NITROGEN FIXATION

Volume I Ecology

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W. J. BROUGHTON

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Preface

Nitrogen and phosphorus availability limit photosynthesis and therefore plant and animal productivity in many terrestrial and aquatic environments. Unfortunately for all concerned it is difficult to estimate not only the levels available to plants, but the absolute amounts of both elements. Yet just as atomic absorption spectroscopy revolutionized other aspects of mineral nutrition, the introduction of the acetylene reduction method for estimating nitrogen fixation (in the 1960s) sparked renewed interest in ecological aspects of nitrogen accretion. Coinciding as it did with recognition of the need to conserve energy and husband the environment, a great burst of research activity resulted. Laboratories, whether well equipped, or possessing little more than a gas chromatograph, began to examine plant, or soil, or water, or even coral and wood samples for tell-tale ethylene peaks on their recorders. On this evidence, nitrogen fixation was reported from such widely different habitats as the tundra and thermal springs.

The seeds of this book sprang from a UNESCO commission to C. A. (Lex) Parker and myself to prepare a chapter for the fifth 'Global Impacts of Applied Microbiology' Conference (Bangkok, 1977). In accepting this task, we assumed that in the decade since the introduction of the acetylene-reduction assay there would be an abundance of ecologically significant data on nitrogen fixation. For this reason, we optimistically entitled the chapter 'Microbial contributions to world nitrogen economy'. Our 118-page manuscript (which, like the present work, did not consider grain legumes) bears testament to the explosion in interest generated by this cheap and readily available technique. Assay of acetylene reduction is not without problems, however, the most serious of which concern the relationship between the amount of acetylene reduced and nitrogen fixed. Unfortunately there is no simple way to overcome this except by comparing the rates of acetylene reduction with the classical means of estimating nitrogen fixation, and integrating these data over considerable periods of time. Our review was finished by the end of 1975, and although it was never properly published, we felt then that understanding of nitrogen cycling in various ecosystems had reached the point where it deserved broader treatment than we were able to give it. This volume, to which experts from all over the world have contributed, is an attempt to do just that.

Obviously, much thought and effort by various people have con-

tributed to this, the first volume in the series. Foremost among those I must thank are the fifteen authors: I shall be ever grateful for their unstinting help. In the period from conception to execution (almost four years) I was attached to four institutions, each of which unquestioningly extended their facilities. Special mention should be made of Professor Satwant S. Dhaliwal and of Cik Rohani binte Muslim of the University of Malaya, Kuala Lumpur for providing the initial facilities and for typing all the early correspondence respectively; to Professor Fritz Lenz and Fräulein Antonia Maria Schäfer of the University of Bonn, who allowed the momentum to continue and for preparing the index; to Professor Ab van Kammen and to G. Wil Landeweerd of the Agricultural University, Wageningen for allowing me to devote some of the precious time I had in The Netherlands to this subject; and to Professor Jeff Schell and Fräulein Elisabeth Schölzel of the Max-Planck-Institut für Züchtungsforschung in Köln for guiding the process to completion. Sustenance for the editor over these years came from the University of Malaya, Kuala Lumpur, the Alexander von Humdoldt-Stiftung, Bonn, the International Agricultural Centre, Wageningen, and the Max-Planck-Gesellschaft, München.

W.J.B.

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1 Photosynthetic micro-organisms

P. FAY

1.1 Introduction

Representatives of the two groups of photosynthetic prokaryotes, the blue–green algae and photosynthetic bacteria, display a unique and remarkable autotrophic capacity to assimilate both carbon dioxide and elemental nitrogen, and convert them into cell material.

Blue–green algae (Cyanophyta, Cyanobacteria) and photosynthetic bacteria show a typical prokaryotic cellular organization which incorporates an elaborate cytoplasmic membrane system, the site of photosynthetic activity. The two groups differ fundamentally in their type of photosynthesis: being accompanied by oxygen evolution (oxygenic) in blue–green algae, and not associated with oxygen evolution (anoxygenic) in photosynthetic bacteria.

