ALGAL NITROGEN FIXATION ON SOLID SURFACES AND TEMPERATE AGRICULTURAL SOILS

A Thesis Presented by

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ABSTRACT

This thesis examines various factors controlling algal nitrogen fixation and nitrogen release in temperate agricultural soils.

New apparatus for investigating nitrogenase activity on uniform soil grown algal crusts and for the *in situ* estimation of nitrogen fixation in the field is described.

The effects of oxygen concentration and temperature on acetylene reduction by soil grown cultures of *Nostoc ellipsosporum* in the light and in the dark suggest that ATP generated by oxidative phosphorylation contributes to fixation even in the light. The oxygen concentration giving optimum nitrogenase activity depends on the intensity of illumination, largely because of the continuing contribution from dark fixation.

The oxygen dependant acetylene reduction continued throughout the night in the field at 20% of the midday rate. This proportion is dependant on daytime light intensity and day/night temperature drop.

The release of nitrogenous compounds by soil and sand grown cultures of *N.ellipsosporum* was investigated in the laboratory. Algal cultures growing on sand released only 2% of the total nitrogen compared with 10% released by liquid cultures. Freezing to below -3°C, drying to less than 4% moisture or pathogenic infection all cause extensive cellular lysis with a concomitant loss of nitrogenase activity, followed, after several days, by a surge in ammonia concentration.

The recovery of nitrogenase activity after re-wetting was investigated in the field after a period of dry weather. All samples achieved a steady rate of nitrogenase activity after 400 minutes irrespective of the magnitude of recovery.

Nitrogenase activity in the field was monitored at weekly intervals using an *in situ* technique over a two year period on Broadbalk, one of the Rothamsted classic plots sown to winter wheat. The seasonal fixation rates varied from 1.4 to 28 Kg/ha depending on the fertilizer and herbicide treatment. The greatest seasonal fixation was given by

non-herbicide plots receiving 48 Kg N/ha of applied nitrogen.

The effect of various species of algal inoculum was investigated on 60 plots sown to winter wheat with and without irrigation and added nitrogen. A significant increase in fixation was produced and the most effective treatment was a liquid application of N. ellipsosporum. Both the N. punctiforme and Anabaena cylindrica were more effective when applied as dried sand cultures.

The most important single factor limiting algal development in field experiments was soil moisture.

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1. INTRODUCTION

(a) BIOLOGICAL AND INDUSTRIAL NITROGEN FIXATION

Although nitrogen constitutes 79% of the Earths atmosphere it is directly available to only a few species of nitrogen fixing organisms which have the ability to reduce elemental nitrogen to more reactive compounds which can be used in cellular metabolism.

The nitrogen made available by the primary fixers can pass to other organisms to be cycled through the biosphere. Some is lost as oceanic and fresh water sediments and some is recycled by denitrifying bacteria which convert nitrates to gaseous nitrogen, so that the quantities available to the biosphere have probably remained relatively constant until man's intervention in the cycle. (Delwiche 1970).

This intervention has taken two forms. First there has been a bias in agricultural practice towards the use of nitrogen fixing crops such as legumes and more recently the exploitation of blue-green algae in rice cultivation, and secondly an enormous increase in the use of industrially fixed nitrogen. The use of nitrogen in crop production has become increasingly important as larger and larger crop yields are demanded from cultivated land to support the World's exponentially growing population.

The crop yields which can be obtained using biologically fixed nitrogen alone are normally lower than those produced by the addition of artificial fertilizer. This is particularly so in some areas of western agriculture where very large quantities of inorganic nitrogen are used because high yields are demanded from limited areas of agricultural land. However, the exploitation of biologically fixed nitrogen as a substitute for, or supplement to, inorganic fertilizers is of great importance where the areas of land are large enough to make a low yield acceptable, or where the cost of other fertilizers is prohibitive.

The groups of organisms which are generally accepted as fixing nitrogen are tabulated below (table 1.1, after Stewart 1966).

The blue-green algae, which are a widely distributed group, developing in certain habitats in prodigious numbers, have recently stimulated particular interest because they offer an exploitable potential for fixing both nitrogen and carbon under atmospheric oxygen conditions.

Table 1.1 Nitrogen fixing organisms (After Stewart 1966)

A. Symbiotic forms.

1) Rhizobium spp. These bacterial symbionts occur within the root nodules of leguminous plants.

2) Actinomycetes. It is believed that the nitrogen fixing associates of certain non leguminous plants, such as Alnus, Myrica gale, and Hippophae ramnoides, are actinomycete.

3) Blue-green algae. The best known association is that with fungi to form lichens. Associations are also recorded with the following organisms;

Byophytes, e.g. Blasia
Pteridophytes, e.g. Azolla
Gymnosperms, e.g. Encephalatos
Angiosperms, e.g. Gunnera

B. Free living forms.

1) Bacteria. These include both photoautotrophs such as Chromatium and heterotrophs such as Azobacter and Clostridium.

2) Blue-green algae. The largest numbers of nitrogen fixing species are members of the order Nostocales with a few fixing member in the stigonematales. A more complete description is given later.

(b) EARLY DISCOVERY OF FIXATION BY BLUE-GREEN ALGAE

The fixation of molecular nitrogen by blue-green algae was first noted by Frank (1889) who observed an increase in bound nitrogen in soil which had supported algae. This observation was substantiated by Beijerinck (1901) and Heinze (1906) but the experimental results were questioned on the grounds that the algal cultures used were not bacteriologically pure.

Axenic cultures were later obtained by Pringsheim (1914) and Maertens (1914) but neither were able to demonstrate nitrogen accumulation, and it was not until 1928 that pure cultures of blue-green algae were convincingly shown to assimilate molecular nitrogen, (Drewes 1928). This result was later substantiated by a number of other workers (for example Allison et al 1937, De 1939, Fogg 1942, Watanabe 1951, and Cameron and Fuller 1960). Further direct evidence was obtained by various workers who demonstrated the uptake of the heavy isotope ^{15}N. (Burns et al 1946, Watanabe 1952, 1965 and Mayland and McIntosh 1966).

More recent studies on nitrogen fixation have been made using the acetylene reduction technique which followed from the discovery that the nitrogenase enzyme system will reduce acetylene to ethylene. (Shollhorn and Burris 1966, Dilworth 1966). This technique, which is far more sensitive than any of those used previously, has led to a vast increase in the spectrum of known nitrogen fixers.

With the exception of Gloeocapsa all the species recorded as fixing nitrogen under aerobic conditions are heterocystous filamentous forms. They include various species of Anabaena, Anabaenopsis, Aulosira, Calothrix, Cylindrospermum, Fischerella, Gloeotrichia, Chlorogloea, Hapalosiphon, Mastigocladus, Nostoc, Scytonema, Tolypothrix and Westiellopsis. A number of non-heterocystous forms belonging to the same order have been shown to fix nitrogen only under micro-aerophilic conditions. These include species of Lyngbya, Oscillatoria and Plectonema One unicellular species, Gloeocapsa, belonging to the order Chroococcales is also recorded as fixing nitrogen aerobically (Fogg et al, 1973, Mishustin and Shilnikova 1971).

