Water Pollution Microbiology

Volume 2

Edited by

RALPH MITCHELL

Water Pollution Microbiology

Volume 2

Edited by

RALPH MITCHELL

Gordon McKay Professor of Applied Biology
Division of Applied Sciences
Harvard University, Cambridge, Massachusetts

A WILEY-INTERSCIENCE PUBLICATION

John Wiley & Sons
New York • Chichester • Brisbane • Toronto

Library of Congress Cataloging in Publication Data: (Revised)

Mitchell, Ralph, 1934–
Water pollution microbiology.

Includes bibliographies.
1. Water—Microbiology. 2. Water—Pollution.
3. Sanitary microbiology. I. Title.
QR48.M58 576.1'6 73-168641
ISBN 0-471-01902-X

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

Preface

The intention of the editor in producing these volumes was to advance both teaching and research in water pollution control. For many years the role of microorganisms in the dynamics of polluted waters was ignored. Five years have passed since the publication of Volume 1 of *Water Pollution Microbiology*. During that time we have seen the rapid development of the discipline of microbial ecology. The gap between microbiologists and engineers has narrowed significantly, and modern biological concepts are being increasingly applied to water pollution control.

In this volume, I have attempted to provide the reader with these new concepts. A number of chapters demonstrate the developing synthesis between microbial ecology and environmental engineering in water pollution control. I have invited the most competent people in the field not only to describe these developments but also to contribute critical assessments.

I express thanks to Susan Mitchell for her invaluable editorial assistance in the preparation of this volume. The editor wishes to thank Viking Press for permission to quote from *Martin Buber: An Intimate Portrait*, by Aubrey Hodes.

RALPH MITCHELL

Cambridge, Massachusetts
February 1978

Contents

1 Nitrogen Fixation in Eutrophic Lakes

Alexander J. Horne
Division of Sanitary Engineering
University of California
Berkeley, California

1-1. The Process and the Organisms

The phenomenon of N_2 fixation probably occurs everywhere but is important in only a few environments. Eutrophic lakes provide suitable conditions for high rates of N_2 fixation, although there is no obvious similarity with other N_2-fixing areas such as clover fields. The process of N_2 fixation is confined to some procaryotes, whose ecology dictates the conditions needed for significant rates of fixation.

Many sanitary engineers and reservoir managers and a surprisingly large number of ecologists regard lake N_2 fixation as an unmitigated nuisance—a concomitant of human development and an indicator of pollution (1). This is not so. The process of N_2 fixation is an integral part of the natural nutrient budget of most eutrophic lakes. However, given