Both groups comprise a great variety of unicellular and simple multicellular forms, and display a whole spectrum of nutritional patterns from obligate autotrophy, through mixotrophy, to a more pronounced heterotrophic ability. On the basis of their pigment complement and of the nature of electron donors for photosynthesis, photosynthetic bacteria are classified traditionally in three main groups: the green sulphur bacteria (Chlorobacteriaceae or Chlorobiineae), the purple sulphur bacteria (Thiorhodaceae or Chromatiaceae), and the purple non-sulphur bacteria (Athiorhodaceae or Rhodospirillaceae) (Pfennig 1977). Blue–green algae are more uniform with respect of the organization and function of the photosynthetic system, and more diverse in terms of morphology, physiological characters, and natural distribution (see Fogg, Stewart, Fay, and Walsby 1973).

Information on general aspects of the biology of photosynthetic bacteria and blue–green algae may be sought in the books of Kondrat'eva (1965), Carr and Whitton (1973), and Fogg *et al.* (1973) and in the review articles of Pfennig (1967, 1975, 1977), Wolk (1973), and Stanier and Cohen-Bazire (1977). Nitrogen fixation in photosynthetic micro-organisms has lately been reviewed by Fogg (1974) and

Stewart (1973, 1977), and the ecology of nitrogen fixation by blue–green algae by Mague (1976). The proceedings of a recent symposium on the environmental role of nitrogen-fixing blue–green algae and asymbiotic bacteria include original reports as well as review articles (Granhall 1978).

1.2 The natural occurrence and importance of nitrogen-fixing photosynthetic prokaryotes

Photosynthetic bacteria and blue–green algae commonly occur in a great variety of natural habitats and are often abundant in cultivated lands. They seem to utilize preferentially combined (inorganic or organic) forms of nitrogen, and many are able to respond with the synthesis of nitrogenase to a temporary or prolonged shortage of combined nitrogen in their environment, thus enabling them to utilize the enormous store of elemental nitrogen in the atmosphere. The ability to fix nitrogen confers on these photosynthetic prokaryotes a significant competitive advantage over other (prokaryotic and eukaryotic) photosynthetic micro-organisms which are fully dependant for their growth on the availability of combined nitrogen in the environment.

Although their metabolism is based on photosynthetic carbon dioxide fixation, many photosynthetic bacteria and blue–green algae can in addition use simple organic substances as sources of carbon and energy. Depending on species, assimilation of organic substrates may take place only in the light, or both in light and dark, but is invariably enhanced by illumination. The basic requirements of nitrogen fixation (ATP, reductant, and carbon skeletons) can thus be produced equally in light and dark metabolic processes. Apart from a few environmental influences, like the partial pressure of oxygen or the concentration of combined nitrogen in the medium, which could directly and specifically affect the activity and synthesis of nitrogenase, most environmental factors act through their effects on photosynthesis and other metabolic and biosynthetic processes.

Distribution

The distribution of photosynthetic bacteria in nature is determined by their photosynthetic mode of nutrition and their sensitivity towards free oxygen. Photosynthetic bacteria occupy an ecological niche which provides reducing conditions and to which sufficient light can penetrate for photosynthesis. Such conditions are present at the mud surface in shallow waters, or in the upper parts of the hypolimnion in deeper lakes (see Pfennig 1967, 1975), apart from certain more peculiar habitats like small polluted ponds or sulphur springs. In general, reducing conditions in such an environment are maintained by the

action of sulphate-reducing bacteria, while carbon dioxide required for photosynthesis is released during fermentation of organic substrates by the anaerobic microflora, and elemental nitrogen is liberated by the action of denitrifying bacteria.

Blue–green algae, on the other hand, are distinctly aerobic micro-organisms, though they are able to survive in microaerobic or anaerobic environments (Stewart and Pearson 1970; Castenholz 1976, 1977). Moreover, several species can perform anoxygenic photosynthesis with sulphide as the electron donor in a photosystem I driven reaction, in a similar way to photosynthetic bacteria (Cohen, Padan, and Shilo 1975; Garlick, Oren, and Padan 1977). More commonly, blue–green algae are typical of well aerated aquatic and terrestrial habitats, and are the principal agents of biological nitrogen fixation in fresh and sea waters (see Fogg 1971, 1978; Stewart 1971).