(c) ALGAL POPULATION

Blue-green algae are a widely distributed group which have been recorded as fixing N_2 in both fresh and salt water at extremes of temperature varying from close to freezing point to 50°C in thermal springs (Stewart 1970). They are found in large numbers colonizing tropical, temperate and arctic soils (Holm-Hansen 1963, Hirano 1965, Fogg and Stewart 1968, Jurgensen and Davey 1968, Cameron et al 1965) as well as deserts (Mayland, McIntosh and Fuller 1966, Cameron and Blank 1966) and sand dune slack regions (Stewart 1965).

The earliest systematic study of soil algae was that published by Bristol Roach in 1927. This paper is of particular relevance since it describes plots at Rothamsted Experimental Station which are identical with those examined in this thesis. Bristol Roach estimated that a total of about 60,000 algal cells occurred per gram of topsoil. Blue-greens were not included in the numerical estimate because the serial dilution method employed did not work well for this group. The author points out that samples taken from an area upon which there was a continuous lamina of blue-green algae about 1 mm thick yielded estimates of no more than 30 individuals per gram of soil. Shtina (1961) working on Russian soils estimates that a gram of acid podsol soil contains 5000-30,000 algae; a gram of virgin soddy Podsol contains 220,000 and a gram of cultivated soddy podsol contains 3000,000 with as many as 3×10^6 algae occurring in the surface films.

(d) ECOLOGICAL PARAMETERS CONTROLLING DISTRIBUTION

1) pH

The species composition and distribution of these algae is extremely varied. John (1942) observed that blue-green algae grow most readily in alkaline conditions and that many have a preference for calcareous soils. Lund (1947) substantiated these findings and concluded that blue-green algae are characteristic of soils which are not base deficient, which are rich in phosphates, and which give a positive test for nitrates. The currently accepted nitrogen fixing species listed by this author include

A.variabilis, Cylindrospermum licheniforme, Tolypothrix tenuis, Nostoc muscorum and N.paludosum.

Granhall and Henriksson (1969) in a survey of Swedish soils, found nitrogen fixing blue-green algae in 19% of soil samples with a pH between 5 and 6, 61.5% of soils with a pH between 6 and 7, 76% of soils with a pH between 7 and 8 and all soil samples with a pH in excess of 8. No blue-green algae were found in the pH range 3-5 but Stewart (1969) notes the occurrence of Anabaena, Hapalosiphon and Stigonema on acid bogland soils at a pH of 4.0. Substantial differences are reported in the range of pH tolerance of different species although the cell-free nitrogenase enzyme operates most efficiently near to 7.0 (Burris 1966). Most authors agree that growth and nitrogen fixation are optimal in the range 7.0-8.5.

Taha (1963) investigated the effect of pH on the growth of three algal species. He found that A.variabilis would grow at pH values between 4.5 and 8 while Hapalosiphon fontinalis and Calothrix elenkinii were rather less tolerant growing over the range 6.0-8.5 and 6.0-9.0 respectively, although all these species showed optimum growth between 7.0-8.0.

Nostoc muscorum is variously reported as growing over a pH range of 5.7-9.0 (Allison et al 1957) 6.0-9.0 (Kratz and Myers 1955) and at pH values as low as 4.0-4.9 in the absence of nitrate nitrogen (Walp and Shopback 1942). This difference may be attributable to the ionic ratios in the growth medium (Fogg 1956). The effect of pH on growth is due not only to the interaction between H^+ ion concentration and cellular metabolism but also the interaction between this H^+ ion concentration and the growth medium. The equilibrium

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^-$$

is affected, as are the solubilities of many heavy metals which tend to precipitate in hydroxides under alkaline conditions. According to Uspenkii (1926) the quantity of iron present in many habitats is such that, under acidic conditions the prevailing flora must be tolerant to both a low pH and a high concentration of iron.

(2) Soluble Nitrogen

Although high concentrations of soluble nitrogen inhibit nitrogen fixation (see section 7) those levels encountered in most natural ecosystems are normally insufficiently high or persistant to supress fixation. Stewart 1969 observes that naturally occurring populations of Nostocales and Stigonematales invariably show the presence of heterocysts and most populations tested give positive results for ^{15}N uptake if the algae are metabolically active. The presence of low levels of combined nitrogen may be advantageous under some circumstances in promoting healthy growth , and thus more vigorous nitrogen fixation (Dugdale and Dugdale 1965). This view is supported in part by the observations on Broadbalk described in this thesis (see sections 7 and 8, the effect of added nitrogen).

(3) Molybdenum and Iron

An interesting nutritional requirement of blue-green algae, and indeed all nitrogen fixing organisms, is that for Mo which appears to be required in quantities as large as 0.2 ppm for optimal nitrogenase activity (Wolfe 1954, Okuda, Yamagachi and Nioh 1962) although Wolfe (1954) notes that the requirement of A. cylindrica is only 0.1 ppm when it is grown on nitrate. Both Mo and Fe are present in the nitrogenase complex (Burris 1969) but the Fe requirement is satisfied at 0.02 - 0.05 ppm (Burk, Lineweaver and Horner 1932) and this level is generally exceeded in most habitats (Stewart 1969). Mo on the other hand may be limiting. De and Mandal (1956) showed that the addition of 0.3 Kg Mo per hectare to Indian paddy soils produced substantial increases in nitrogen fixation, in some cases exceeding 23%.

(4) Interaction with Bacteria

Experimentation with pure algal cultures is of obvious value in determining specific physiological functions and reactions, but the

applicability of these results to field situations is dubious in many respects because of the interaction between the algae and other members of the micro-environment.

Bacteria which form a numerous and diverse flora associated with most naturally occurring algal populations are of particular importance since they may utilize and modify the various organic compounds released by the algae, as well as altering the fixation rates and general physiology of the algae *per se*. Bunt (1960) noted that the non-nitrogen fixing bacteria *Caulobacter* living in the mucilage of *Anabaena* almost doubled the rate of nitrogen fixation. A similar increase is noted when *Nostoc calcicola* is cultured with nodule bacteria (Bjalfve 1962). Bacteria may also improve algal growth by providing CO_2. Lange (1970) showed that under CO_2-limiting conditions in the presence of bacteria, the growth of blue-green algae is enhanced by the addition of sucrose.

The distribution of algae and the availability to higher plants of fixed nitrogen is also affected by bacterial and viral pathogens which cause the death of blue-green algal populations (Daft and Stewart 1971, Daft, Begg and Stewart 1970) with a concomitant release of bound nitrogen (see section H6.5)

(5) Light intensity and heterotrophic growth

A number of species of blue-green algae have been shown to grow and fix nitrogen heterotrophically in the dark in the presence of a suitable substrate. *N.muscorum* for example, will grow slowly in the dark on elemental nitrogen in the presence of glucose or sucrose (Allison *et al* 1937). *Chlorogloea fritschii* fixes nitrogen with sucrose and to a lesser extent with glucose (Fay and Fogg 1962, Fay 1965) while *Anabaenopsis circulans* fixes nitrogen most efficiently in the presence of glucose and less well with fructose, sucrose and maltose (Watanabe and Yamamoto 1967).