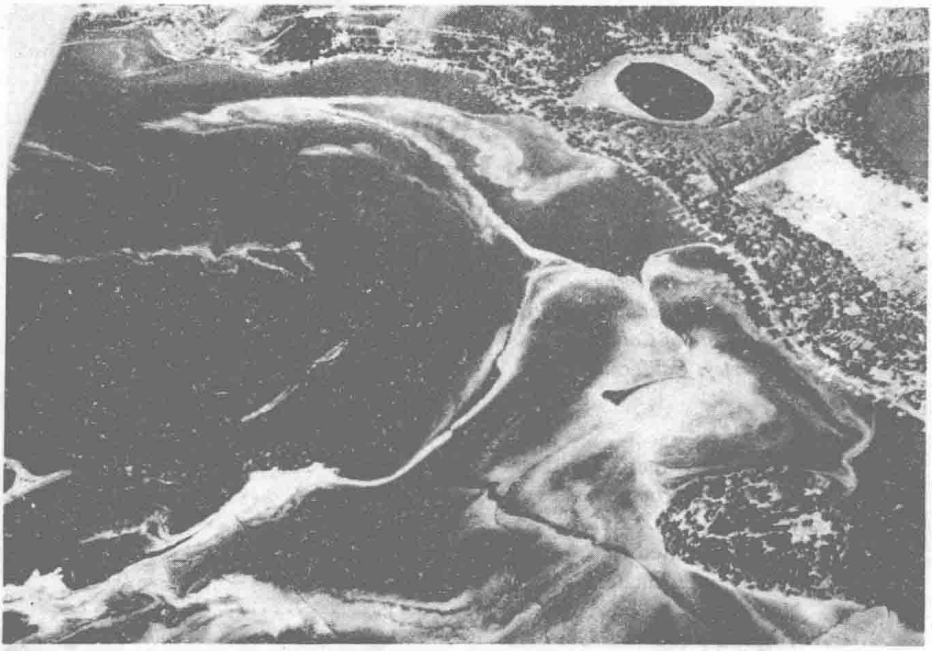

Figure 1.1 Large surface bloom showing spatial variations and recreational disadvantages of a nitrogen–fixing blue-green alga *Aphanizomenon flos-aquae* in its spring bloom, in eutrophic Clear Lake, California. Surface chlorophyll *a* concentrations range up to 50,000 μg/liter. The white areas show recently dead algae.

suitable stimulation in the form of excess phosphate, iron, warm water, and high sunlight, N_2 fixation can render mesotrophic or eutrophic lakes more objectional for many uses. Blue-green algal blooms, which often owe their large size to N_2 fixation, can decay on a grand scale and are not to be trifled with (Fig. 1.1).

Nitrogen fixation may be defined biologically as that process whereby an organism can grow indefinitely with only N_2 gas as a nitrogen source. More biochemically, N_2 fixation is defined as the process whereby the very stable triple bond of nitrogen gas is completely split by an enzymic process. The process requires an enormous amount of energy to split the $N \equiv N$ bonds, and is the most energetic reaction in the biologic world. The nitrogen molecule is also split in the Haber-Bosch process, used for NH_3 manufacture, at temperatures of 500°C and pressures of 250 atmospheres. Microorganisms accomplish the same process at 1 atmosphere and temperatures of 0–40°C!

The ability to fix N_2 is confined to a few genera of blue-green and a larger (and growing) number of bacterial genera. In the aquatic environment we are mostly concerned with blue-green algae. The main genera con-

cerned in lakes are *Aphanizomenon, Anabaena, Gleotrichia, Nodularia,* and *Nostoc* in that order of importance, although some other blue-green algae may be significant locally. That photosynthetic procaryotes dominate is mainly due to the enormous energy demand of the N_2-fixing process. Heterotrophic bacteria obtain energy from organic substrates which generally contain nitrogen, rendering N_2 fixation unnecessary. This logic does not, of course, apply to chemotrophic or photosynthetic bacteria which are occasionally important in N_2 fixation in eutrophic lakes (2).

The reason for the restriction of N_2 fixation to blue-green algae or photosynthetic bacteria in lakes is not hard to find if we examine the process carefully at the cellular level. Naturally, high energetic requirements pose limits, but it is conceivable that slower rates of N_2 fixation could go on in all plant cells with lower energy inputs. This does not occur because there is a discontinuity in the process—a minimum energy input which is still very high. The enzyme responsible for N_2 fixation, nitrogenase, which sequentially supplies all six electrons required to reduce N_2 to NH_3, is oxygen labile. This sensitivity poses severe problems for plant cells, which release oxygen from photosynthesis. There are three solutions to the problem, one of which is to alternate photosynthesis with N_2 fixation. The alga *Gleocapsa* (3) utilizes this technique, but it is an inefficient process. The second method is to photosynthesize without using water as the terminal electron acceptor and not produce O_2 as do the photosynthetic bacteria. Their habitat is thus severely restricted to anoxic zones. The best method is to construct a special anoxic cell to fix N_2, one of the few compartmentalizations in the procaryotic world. Compartmentalization of processes is a reason for the success of eucaryotes, but apparently separation of oxic and anoxic processes in the same cell is impractical.