Freshwater habitats

There is very little information available on the contribution of photosynthetic bacteria to the nitrogen economy of fresh waters (Stewart 1968; Knowles 1976). Blue–green algae are thought to be primarily responsible for nitrogen fixation in temperate and tropical lakes (Fogg 1971). Even so, nitrogen fixed by blue–green algae may constitute only a small proportion of the total nitrogen income, as shown by Horne and Fogg (1970) in some English lakes. Nitrogen-fixing activity is associated with dense populations of heterocystous planktonic (gas vacuolate) forms, mainly of the genera *Anabaena, Gloeotrichia,* and *Aphanizomenon* (Dugdale, Dugdale, Nees, and Goering 1959; Dugdale and Dugdale 1962; Goering and Nees 1964; Stewart, Fitzgerald, and Burris 1968; Ogawa and Carr 1969; Granhall and Lundgren 1971; Horne and Viner 1971; Horne 1972).

Benthic blue–green algae associated with macrophytes and growing over littoral sediments may be responsible for the occasional high rates of nitrogen fixation measured in oligotrophic lakes (Moeller and Roskoski 1978). They may be a significant source of nitrogen but little is known of their distribution and contribution to the nitrogen budget in oligotrophic lakes.

Nitrogen fixation in thermal springs was found to be related to the occurrence and abundance of *Calothrix* and *Mastigocladus* species (Fogg 1951; Stewart 1970).

Marine habitats

It is rather puzzling that heterocyst-bearing blue–green algae are generally absent or insignificant in the plankton of the open oceans, considering that sea waters are alkaline and generally poor in available combined nitrogen (Fogg 1978). Significant nitrogen-fixing activity is

mainly associated with the often spectacular blooms of the non-heterocystous marine *Oscillatoria (Trichodesmium)*. Although nitrogen-fixing activity in natural populations of *Oscillatoria* was demonstrated conclusively by several workers using both the isotopic (^{15}N) and the acetylene-reduction assays (Dugdale, Menzel, and Ryther 1961; Goering, Dugdale, and Menzel 1966; Bunt, Cooksey, Heeb, Lee, and Taylor 1970; Taylor, Lee, and Bunt 1973; Carpenter and McCarthy 1975), the evidence for *Oscillatoria* being the agent responsible remains inconclusive. Owing to the repeated failure to grow the alga in pure culture, it is yet not possible to test nitrogen-fixing activity in the absence of bacteria. To add further to the confusion, reports concerning nitrogen-fixing ability of bacteria isolated from *Oscillatoria* blooms are unfortunately conflicting (Maruyama, Taga, and Matsuda 1970; Maruyama 1975; Carpenter and McCarthy 1975).

Nitrogenase activity measured in planktonic populations of the marine diatoms *Rhizoselenia* and *Chaetoceros*, is almost certainly associated with the presence of a heterocystous blue–green algal endosymbiont, *Richelia intracellularis* (Mague, Weare, and Holm-Hansen 1974; Venrick 1974).

Heterocyst-bearing cyanophytes (*Nostoc, Calothrix, Dichothrix, Rivularia, Scytonema*) and non-heterocystous forms (*Oscillatoria, Schizothrix, Lyngbya*) are common in the intertidal region of coral reefs. They probably contribute significantly, through both carbon and nitrogen fixation, to the high productivity of reef communities (Wiebe, Johannes, and Webb 1975; Burris 1976; Capone, Taylor, and Taylor 1977).