Khoja and Whitton (1971) incubated 24 strains of algae in the dark with 0.01M sucrose, and found that 17 species grew satisfactorily. Those which fix nitrogen in the dark, albeit slowly, include *Anabaenopsis*

circulans, Nostoc commune, N. ellipsosporum, N.punctiforme and Scytonema. Anabaena cylindrica and A, variabilis both failed to grow. Tolypothrix tenuis would grow heterotrophically only in the presence of combined nitrogen. This anomaly is in agreement with the reports of Kiyohara et al (1960).

The reported rates of heterotrophic nitrogen fixation are generally low and many species appear to be obligate phototrophs. The blue-green algae as a group are considered by Fogg et al (1973) to be primarily photo-autotrophic organisms. This view is substantiated by their distribution in the soil where they are far more prolific,at least in a physiologically active state,in the upper layers.

Those distributed below the soil surface have been commonly enumerated and identified after samples have been enriched by phot-autrophic growth in a suitable medium (for example Bristol Roach 1927, Shtina 1961).

This method gives no qualitative indication of the state in which the algae occur in the soil and the quantitative information inferred from the dilution counts may be biased toward species which culture readily. Such counts are artificially high by comparison with surface estimates because of the difficulties involved in breaking down the algal filaments encountered in surface crusts (see Bristol Roach 1927). The algal filaments or colonies grown from subterranean soil samples may have come from isolated akinetes which would readily separate in the culture medium.

The contribution of heterotrophically growing algal populations to the nitrogen economy of the soil is, in any case, probably small since, as Stewart (1969) observes, the quantities of carbohydrates required for nitrogen fixation are large and there is intense competition for that which is available not only between nitrogen fixing forms, but also between nitrogen fixing and non-nitrogen fixing forms. Under these conditions it is doubtful whether there is sufficient readily oxidizable carbohydrate present in the soil to support high levels of nitrogen fixation.

Algae buried by heavy rainfall grow back to the surface quite rapidly (see section 3) and, as Lund (1967) observes, facultative heterotrophy may be of value in maintaining algal populations buried in the soil, but it is of little value in maintaining production as a whole.

(e) BIOCHEMISTRY OF ALGAL NITROGEN FIXATION

The configuration of atomic nitrogen may be represented as $1S^2$, $2S^2$, $2P^3$. There are thus five bonding electrons in the outer shell and the stability of the diatomic N_2 can be attributed to the sharing of three electrons to give a triple bonded molecule whose constituent atoms have the stable outer octet of electrons characteristic of the noble gases.

On the molecular orbital theory the triple bond is formed by the π overlap of the P_z and P_y atomic orbitals and σ overlap of the P_x orbitals to give an extremely stable unreactive molecule with a total bond energy of 225K cals. per mole.

The drastic conditions required by the available commercial methods for the fixation of N_2 make the operation of the nitrogenase enzyme all the more remarkable. The catalytic hydrogenation of N_2 in the Haber process, for example, requires a temperature of $500^{\circ}C$ and a pressure of several hundred atmospheres.

The only reaction of N_2 known to occur in the laboratory in aqueous solution under normal conditions, is the formation of transition metal-N complexes, and these are of particular significance with regard to the initial uptake of N_2 by nitrogenase. (Chatt and Richards 1969).

Nitrogenase in photosynthetic organisms.

Nitrogenase can be most effectively extracted from exponentially growing cells of nitrogen fixing organisms ruptured under anaerobic conditions in a pressure cell. The process is normally carried out under Ar, H_2 or N_2 and the pre-incubation of the sample in a nitrogen

free atmosphere often increases the yield by depleting the pool of intracellular NH_3 (Stewart 1973).

The enzyme system consists of at least two components; an iron containing protein and an iron molybdenum containing protein. The Fe protein is unstable in the absence of its complement, but the two proteins in combination are relatively stable and will, under suitable conditions, effect all the reactions associated with in vitro systems. These have been separated in a variety of ways most of which are based on protamine sulphate precipitation or chromatography using columns of DEAE cellulose or Sephadex. The fractions can be eluted from the column as brown solutions with KCL, the Fe-Mo fraction coming off first (Burris 1969). Further separation has been effected by disc gel electrophoresis (see Stewart 1973).

The estimated molecular weights and metal contents of the two proteins vary considerably, possibly because of the different separation techniques used in their preparation. Vandecasteele and Burris (1970) working with extracts of Clostridium pasteurianum suggest that 1 atom of Mo and 15 atoms of non-haem Fe are present in a Mo-Fe protein of molecular weight 160,000. Dalton and Mortenson (1970) report 2Mo and 3 non-haem Fe to be present in a unit of molecular weight 200,000.

Nitrogenase is an extremely unspecific enzyme which will reduce a wide variety of substrates in addition to N_2. These include N_2O, N_3^-, alkynes, cyanides and isocyanides. These substances are characterized by a triple bond and with the exception of alkynes all have one or more non-bonding electrons.

Cell free systems require energy, reductant and a divalent cation such as Mg^{++} although Co^{++} or Mn^{++} but not Ca^{++}, Cu^{++} or Zn^{++} may be used (Haystead and Stewart 1972).

In addition, the nitrogenase of whole organisms requires carbon skeletons for the removal of reduced products, although there is no such requirement when the enzyme is reducing substrates such as acetylene.

The energy requirement is met by ATP which must be provided, for the efficient functioning of *in vitro* systems, at a low steady state concentration. An ATP generating system such as creatine phosphate/ creatine phosphokinase is normally used. Sodium dithionite is commonly used as the reductant in cell free systems (Stewart 1973) although pyruvate has been used in earlier work as the source of both reductant and ATP (Carnahan *et.al.* 1960). Dithionite is the only reductant which is known to couple directly to nitrogenase without the intervention of an electron carrier such as ferredoxin, flavodoxin or methyl viologen. (Hardy and Burns 1968).

In photosynthetic organisms the source of *in vivo* reductant has been disputed. The direct photosynthetic provision of reductant to nitrogenase is supported by the fact that there is a general correlation between photosynthesis and nitrogen fixation and by early stochiometric studies, which suggested that the electrons generated by photosystem II directly provide the reductant for algal nitrogenase (see Stewart 1973). Cox (1966), however, using manometric measurement of N_2 uptake has subsequently shown that the stochiometric relationship breaks down in nitrogen starved cells of *Anabaena cylindrica*.

Cox and Fay (1969) note that N_2 fixation is unaffected by the addition of CMU (p-chlorophenol-1, 1-dimethyl urea) at a concentration which inhibited CO_2 fixation by more than 90% and conclude that nitrogen fixation is independent of reducing potential generated in non cyclic electron flow from photosystem II. Although this evidence does suggest a lack of direct interdependance between the two processes Lex *et al* (1972) showed that nitrogenase activity does become DCMU (dichlorophenol-1, 1-dimethyl urea) sensitive under conditions which stimulate photorespiration. These authors suggest that there is an indirect competition between photo-respiration and nitrogen fixation for the same reductant pool.

The action spectrum for nitrogenase activity suggests that photosystem I only is involved (Fay 1970) and microspectroscopic studies show that the

heterocysts lack the pigments of photosystem II. If, as other evidence suggests, the heterocysts are indeed the primary sites of nitrogen fixation, reducing power photosynthetically generated in the vegetative cells must pass into the heterocysts via an intermediate reductant. (Fogg and Than-Tun 1960, Stewart 1973).