Virtually all N_2 fixation by blue-green algae in lakes is carried out in specialized cells called "heterocysts" (4). These are modified vegetative cells with a thickened cell wall and a clear-looking appearance even under the light microscope (Fig. 1.2a). When viewed by the scanning electron microscope (Fig. 1.2b), the cells are also very different from vegetative cells. The heterocyst's obvious, easily counted presence is useful in assessing the potential of N_2 fixation to "pollute" lakes. There is still some dispute about exactly how the heterocyst provides a suitable site for N_2 fixation in oxygenated lake waters, but the outline is clear. The heterocyst contains only photosystem I and has lost much of the pigment system and photosystem II (5, 6) by reducing the manganese concentrations (7). Thus, no oxygen is produced since manganese is vital in the first stage of transfer of electrons from water in photosystem II. The rate

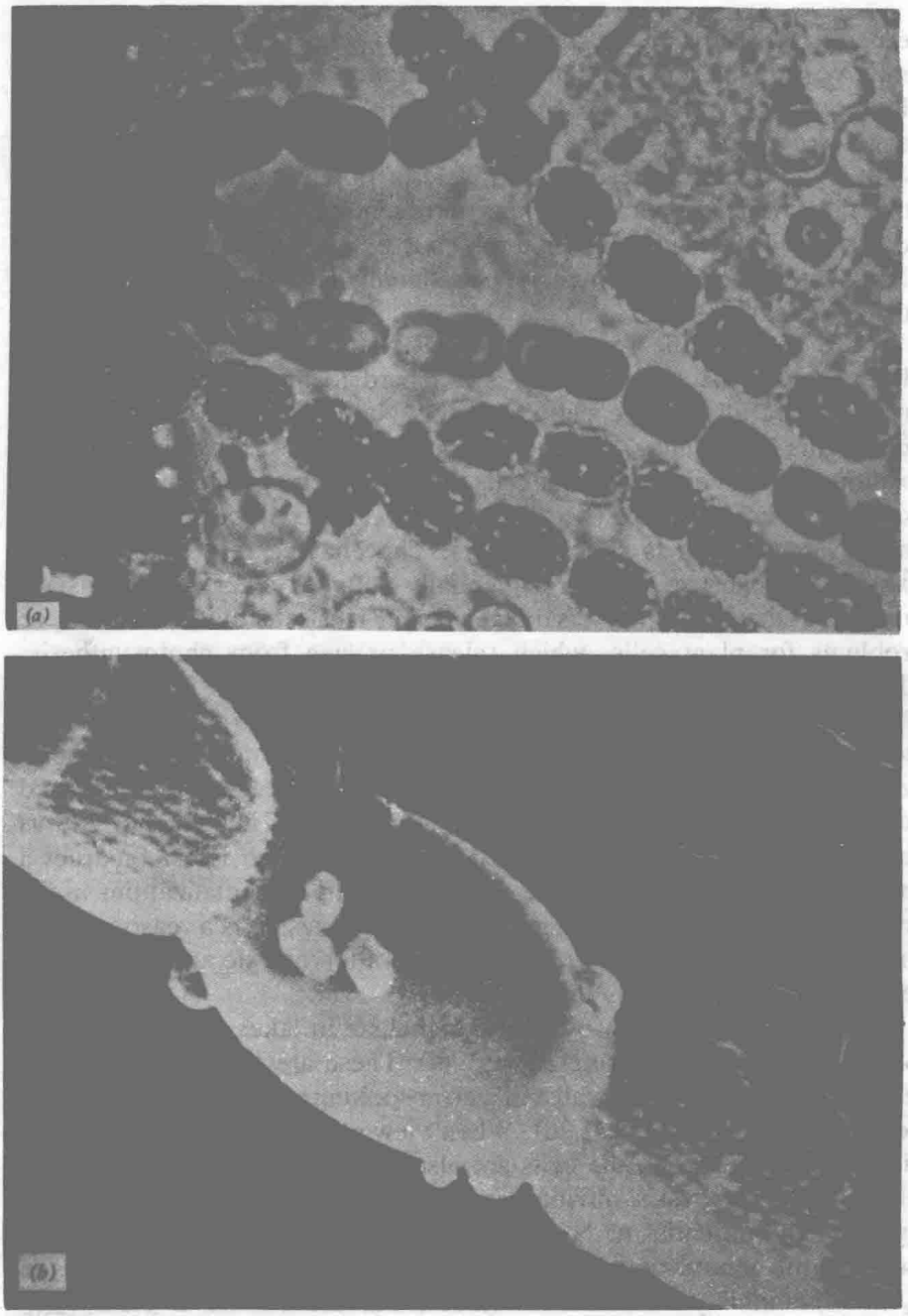

Figure 1.2 Heterocysts — the site of N_2 fixation for most blue-green algae. (*a*) The large clear cell in the lower left-hand corner is a heterocyst. The other cells are dark due to gas vacuoles. (*b*) A single heterocyst of *Aphanizomenoa* with adjacent vegetative cells. Small blocks are bacteria (Courtesy H. W. Paerl).

of respiration in the heterocyst is probably increased using reducing energy supplied by several adjacent vegetative, actively photosynthesizing cells (8). The thick heterocyst cell wall contains lipids which may also play a role in reducing the ingress of oxygen. That such a system might also reduce N_2 inflow is not important since saturation of nitrogenase occurs at only 20% N_2.

No oxygen is produced in the heterocyst, and any oxygen diffusing inward is consumed, which produces an anoxic zone allowing the nitrogenase to function. The enzyme's major energy source is external, consisting of the adjacent vegetative cells which supply simple carbohydrates and in turn are supplied with simple organic nitrogen compounds and/or ammonium ions.

The only other parameter of concern to studies of eutrophic or polluted lakes is the composition of the nitrogenase. It is a metalloprotein consisting of two parts, one containing iron and the other, both iron and molybdenum. Under some circumstances, iron may become rate limiting for N_2 fixation in eutrophic lakes (9).

