURE 3.—Sampler Drop Plate.

return the spring-loaded plate to full tension against the bag. The slide bar should be held in this position until ready to sample. The sampler should be lowered slowly to prevent water resistance from lifting the perforated bottom plate, causing it to slide upward and permitting the bag to fill with water. When the counter-balanced hook is used for remote operation, the lowering line should be slackened after the sample has reached bottom to ensure that the hook has swung free.

When operating the sampler by hand in shallow water, the sampler is pushed into the bottom sediment with the rod attachment, then the closing cords are pulled to cut off the sample and secure it for retrieval. Tension on the closure cords, applied during retrieval by weight of the sampler is sufficient to keep the bag closed.

SPECIAL PRECAUTIONS

Because the nylon cord frays easily, all burrs and sharp edges should be removed from parts with which it comes in contact. The slide will bind on the two solid rods if all four lips of the slide holes are not rounded and polished. To ensure smooth operation of the bag-opening mechanism, the spring-loaded plate and the bar behind it should be polished and their edges rounded.

The perforated bottom disk shown in Figure 1 may be unsatisfactory for repeated operation from a boat because it requires a slow descent. If the bottom disk is unperforated, the water resistance of the disk may be reduced by using a smaller one or by using material having larger perforations, such as heavy hardware cloth. In instances where very soft sediments are encountered, a solid sheet metal disk has been used; this requires an extremely slow descent. In soft sediments, the depth to which the nose piece penetrates can be limited by attaching a Hoffman tubing clamp on one of the solid rods as a slide stop.

Although the sampler was designed for use in relatively shallow waters, it will operate at considerable depths (up to at least 200 feet); however, at depths greater than 50 feet, there is a chance that the Whirl-pak bag may rupture as it is returned to atmospheric pressure. When a sample is retrieved

from depths greater than 50 feet, the closing loops must be opened cautiously and the mouth of the bag pointed away from the operator lest internal pressure sprays the material over the operator.

REFERENCES

(*1*) Van Donsel, D. J., and E. E. Geldreich. 1971. Relationships of Salmonellae to Fecal Coliforms in Bottom Sediments. Water Research *5*: 1079–1087.

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