Cox (1966) showed that nitrogen fixation is affected more by respiratory inhibitors (KCN and chloropromazone) than by photosynthetic inhibitors (CMU). Although this finding may support the view that a dark generated reductant becomes limiting, the data submitted in section 3 of this thesis suggests that such inhibition could operate via a limitation in available ATP.

Several possible sources exist for dark generated reductant. Pyruvate, used by Carnahan et al (1960) with cell free extracts of Clostridium pasteurianum was the first known electron donor for nitrogen fixation. Although the phosphoroclastic cleavage of this compound provides both reducing power and ATP its position as an electron donor for in vivo systems is now questioned on several grounds. Its molar efficiency in supporting nitrogen fixation is low. Mortenson and Sizelove (1963) working with extracts from C.pasteurianum found that as many as 100 moles of pyruvate are required for each mole of N_2 reduced, although, as Stewart (1973) observes this could be due to a limitation in ATP rather than reductant.

Smith, Noy and Evans (1971) working with extracts of A. cylindrica showed that pyruvate or isocitrate could reduce NADP which, in turn, transferred electrons to nitrogenase via ferredoxin, but observed that the rate obtained was only about 5% of that given by light generated reductant. Bothe et al (1974) found that cell free extracts from the same organism catalysed a ferredoxin dependant pyruvate decarboxylation and noted that the activity of the pyruvate: ferredoxin oxidoreductase was five times higher in material which was growing on N_2 than that of material growing on ammonia. The authors conclude that

the physiological role of the reaction is to generate ferredoxin for nitrogen fixation.

The pentose phosphate pathway would also seem to be an important possible source of reductant in blue-green algae. Evidence suggests that this pathway is operative in glucose oxidation in Tolypothrix tenuis (Cheung and Gibbs 1965) and A.cylindrica (Wildon and Rees 1965). Bothe (1971) showed that in extracts of A.cylindrica a glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, ferredoxin and ferredoxin NADP reductase system gave, in the light, about 20% of the activity associated with the intact filaments. The functioning of this system in whole cells in the light is, however, questioned by Pelroy et al (1972) who believe that the pentose phosphate pathway is switched off by ribulose diphosphate generated in the light by the Calvin cycle.

The tricarboxylic acid cycle has been shown, in many blue green algae, to lack 2-oxaloglutarate dehydrogenase and succinyl CoA synthetase but the enzymes necessary for the glyoxalate shunt are present. (see Stewart 1973) Pelroy et al (1972) found that, in the dark, cells of Aphanocapsa converted acetate to CO_2 and cellular material, presumably via the tricarboxylic acid cycle, at a rate which represented only about 1% of that when glucose was utilized via the pentose phosphate pathway. Since these authors believe that the pentose phosphate pathway shuts down in the light, it is possible that the role of the tricarboxylic acid cycle is larger under these circumstances. Stewart (1973) observes that its role, may, in any case, be important in providing carbon skeletons, possibly 2-oxoglutarate, for the uptake of newly fixed ammonia.

The energy requirement of nitrogen fixation by blue-green algae is met by ATP produced primarily by oxidative and photosynthetic phosphorylation. Little or no contribution from oxidative phosphorylation can occur in photosynthetic bacteria which fix N_2 only under anaerobic conditions.

The involvement of photosynthetic ATP production in the nitrogenase activity of blue-green algae is supported by the correlation between photosystem I and nitrogen fixation (Fay 1970). Cox and Fay (1969)

observe that even in the presence of CMU, under nitrogen starved conditions where large quantities of substrate are available, there is an increase in acetylene production with increasing light intensity.

Oxidative phosphorylation is implicated in the fixation process since those algae which fix N_2 in the dark do so at an appreciable rate only under aerobic conditions (See Section 3c). The results obtained in this thesis suggest that oxidative phosphorylation also plays an important role in the light by producing ATP in addition to that produced by photosynthetic phosphorylation.

The energy provisions for nitrogen fixation in cells of A.cylindrica and N. ellipsosporum are discussed further in section 3.

(f) ALGAL NITROGEN FIXATION IN RICE CULTIVATION

Watanabe and Yamamoto (1971) observe that the wet, warm and often alkaline conditions which are prevalent in paddy soils for a large part of the season offer an ideal environment for blue-green algae which can develop under these conditions in prodigious numbers.

The exploitation of atmospheric nitrogen fixed by algae under these circumstances has attracted a great deal of interest, particularly since the rice crops which benefit from algalization provide the staple diet for a large proportion of the worlds population which is often economically unable to obtain the quantities of artifical nitrogenous fertilizer used in western agriculture.

The earliest observations on the beneficial effects of algae on rice yield were those of De (1939) who showed that the nitrogen required for rice harvests, which were obtained from the same plots year after year without the addition of fertilizer, was derived from the algae which grew around the roots of the crop. A considerable amount of subsequent work has been carried out in India (see Singh1961), Japan (see Watanabe 1966), Russia (see Mishustin and Shil'nikova 1971) and the Philippines (see MacRae and Castro 1967).

The algal species which contribute to nitrogen fixation vary depending on the conditions. Singh (1961) considers that the most important single

contributor in Indian rice fields is Aulosira fertilissima which is both abundant and efficient as a nitrogen fixer. Watanabe and Yamamoto (1971), who have used Tolypothrix tenuis in large numbers of field experiments in Japan, consider this to be the most effective nitrogen fixer, while Shang Hao is cited by Mishustin and Shli'nikova (1971) as finding Anabaena azotica to be the most important species under Chinese paddy conditions.

Several authors also note that species succession occurs during the growing season. Mishustin and Shli'nikova (1971) observe, in certain Russian paddy soils, an early development of green algae such as Spirogyra, Zygnema and Oedogonium, followed, as the plant canopy closed over, by blue-greens which appeared to flourish despite almost complete shading. Singh (1961) who notes a similar succession in Indian paddy soils, further subdivides the emmergent species of blue-green algae. Anabaena appears to develop first followed by Cylindrospermum, Tolypothrix and Fischerella. These are then superceded by an abundant growth of Aulosira.

The effect of inoculating rice crops with various species of algae has been investigated by a number of workers although Mishustin and Shil'nikova (1971) and Veukataraman (1973) point out that this may not be necessary under all circumstances because of an abundant natural flora. It is, however, possible for algal inoculations to have a beneficial effect even in the presence of a large indigenous flora, both by biasing the population towards an effective nitrogen fixing species, and by producing an algal bloom earlier in the growing season.

Watanabe (1962), reviewing much of the work done in Japan, estimated that the average increase due to blue-green algae was between 20 and 25 Kg N/ha. The exact benefit produced appears to be dependant on the soil type, the chemical composition of the soil water and the fertilizer used, as well as the algal species and climate. De and Mandal (1956) estimated that the fixation in a range of unfertilized Indian soils varied between 13.8 and 44.4 lb N / acre (16.7 to 53.7 Kg N/ha).

The effectiveness of algal inoculation appears to increase in successive years of application. Watanabe (1962) investigating the effect of inoculation with Tolypothrix tenuis on about 40 rice fields located in various parts of Japan observed a mean increase of crop yield in the first year of 2%, in the second year 8%, in the third year 15.1% and in the fourth year 10.6%.