There are some occasions when oxygen may be lowered sufficiently to allow N_2 fixation without the presence of heterocysts. It is probable that the centers of some colonial blue-green algae (*Trichodesmium, Aphanizomenon*) are virtually anoxic allowing the continually produced nitrogenase of vegetative cells (10) to function (11, 12).

1-2. The Habitat: Eutrophic Lakes

It is in eutrophic lakes where N_2 fixation reaches its apogee, although the process is often of significance in meso- and oligotrophic lakes (13). Unfortunately, there is a little more difficulty in defining a eutrophic lake than there is in defining N_2 fixation. Eutrophic (lit. = well nourished) can be defined as well supplied with essential nutrients, which is hardly true if nitrogen is so scarce that organisms must resort to N_2 fixation. Also, for most of the growing season, nutrients are present in very low concentrations in eutrophic lakes. The large phytoplankton crops rapidly utilize any nutrient inflows from rivers, rain, animal excretion, benthic releases, and even sewage pollution (Table 1.1).

However, most people recognize a eutrophic lake when they see one since it is frequently cloudy with suspended algae, filled with fish, and usually rather warm. My own definition of a eutrophic lake is one where the oxygen level rises above 150% saturation for most nonstormy days in a couple of summer months. This condition requires a good supply of most nutrients, and a large, healthy algal crop, and incidentally provides an oxygen toxicity problem for the nitrogenase of blue-green algae.

Table 1.1 Typical Concentrations of Nitrate and Ammonia During Blooms of N_2-Fixing Blue-Green Algae[a]

Lake	Trophic Status	NO_3–N Range	NH_4–N Range	Reference
Clear L., California				4, 9, 41
Upper arm	e	2–15	16–29	4, 9, 41
Oaks arm	e	6–9	15–63	4, 9, 41
Lower arm	e	6–22	29–167	4, 9, 41
Upper Klamath L., Oregon	e	0–200	0–1800	57
L. Erken, Sweden	e	1–44	3–100	48
L. George, Uganda	e	0–10	0–10	16
L. Blackwell, Oklahoma	m(?)	10–40	0–20	59
Green Bay (L. Michigan)	e–m	40–90	50–400	24
Esthwaite Water, U.K.	e	100–400	10–40	13
L. Windermere, U.K.				
N. Basin	o	180–450	5–15	13
S. Basin	m	150–300	5–50	13
L. Arlington	e	0–340	0–60	60
L. Hubbard	m?	160–240	30–250	60

[a]Eutrophic = e, mesotrophic = m, oligotrophic = o.