Epiphytic blue–green algae, like *Dichothrix*, common on *Sargassum* in the Western Sargasso Sea, and *Calothrix*, found on sea grasses in the Gulf of Mexico, have been shown to fix nitrogen, but their importance is doubtful (Carpenter 1972; Carpenter and Cox 1974). Blue–green algae, especially *Calothrix scopulorum* and *Rivularia* spp, are widely distributed and often abundant in the supralittoral fringe of temperate oceans, and their contribution to fixed nitrogen is probably more significant (Allen 1963; Stewart 1965, 1967*a*, 1971; Wärmling 1973).

Henriksson (1971) recorded light-dependent nitrogen fixation in marine sand containing photosynthetic bacteria. Several strains of purple non-sulphur bacteria were isolated from inshore muds in the Irish Sea and found to display nitrogenase activity (Wynn-Williams and Rhodes 1974). Photosynthetic bacteria, which occur in an unusual situation at the interface between an anaerobic bottom layer of sea water and an aerobic top layer of fresh water in a Norwegian lake, were shown to fix nitrogen but rates were relatively low (Stewart 1968). However, in most brackish waters and salt marshes tested, nitrogen-fixing blue–green algae (*Anabaena, Calothrix, Nodularia, Nostoc, Scytonema,*

Tolypothrix) are the dominant forms (Stewart 1965, 1967*a*, 1967*b*).

Terrestrial habitats

Blue–green algae are frequently encountered in temperate soils though they are less common than green algae or diatoms probably because of the acidic reaction of most of these soils. In forest and grassland soil, nitrogenase activity is associated mainly with the presence of heterotrophic bacteria (Jurgensen and Davey 1971; Paul, Myers, and Rice 1971). Light-dependent nitrogen-fixing activity was measured in a variety of terrestrial habitats in Scotland but the total nitrogen input which results from this activity is probably of minor importance (Stewart, Sampaio, Isichei, and Sylvester-Bradley 1978). Nitrogen fixation by blue–green algae may be more important to soil fertility in the neutral and alkaline soils of Sweden (Granhall and Henriksson 1969).

Blue–green algae are almost ubiquitous in tropical soils and often display lavish growths in waterlogged fields. The most abundant species belong to the genera *Anabaena, Anabaenopsis, Aulosira, Calothrix, Cylindrospermum, Gloeotrichia, Hapalosiphon, Nostoc, Scytonema, Stigonema* and *Tolypothrix* (Singh 1961; Watanabe and Yamamoto 1971; Venkataraman 1975; Stewart, Sampaio, Isichei, and Sylvester-Bradley 1978). Durrel (1964) found that blue–green algae represent a large proportion of the soil microflora in the Caribbean islands and in Central and South America. Soil crust samples taken from a great variety of terrestrial habitats in Nigeria showed without exception significant nitrogenase activities, when moistened and tested under simulated field conditions (Stewart *et al.* 1978).

In paddy fields, nitrogen fixation is significant only under flooded conditions, and decreases rapidly after drainage (Ishizawa, Suzuki, and Araragi 1975). While heterocystous blue–green algae are mainly active in surface waters, photosynthetic bacteria appear to be primarily responsible for nitrogen fixation at the anaerobic mud surface (Materassi and Balloni 1965; MacRae and Castro 1967; Kobayashi, Takahashi, and Kawaguchi 1967; Kobayashi and Haque 1971; Kobayashi 1975; Ishizawa *et al.* 1975).

Blue–green algae are common inhabitants also of soils in the polar region (Jurgensen and Davey 1968). They were found to make up a large proportion of the terrestrial algal flora in the Antarctic (Hirano 1965). Fourteen out of 42 sites tested in Signy Islands showed [^{15}N]-nitrogen-fixing activity (Fogg and Stewart 1968). Among the potential (heterocyst-bearing) nitrogen-fixing species, *Nostoc commune* was found to be the most abundant. It was estimated that nitrogen fixation by blue–green algae may contribute an annual increase in total nitrogen of about 7 per cent to the Antarctic habitat (Horne 1972).

Blue–green algae, especially those epiphytic on mosses, were found to be the dominant nitrogen-fixing (acetylene-reducing) micro-organisms in the Arctic meadows and peats (Jordan, McNicol, and Marshall 1978).