A similar increase in the second year was noted by Iha et al (1965) who also noted that the time of application is important. Tolypothrix tenuis was more effective in the first year of inoculation when it was applied 20 days before the crop was sown, rather than at the time of sowing. The time of application appeared to have little effect in the second year.

These authors also found that the addition of phosphate was beneficial. The rice yield in the first year was increased from 3070 Kg/ha to 3614 Kg/ha by the addition of 40 Kg/ha phosphate whereas similar additions to uninoculated control plots reduced the yield.

De and Mandal (1956) note an increase in fixation from 48.8 Kg N/ha to 69.0 Kg N/ha associated with the addition of phosphate and observe that the fixation could be increased still further, to 77 Kg N/ha, by the addition of small quantities of Mo, a growth requirement specific to nitrogen fixing organisms.

Estimated fixation values are greater in the presence of a rice crop than in its absence. De and Mandal (1956) consider that this is due to the algal utilization of CO_2 produced by the plant root systems.

The levels of crop sustained by algae alone may be relatively low and although these yields can be improved by the addition of organic fertilizer, phosphates and trace elements, it may be desirable to increase it still further by the addition of supplementary inorganic nitrogen. Subramanyan et al (1964) investigating the effect of Tolypothrix tenuis on rice yield found that the increase associated with the algae did not become manifest against the background nitrogen level caused by the addition of 20 Kg / ha N as

ammonium sulphate, although a 30% improvement over the control values were produced in the absence of added N. Venkataruman (1972 and 1973), however, noted an increase in crop yield of about 10% due to algalization even in the presence of high levels of soluble nitrogen and suggested that the improvement in the crop under these circumstances may be due to the production of growth substances and vitamins rather than soluble nitrogen.

(g) ALGAL NITROGEN FIXATION IN TEMPERATE SOILS

Although the role of blue-green algae in the nitrogen economy of a wide range of different ecosystems has been investigated, information about their effect on temperate soils has been scarce despite the fact that their potential in such habitats was first noted by Frank as early as 1889.

The blue-green algae subsequently found in temperate soils (for example Bristol Roach 1927, John 1942, Lund 1947) were presumably physiologically able to fix N_2 since the heterocysts used to identify many of the species are normally indicative of active nitrogen fixation (Stewart 1966, 1969) although Stokes (1940) and later Beck (1968) suggested that the quantitative importance of algal nitrogen fixation is small.

A number of investigators have reported the occurrence of nitrogen fixing algae in Russian soils. Shtina (1961) and Gollerbakh and Shtina (1969) report that potential nitrogen fixing species are often abundant in soddy podsol although they are less important in arable soils. Prykhod'kova (1969, 1971) carried out a study of nitrogen fixing algae in the steppe regions of the Ukraine and reported that blue-green algae were abundant in 40 out of the 50 soil samples examined. The most commonly occurring nitrogen fixing forms were N.commune, N.punctiforme and Calothrix elenkinii.

The first systematic study of the nitrogen fixing potential in temperate regions was that of Granhall and Henriksson (1969) who studied the occurrence of heterocystous nitrogen fixing algae in Swedish soils. They

concluded that nitrogen fixing forms were abundant in most soils which were moist, low in combined nitrogen and very slightly acid or alkaline. Nitrogen fixing species were found in all soil samples with a pH in excess of 8.0 and 76% of soil samples with a pH between 7.0 and 8.0.

Henriksson (1969) subsequently investigated the in situ fixation in more than 1000 soil samples from various localities using the acetylene reduction technique. Fixation was monitored at three sites from April until October in order to obtain estimates of annual fixation. The estimated nitrogen fixation at all three sites continued at a rate in excess of 10 $ngN/cm^2/hr.$ throughout the season irrespective of changes in temperature and light intensity. The highest fixation recorded was 3300 $ngN/cm^2/hr.$ produced by samples from the bottom of puddles in a clay field. The estimated annual fixation rates varied from 0.4 to 5.1 $gN_2/m^2/year$ and the author concludes that blue-green algae can contribute to the nitrogen economy of temperate soils. Although these results are in general agreement with those described in this thesis, there is a significant point of difference. The fixation values are reported by Henriksson as remaining relatively constant despite changes in temperature (9-35°C) and the decrease in light intensity during the short Swedish nights. However, the work described here (section 4) shows that the fixation rates vary substantially with both factors.

(h) AIMS OF INVESTIGATION

The present study was carried out in an attempt to evaluate the contribution of algal nitrogen fixation to temperate agricultural soils in this country. Little or no previous work has been done on this topic.

The investigation involves both monitoring the seasonal nitrogenase activity in the field under various conditions, and the laboratory studies of physiological factors encountered in the field which might affect algal growth, nitrogen fixation and nitrogen release. The field work, carried out at Rothamsted Experimental Station, was of two types. First inoculation

of various algal species onto suitably arranged test plots sown to winter wheat and the subsequent estimation of nitrogen fixation and crop yield; and secondly the seasonal monitoring of nitrogenase activity over a two year period under wheat.

In the latter investigations Broadbalk, one of the Rothamsted classic plots, was uniquely useful. Parts of this field which have been sown to wheat since 1843 provide a series of plots of similar soil type and drainage, with fertilizer treatments which vary from nothing at all through a number of combinations of N, P and K to farmyard manure. Each plot has received the same fertilizer treatment for 130 years, and the record of crop yields are available over this period.

SECTION 2 MATERIALS AND METHODS

A. ISOLATION AND PURIFICATION OF ALGAE FROM THE FIELD.

B. CULTURE OF ALGAE IN THE LABORATORY.

1) Liquid Cultures.

2) Algal Cultures on Solid Surfaces.

C. ESTIMATION OF NITROGENASE ACTIVITY

1) Introduction

2) The Acetylene Reduction Technique for Assaying Nitrogenase Activity.

3) The Statim Block and Fibre Optic Technique

4) The Estimation of Nitrogen Fixation in the Field.

2. MATERIALS AND METHODS

A. ISOLATION AND PURIFICATION OF ALGAE FROM THE FIELD.

A number of methods exist for the isolation and purification of blue-green algae. These include the use of antibiotics (Droop 1967, Goldweig-Shelubsky 1951, Reich and Khan 1964, Spencer 1952, Tchan and Gould 1961, Pintner and Provasali 1958 and Kiyohara 1963), ultra violet irradiation (Allen 1952, Geroloff, Fitzgerald and Skoog 1950 and Watanabe 1959), growth at relatively high temperatures (Wierringa 1968 and Allen and Stanier 1968), micromanipulation (Pringsheim 1946, Weidman, Walne and Trainor 1964), ultrasonication and repeated dilution (Brown and Bischoff 1962) and phototactic growth through nitrogen free agar (Bunt 1961a, Van Baalen, 1962). Several of these techniques were tried but the simplest and most effective was found to be ultrasonication followed by micromanipulation. The procedure used was basically similar to that of Pringsheim (1946).

The first stage in purification involved the production of an enriched culture. Approximately 1 sq. cm of the surface soil crust was mixed with a few drops of N-free culture medium (Allen and Arnon 1955) in a test tube using a glass rod and the resulting paste suspended in 25 ml of culture medium. A series of dilutions were prepared from this suspension and these were used to inoculate N-free agar plates (Bunt 1961a). These plates were incubated at 1800 lux and room temperature for about 10 days. Suitable discrete colonies were then removed from the agar surface and used to inoculate 25 ml aliquots of N-free medium in small sterile conical flasks. After 20 days growth in a shaking incubator some of these flasks contained a healthily growing culture which was, in many cases, ~~uni-algal~~. of a single algal species.