1-3. Assay Methods

Two dramatic improvements in the methodology of N_2 fixation measurement allow me to write this chapter: the use of ^{15}N and acetylene. Prior to 1950, only otherwise inexplicable increases in algal nitrogen content or lake nitrogen outflows would have allowed a prediction of the presence of N_2 fixation in lakes. Both these budget methods are subject to large errors—small daily increases in the total nitrogen of cells or uncertainties in the quantities of nitrogen in the many small streams or sheet water flows at the lake edges.

The use of the heavy isotope of nitrogen, ^{15}N, permitted investigators to mimic the pathways of ^{14}N with great precision and certainty, since nitrogenase does not discriminate ^{14}N from ^{15}N (14). The ^{15}N is stable (i.e., not radioactive), and thus a costly mass spectrometer to distinguish $^{15}N_2$ from $^{14}N_2$ is required. The process of conversion of nitrogen for admittance to the mass spectrometer is tedious, and the initial preparation of the isotopes by differential solubilities is long and expensive. Nevertheless, $^{15}N_2$ determination remains the only certain absolute method by which N_2 fixation can be measured in lakes and has given good results in whole lake studies (13, 15, 16).

The use of acetylene ($HC{\equiv}CH$) which nitrogenase reduces to ethylene ($H_2C{=}CH_2$) at a rate three to four times as fast as the reduction of $N{\equiv}N$ to

N– has revolutionized lake N_2 fixation studies. The method discovered in the early 1960s (17, 18) and pioneered in lakes by Stewart et al. (19) requires only cheap acetylene and relatively inexpensive gas chromatography for analysis.

Several hundred acetylene reduction bioassays can be carried out for the cost of one $^{15}N_2$ analysis and in much less time. Horne and Fogg (13) first fully assayed whole-lake N_2 fixation using less than 250 ^{15}N measurements. Only a few years later, 2500 acetylene reductions were carried out in a similar but more intensive survey (4). The speed and cost of the acetylene reduction technique far outweigh its drawbacks as an indirect method in normal aquatic situations, but care should be taken in anoxic sites or where diffusion paths are restricted, for example, waterlogged soils (20). Because acetylene reduction is not a direct estimate of N_2 fixed and measures nitrogenase (N_2-ase) activity, estimates made using acetylene reduction should properly be called nitrogenase activity, but this distinction is not always observed in the literature.

Finally, mention should be made of the recent use of ^{13}N, a radioactive isotope with a very short half-life (10 minutes) which restricts its use to lakes a few yards from cyclotrons or to bioassays in the laboratory. Despite this restriction, some useful studies of the role of the heterocyst vis-à-vis the vegetative cell in N_2 fixation have been made (21, 22).

1-4. Conditions Required for N_2 Fixation in Eutrophic Lakes

All else being limnologically normal, N_2 fixation occurs in lakes in sunny periods when the water is warm (15–30°C), provided that nitrate and especially ammonia are low and phosphate and iron are relatively high. Under such conditions, heterocysts are usually induced in planktonic blue-green algae, and N_2 fixation proceeds.

A. Temperate Climates. In lakes with seasons, these conditions normally occur in summer and autumn, since winter is a period of little plant growth but high input of plant nutrients. Seasonal nutrient cycles for two eutrophic lakes — one northern temperate the other mediterranean, both of which have N_2-fixing algae — are shown in Fig. 1.3. It is important to note that the increase in N_2 fixation is not a uniform upsurge but a series of peaks and troughs even when values are averaged from several sites over a whole lake (Fig. 1.3). No doubt, many lakes give similar seasonal cycles, but unfortunately there are few published reports that combine detailed measurements of nutrient, algal, and nitrogen fixation activities.