Blue–green algae are known to be amongst the first colonizers of arid ground (Fogg *et al.* 1973). They were observed to be actively fixing nitrogen on lava soil in Heimaey, Iceland, only eighteen months after a volcanic eruption (Englund 1976). Tests for nitrogen fixation performed 3½ years after the outbreak indicate that the abundant blue–green algal community, with *Nostoc muscorum* predominating, contributes a substantial part of the nitrogen input to the juvenile lava field (Englund 1978).

1.3 Environmental factors affecting nitrogen fixation

Light

It is self-evident that light is the most important single factor in the natural distribution of photosynthetic micro-organisms though its effect on growth, photosynthesis, or nitrogen fixation is not always simple.

The observation that nitrogen fixation in natural waters or in soils is light-dependent was generally considered to indicate that photosynthetic micro-organisms are the agents responsible (Dugdale and Dugdale 1962; Goering and Nees 1964; Horne and Fogg 1970; Stewart 1965; Jurgensen and Davey 1968; Granhall and Henriksson 1969). Though nitrogen fixation in photosynthetic organisms is principally, but not exclusively, dependent on the energy and reductant generated in the photochemical process, the nitrogenase reaction *per se* is independent of light (Fay 1965; Cox 1966; Cox and Fay 1969). Many blue–green algae can grow and fix nitrogen in complete darkness in the presence of suitable organic substrates (Allison, Hoover, and Morris 1937; Fay 1965; Watanabe and Yamamoto 1967; Khoja and Whitton 1975). This ability appears to be more pronounced in terrestrial than in planktonic forms (Goering and Nees 1964) though nitrogen fixation even in obligate phototrophic species, like *Anabaena cylindrica*, will continue in the dark at the expense of endogenous substrates produced in the previous light period, and of ATP and reductant generated in respiratory processes (Cox 1966). Planktonic populations and soil algae continue to fix nitrogen in the dark for a limited period; such dark nitrogen fixation being greater and lasting longer in algae previously exposed to bright light for a prolonged period (Fig. 1.1) (Dugdale and Dugdale 1962; Goering and Nees 1964; Horne and Fogg 1970; Granhall and Lundgren 1971; Stewart, Mague, Fitzgerald, and Burris 1971; Fay 1976; Burris and Peterson 1978; Stewart *et al.* 1978). Light may further

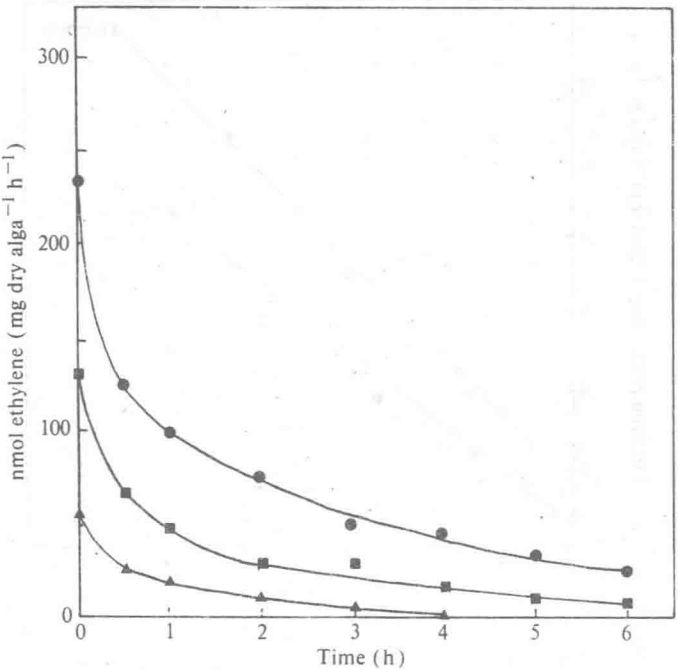

FIG. 1.1. Effect of light pretreatment of 1500 (▲), 3000 (■), and 6000 (●) lux intensity on nitrogenase activity of *Anabaenopsis circularis* in the dark. (After Fay (1976)).

enhance assimilation of organic substances, and this in turn will promote the uptake of elemental nitrogen in algae which exhibit heterotrophic abilities (Fay 1965; Watanabe and Yamamoto 1967; see also p. 22).