Axenic cultures were produced from these enriched cultures by micromanipulation. Firstly the culture was diluted by a factor of about 20, and then ultrasonicated to break the filaments down to 2-4 cell units. A drop of this material was placed on a slide under a phase contrast microscope and a single group of vegetative cells was transferred with a micropipette to a drop of sterile medium on the same slide. This

procedure was repeated six times before the cells were transferred to sterile slopes in universal bottles. In about 90% of the attempts the cells were lost during transfer, but of those that were successfully transferred just under a quarter produced axenic cultures.

The cultures isolated by the author were identified by Mr A. E. George of the Cambridge culture collection and several cultures were purchased from this source.

B. CULTURE OF ALGAE IN THE LABORATORY

1) Liquid Cultures

a) Culture media

The culture medium described by Allen and Arnon (1955) was used for most of the liquid cultures. The media described by Chu (1942) Bunt (1961) and Henriksson (1951) were also used in various experiments (see appendix).

b) Small volumes

(i) Shake cultures.

Small volumes of culture, such as those used as pilot cultures when transferring from agar slopes into liquid culture, were grown in 100 and 250 ml conical flasks attached to a flat bed orbital shaker at room temperature. The flasks were stoppered with cotton wool.

(ii) Bubble cultures.

Routine bubble cultures for experiments were grown in $2\frac{1}{2}$l clear glass Winchesters. The air was filtered through charcoal and passed through rubber tubing which was tapped by inserting a hypodermic needle (see Fig. 2.1). The air from the supply was generally maintained at a constant pressure and the flow to each of the cultures adjusted by changing the size of the hypodermic needle. Axenic cultures may be maintained by inserting a millipore filter into the air line close to the culture vessel. The cultures were illuminated by a variable number of Dayglo fluorescent tubes and routinely diluted in order to maintain a healthily growing log. phase culture. It is of interest that in cultures of N. ellipsosporum and to a lesser extent A. cylindrica, excessive dilution caused an apparent change in growth habit.

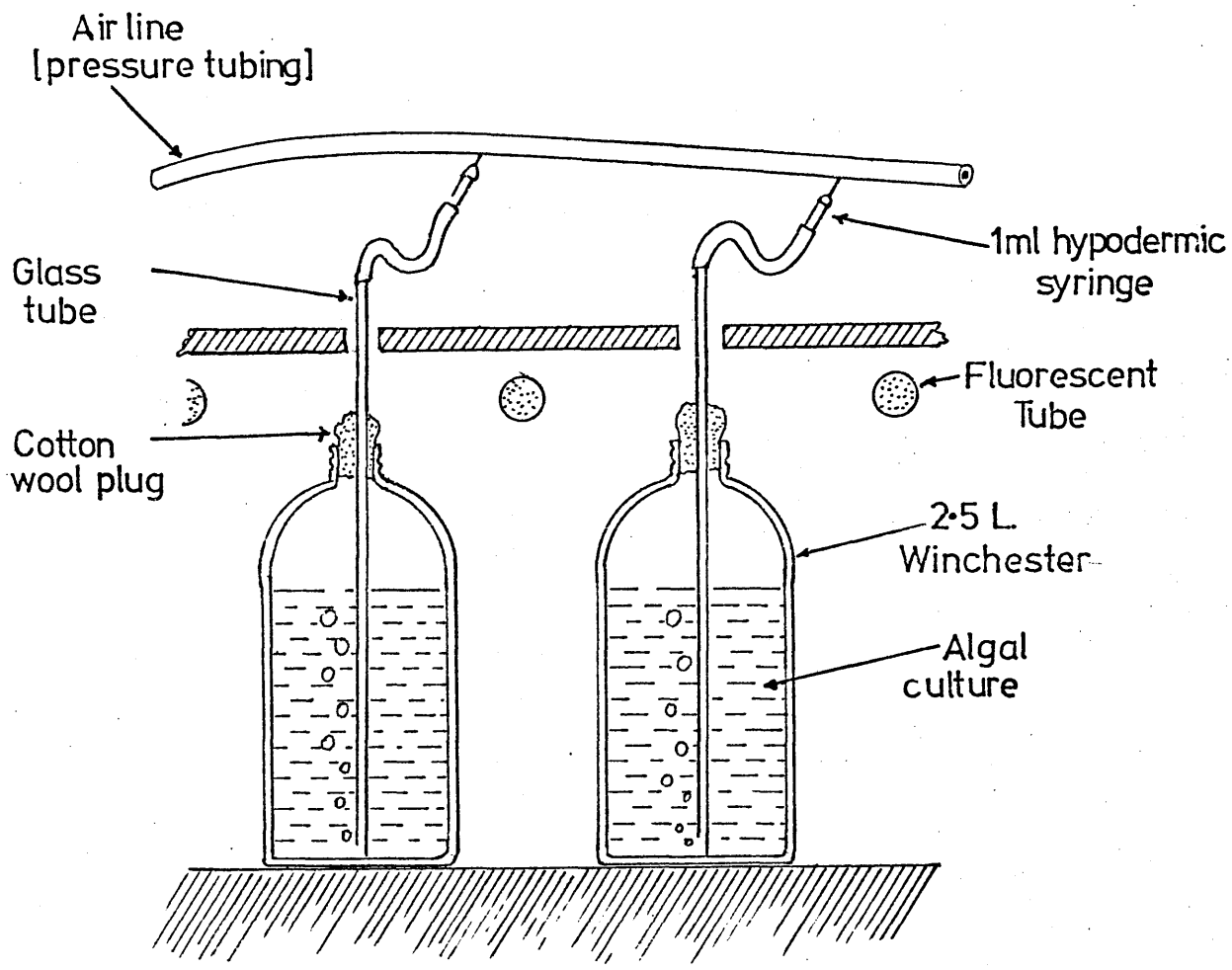


Fig 2·1 Apparatus used for maintaining experimental culture material.

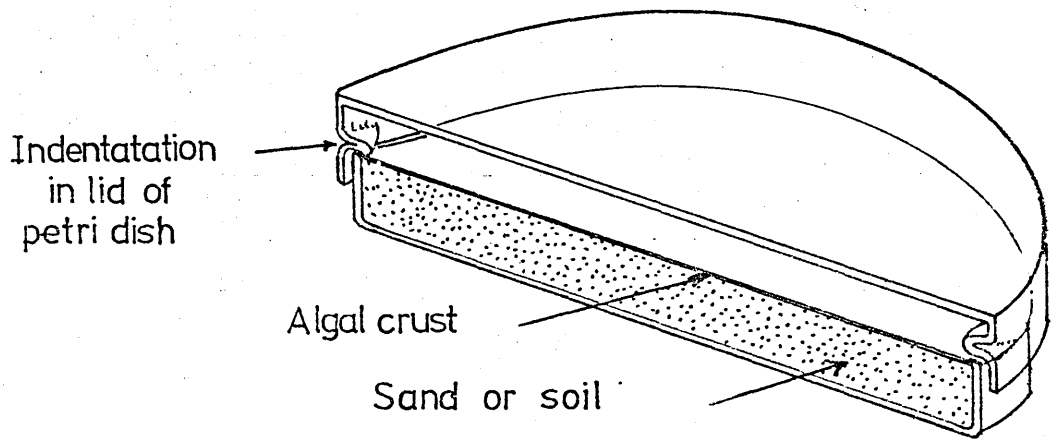


Fig 2·2 stereogram showing petri dish modified for growing algae on solid surfaces

The algae, instead of growing in the bulk of liquid medium, tended to clump together and stick to the walls of the culture vessel giving, after several days, a completely clear solution.