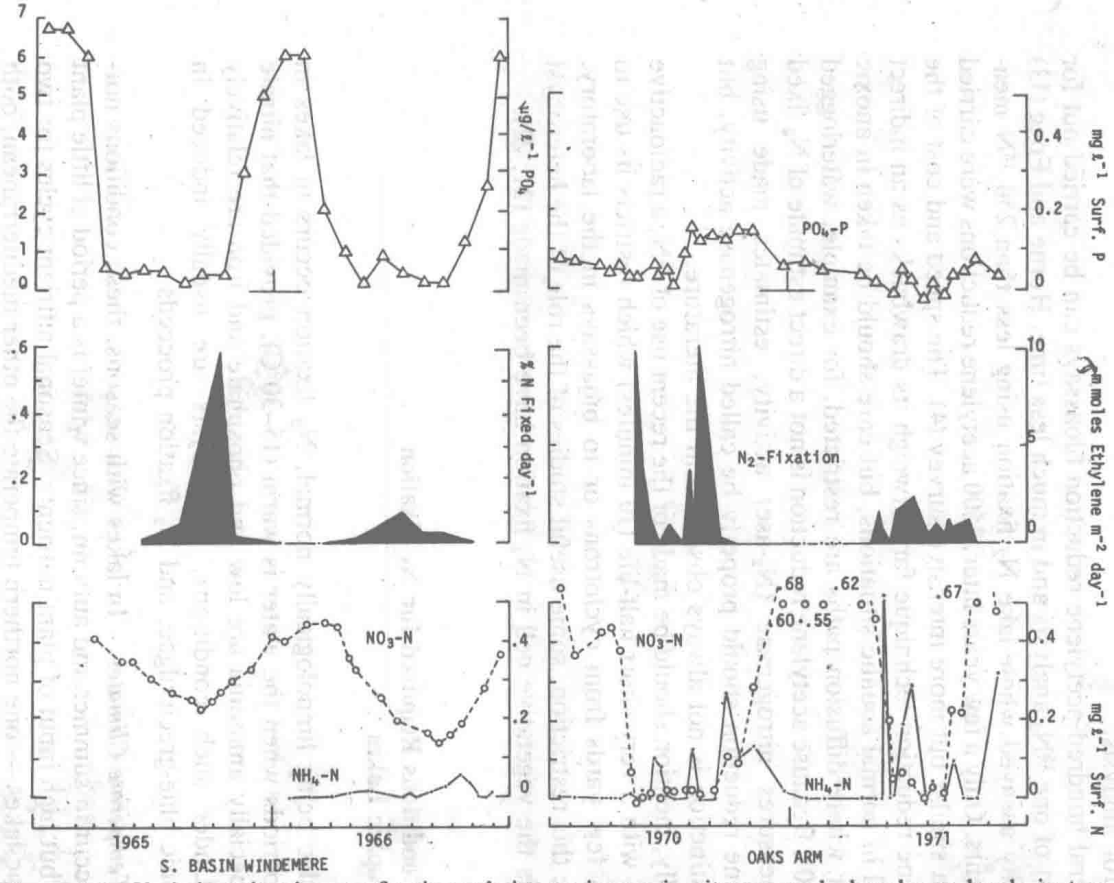

Figure 1.3 Variations in nitrogen fixation relative to inorganic nitrogen and phosphorus levels in two dissimilar lakes, the South Basin of L. Windermere, English Lake District (mesotrophic), and the Oaks Arm of eutrophic Clear Lake, California. Note that the scales of N_2 fixation are not the same. There is much more N_2 fixation in Clear Lake's Oaks Arm. Redrawn from references 4 and 13 with Windermere PO_4 data by courtesy of Mr. J. Heron, Freshwater Biological Association, United Kingdom.

Nevertheless, sufficient data of various types are available to present a broad picture of the effect of nutrients on lake N_2 fixation.