Nitrogenase activity, like the rate of photosynthesis, increases linearly in photosynthetic micro-organisms with the increase of light intensity (Fay 1970) (Fig. 1.2), but it may become depressed at the full intensity of solar radiation to which algae are eventually exposed at the surface of natural waters. Maximum rates of nitrogen fixation in planktonic blue–green algae measured in the subsurface layers (about 2 m below the surface), follow those of photosynthesis (Horne and Fogg 1970; Burris and Peterson 1978). Blue–green algae are more sensitive to high light intensities than diatoms or green algae (Brown and Richardson 1968). They grow best and fix nitrogen at higher rates in tropical rice fields at intensities less than 10 per cent of the full incident light (Reynaud and Roger 1978). Rates of nitrogen fixation measured at the surface of temperate lakes are only about 50 per cent of the maximum recorded in subsurface samples; rates fall off rapidly below the

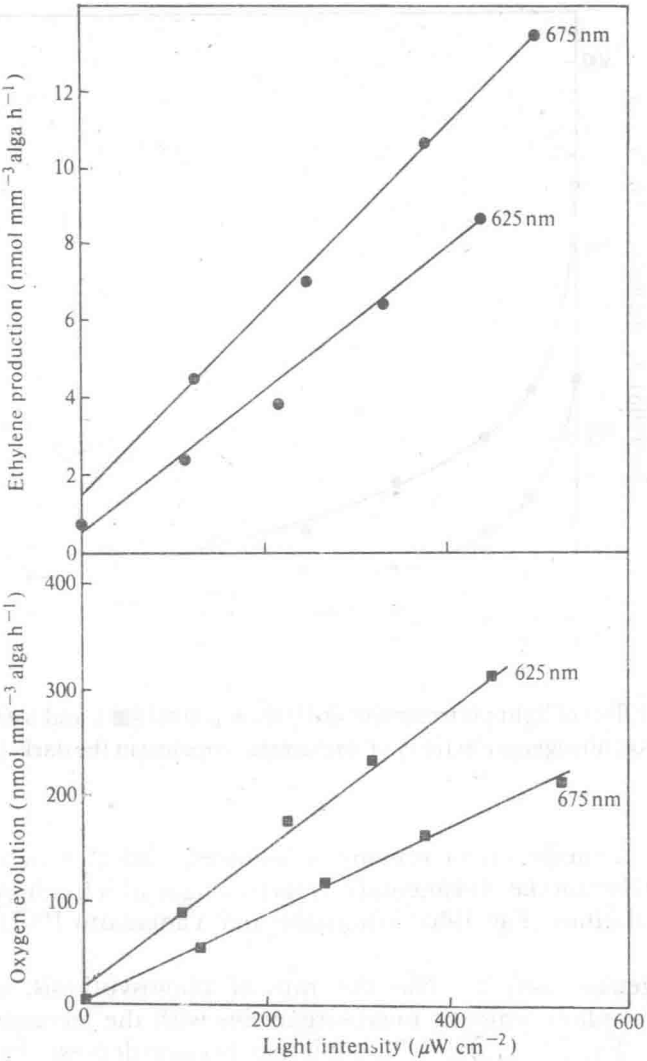

FIG. 1.2. Rates of acetylene reduction (ethylene production) (●) and photosynthetic oxygen evolution (■) by *Anabaena cylindrica v.* light intensity at 625 and 675 nm. (After Fay (1970).)

subsurface zone and become insignificant at a depth of about 5 m below the surface (Horne and Fogg 1970; Granhall and Lundgren 1971). A similar activity profile was obtained from experiments performed in the marine environment with *Oscillatoria* (*Trichodesmium*) or with the *Rhizosolenia–Richelia* symbiosis dominating the plankton