This effect was also noted at low light intensities and it would seem probable that it was caused by some factor released by the cells into the culture medium.

(iii) Large volumes of culture for the inoculation of field plots.

Large volumes of algae were grown in bubble cultures using the 9l glass columns containing 7l of culture shown in Figs. 2.3 and 2.4. For axenic cultures a millipore filter was inserted in the butyl rubber tube and the entire column autoclaved.

For field inoculations requiring large volumes of culture, the algae can be conveniently stored in 40l glass aspirators with a bubbled air supply. Cultures under these conditions remained healthy for 3-4 weeks but growth was extremely slow because the light intensity is limited by the depth of culture.

2. Algal Cultures on Solid Surfaces

a) Agar slopes

N-free agar slopes in universal bottles were used for the maintainance of stock algal cultures. These were kept at room temperature and 900 Lux. The caps were loosened and re-tightened every week to allow gas exchange and subcultured every 3-4 months.

The slopes were prepared by mixing Oxoid No. 1 agar (12g/L) with the medium of Allen and Arnon (1955).

b) Sand cultures

Acid washed quartz sand was rinsed in distilled water and steam sterilized. In order to obtain an even cover a flat surface is necessary. This can be readily achieved in a petri dish having a modified lid, by over filling the base and then scraping off the excess (Fig. 2.2).

Larger cultures were grown in 12" x 18" x 2" enamelled surgical trays covered by a glass plate. The sand was moistened with N-free

Fig 2·3 One of a bank of culture vessels used for producing large volumes of inoculum for field experiments

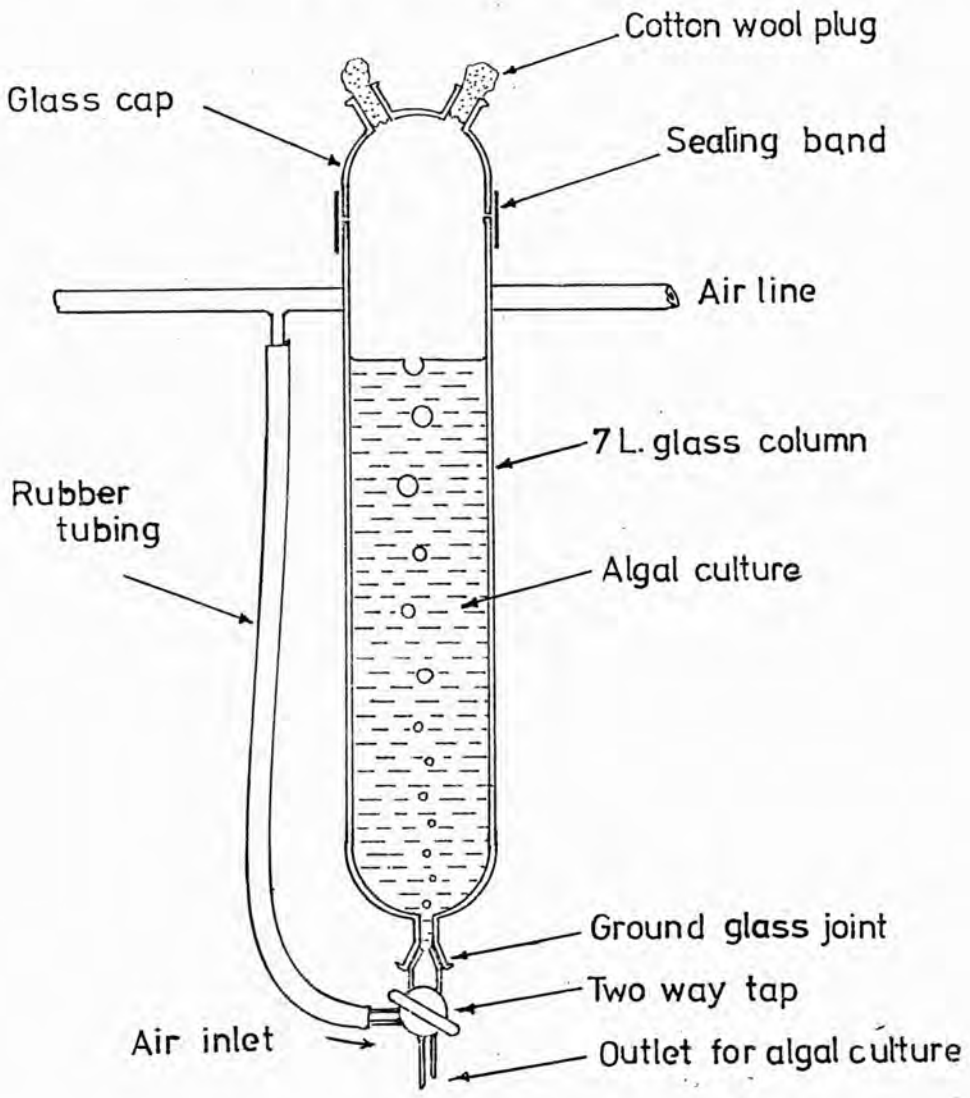


Fig 2·4 ; Empty culture vessels

culture medium and spray-inoculated with the desired algal spp.

Various methods were tried for producing an even inoculum on solid surfaces. A Golden Products super dispenser (B.P. Ltd., 1, Golden Drive, Henlow, Beds) was found to be convenient. This delivered a fixed volume of algae through an adjustable nozzle. A BDH ninhydrin spray is also useful.

Before spraying, the algae were filtered through 1.5 mm glass beads to remove the larger clumps.

c) Soil cultures

Soil from the Rothamsted soil mine at Woburn was used because of its low soluble-N content.

The soil was sieved (2mm mesh sieve) and 8% $CaCO_3$ was added to raise the pH from 6.2 to 7.5. N-free culture medium was added to form a paste which was spread evenly in a petri dish or culture tray and spray inoculated. Excessive wetting during inoculation, causing local concentrations of algae, was avoided by partially drying the tray before treatment. Using this method a dense even cover of algae can be obtained in 10-14 days (see Fig. 2.5). The available methods of sterilizing soil tend to produce a large increase in free N caused by the solubilization of bound N and the lysis of living organisms in the soil. If, for example, Woburn soil is autoclaved the NH_4^+ - N concentration in soil water rises from 5 ppm to over 100 ppm. Aseptic leaching of the soil with distilled water or cation exchange material was ineffective in removing this N. The soil used in all experiments was unsterilized.

The experimental conditions which prevail with respect to the soil flora and fauna were considered to be similar to those occurring in the field.