B. Nutrients. Ammonia, which is surprisingly common in quite high, if patchy, concentrations in eutrophic lakes, is a strong suppressor of both heterocyst induction and N_2 fixation itself. In natural eutrophic lakes, ammonia is released in bursts from disturbances of the sediments (9) which continually release lesser quantities. In deeper lakes, only the fall overturn produces sufficient benthic ammonia to act as a brake on N_2 fixation (Windermere in October 1965 and 1966, Fig. 1.3). By contrast, shallow, polymictic lakes show sudden depressions in N_2 fixation due to surges in ammonia (Fig. 1.3 in late summer–early fall). Other natural ammonia inputs are from zooplankton or fish excretion, while sudden increases are often caused by sudden die-offs of algal blooms. Such an effect, probably caused by the photo-oxidative death of a spring bloom of *Aphanizomenon flos-aquae*, is shown in Fig. 1.4. Rainfall and river inflow also contribute ammonia to lakes, and the persistence of nitrogen in the reduced form can only be attributed to a lack of nitrifying organisms, possibly due to the lack of surfaces in open waters. Artificial inflows, particularly those from secondarily treated sewage (approximately 30 mg/liter NH_4 nitrogen) will, if normally diluted, have similar effects to benthic releases (approximately 100 μg/liter NH_4 nitrogen) once diluted through the whole water column.

The normal method by which nitrogenous compounds reduce N_2 fixation in blue-green algae is indirect. This is a logical result of the separation of functions of the heterocyst and vegetative cells. Ammonia, for example, will enter all cells; but in lake waters it is inconceivable that the incoming ammonia concentration will exceed that of the interior of the heterocyst, so product inhibition of nitrogenase is unlikely. In addition, there is a highly effective mechanism to remove ammonia in heterocysts and to export it to the vegetative cells. We can thus conclude that there is no likely direct effect of ammonia on nitrogenase or the heterocyst. However, ammonia entering the vegetative cell will have an entirely different effect with nitrogen scarce but both energy and carbon skeletons in abundance. Ammonia will rapidly be utilized to form new cellular components directly and thus deprive the heterocyst of its energy source. Without a continual supply of reducing power, the heterocyst becomes more oxygenated and nitrogenase is irreversibly denatured. In the case of the vegetative cells, the natural water supply of ammonia increases the C–N ratio above the critical level and suppresses heterocyst induction (Table 1.2).

Similar effects should be observed for other nitrogen compounds, such

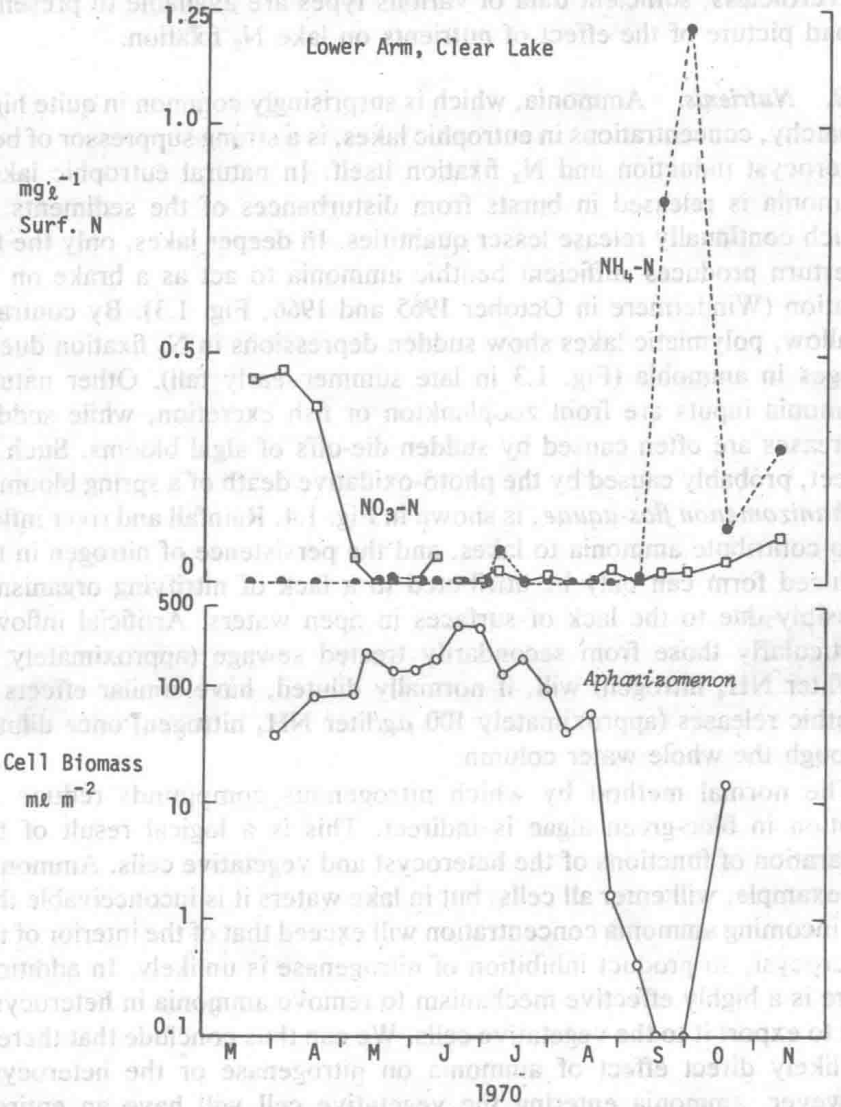

Figure 1.4 Effect of ammonia in the surface waters on the spring N_2-fixing algal bloom in a eutrophic lake (redrawn from reference 4). Such bursts of ammonia depress N_2 fixation.

as NO_3^-, urea, and amines, but these are probably rapidly converted to ammonia once inside the cell. Because of the generally low concentrations of nitrate in lakes during periods of N_2 fixation (Table 1.1), its role is generally to suppress heterocyst induction. This role of nitrate is discussed in detail for spatial and temporal variations in N_2 fixation (Section 1–7).

Table 1.2 Multiple Regression for the Log of Heterocyst Numbers as Dependent Variable in Between-Day Sampling for Relatively Phosphate-Rich Clear Lake[a]

	Analysis of Variance			
	d.f.	Sum squares	Mean squares	*F*
Regression	8	16.78	2.097	20.76
Residual	67	6.769	1.1010	

	Regression Variables			
Variable	Coeff.	Std. Error	*t*	Probability
Const.	−9.666	—	—	—
Aphanizomenon	*0.6613*	*0.1290*	*5.12*	>0.001
NO_3^-	−0.1655	0.0604	−2.74	>0.01
Turbidity	−0.7197	0.3145	−2.9	>0.05
Chlorophyll *a*	0.4206	0.1910	2.20	>0.05
Temperature	3.740	1.913	1.95	>0.1
NH_4^+	−0.1302	0.0913	−1.43	N.S.
PO_4	0.1547	0.1287	1.20	N.S.
Diss. O_2	−0.4251	0.7225	−1.00	N.S.

[a]Coefficient of determinism 71.25, coefficient of multiple correlation 0.8441, standard error of estimate 0.3.79. from ref. 41.

By definition at steady state, only one element can limit growth at one time; but the spatial and temporal variations of algae, their ambient water masses, nutrients, and light ensure that steady states are ephemeral. However, N_2 fixation can only occur when phosphate is present in excess since nitrogenase presents an additional requirement for phosphate (23).

Pollution by sewage normally produces a phosphate excess. In sewage, the P–N ratio of human excreta is increased from 1–7 to about 3–7 due to phosphate detergents. Thus, in oligo- or mesotrophic lakes sewage pollution creates a phosphorus-rich environment, ideal for N_2 fixation if other conditions are met (temperature, light, iron, lack of other N inputs). Under such circumstances, the effects of phosphate can be dramatic. A well-documented example is given by Vanterhoef et al. (24) where phosphate in water carried by the Fox River stimulated N_2 fixation in its plume in Green Bay, Wisconsin (Fig. 1.5).

Care should be taken in predicting the results of waste inflow on N_2 fixation (1). In granitic watersheds, in those with moderate to high sum-