C. ESTIMATION OF NITROGENASE ACTIVITY

(1) Introduction

Four principal methods exist for the estimation of biological nitrogen

Fig 2.5, N ellipsosporum after 10 days growth on soil.

fixation. These are:

a) Measurement of increase in total nitrogen within the system under consideration, normally by Kjeldahl digestion and analysis for ammonia. This method has the advantage that it measures directly the absolute increase in nitrogen, but it is extremely slow since a relatively long growth period must be allowed, and it cannot be applied to situations where there is already a high (insoluble) background nitrogen level, such as is found in clay soils. It does not therefore lend itself easily to in situ field studies.

b) The measurements of ^{13}N uptake. This method is sensitive enough to allow short incubation periods but the half life of ^{13}N is extremely short (10 mins.) and this normally precludes the use of the technique in the field, or, indeed, in laboratories which do not have a ready access to the isotope.

c) Measurement of ^{15}N uptake. ^{15}N is more convenient than ^{13}N in that it is a stable non-radioactive isotope which can be used for field investigations (Stewart 1967a, 1967b). This method is, however, time consuming, expensive and requires the use of a mass spectrometer.

d) The Acetylene reduction assay. This technique is quick, sensitive and inexpensive, and lends itself easily to field investigations. Its principal disadvantage lies in the fact that it is an indirect assay procedure which must be carefully checked for each experimental situation against the results from one of the direct techniques previously described.

2. The acetylene reduction technique for assaying Nitrogenase activity

(a) Theoretical

Shöllhorn and Burris (1966) noted that acetylene inhibited nitrogen fixation and was reduced to an unknown product. Dilworth (1966) showed that this product was ethylene and that it was reduced at the same enzymic site as N_2.

These observations led to the use of this reaction as an assay for nitrogen-fixing potential in root nodules (Koch and Evans, 1966)

nodule extracts (Koch et al 1967) blue-green algae (Stewart et al 1968) and a variety of field and aquatic habitats (Stewart et al 1967; Hardy et al 1968). The assay is based on a similarity in the rate of electron activation and transfer to either C_2H_2 or N_2,

Assuming that $N_2 + 6H^+ \xrightarrow{6e^-} 2NH_3$ and that $C_2H_2 + 2H^+ \xrightarrow{2e^-} C_2H_4$

a theoretical ratio of $1N_2 = 3C_2H_2$ or $1.5C_2H_4 = 1NH_3$ is obtained. This theoretical ratio is seldom obtained and estimates of the ratio vary considerably. Shollhorn and Burris (1967) report ratios close to 1.25 for Clostidium and Azobacter while Klucas (1967) reports an average ratio 1.59 for C_2H_4 produced to NH_3 formed by Azobacter vinelandii. Stewart et al (1968) calculated the ratio for three species of blue-green algae using the ^{15}N technique. The ratios were: Nostoc muscorum 1.8, Anabaena cylindrica 1.4, Anabaena flosaquae 1.6.

The reduction of acetylene is competitively inhibited by N_2 but the affinity of N-ase for C_2H_4 is much greater than its affinity for N_2. The Km for C_2H_2 reduction by N-ase is in the range 0.004 - 0.01 Atm. both in vivo and in vitro (Hardy and Burns 1968, Hardy and Knight 1967, Postgate 1971). The author has shown that, provided the acetylene concentration is maintained above about 5%, the presence of atmospheric N_2 has no effect on the assay and the sample can be incubated in air with added acetylene.

Although the mechanism for C_2H_2 reduction is similar to the initial mechanism of N_2 fixation, the requirements are not identical. For N_2 fixation in vivo N-ase requires ATP, reducing power, and carbon skeletons to mop up the reduced products. The reduction of C_2H_2 does not require C skeletons since the C_2H_4 produced is immediately released by the cell. The acetylene reduction assay has been used extensively in both the laboratory and the field experiments described in this thesis. A wide variety of methods exist for estimating N-ase activity in liquid cultures but little work has been carried out on solid surfaces.

Several new pieces of apparatus have been evolved for the quantitative estimation of N-ase activity in material growing on solid surfaces under controlled conditions in the laboratory, and in the field.

In general terms the nitrogenase assay requires that the test material be incubated in a sealed container under an atmosphere containing 10% C_2H_2 and that, after the desired incubation time (usually 30 mins or 1 hour), the gas phase be analysed for C_2H_4 content.

(b) Gas sampling and analysis

Replicate $\frac{1}{2}$ml sub-samples were taken from the incubation chamber with a 1ml disposable plastic syringe fitted with a 25g needle. These samples can be conveniently stored prior to analysis for periods of up to 24 hours by stabbing the needles into a rubber bung held in a suitably arranged rack.

The C_2H_4 was measured using a Pye 104 gas chromatogram furnished with an H_2/Air flame ionization detector. A 2m glass column packed with 80-100 mesh Rropak N was used at 80°C with N_2 carrier gas flow rate of about 30ml/min. The linearity of the flame ionization detector and amplifier response to C_2H_4 was checked using a continuous dilution method. The response was linear over the range of concentrations encountered in the experiments. The response does change over a period of time due to dirt accumulations on the detector etc., and the instrument was re-calibrated before each batch of analyses using a BOC special gas mix of 100 ppm ethylene in Argon. The BOC welding grade acetylene used for the assays contains small quantities of C_2H_4 as well as methane, phosphine and acetone. The quantities of these contaminants vary from cylinder to cylinder and between samples taken from the same cylinder. In all laboratory experiments the aliquots of acetylene to be injected into each sample were not taken straight from the cylinder but rather from a homogenous volume of acetylene in a football bladder. A short length of rubber pressure tubing was fitted to the neck of the bladder so that gas could be removed with a hypodermic syringe.

In all experiments, allowance was made for the contaminating ethylene by either:-

1) using a blank sample containing acetylene but no algal material.

This sample was incubated with the others and background C_2H_4 measured and subtracted from experimental values.
Or 2) taking two successive readings from each sample and subtracting one from the other. The ethylene concentration after 5 minutes, for example, could be subtracted from the concentration after 1 hour 5 minutes and the result expressed as C_2H_4 production per hour.

The plastic syringes can be re-used after the contaminating ethylene and acetylene have been removed. This can be conveniently achieved by placing them in a vacuum dessicator and alternately evacuating and flushing with air.

(3) The statim block and fibre optic technique

Laboratory measurements of nitrogen fixation with material growing on solid surfaces required controlled temperature and light intensity. Initial experiments were carried out in universal bottles or medical flats capped with subaseals. The required volume of C_2H_2 was injected into the bottle after the appropriate volume of air had been removed, and the bottles were incubated in a water bath. Illumination was provided by fluorescent tubes, photofloods, or a quartz halogen lamp. Although this technique is satisfactory for some experiments, it is inconvenient if large numbers of replicates are required and accurate measurement of light intensity is difficult.

(a) Apparatus

In order to overcome these difficulties the apparatus shown in Figs. 2.6, 2.7 and 2.8 was developed. Light is carried through fibre optic bundles to algal cores to give a known light intensity in a controlled temperature statim block. The fibre optics used (Rank Precision Industries Ltd.) were 1m long with a 6mm dia. light transmitting core (overall diameter = 10mm); they transmit about 80% of the visable light but act as an effective I.R. filter. The spectral adsorbtion curve is shown in Fig. 2.9.

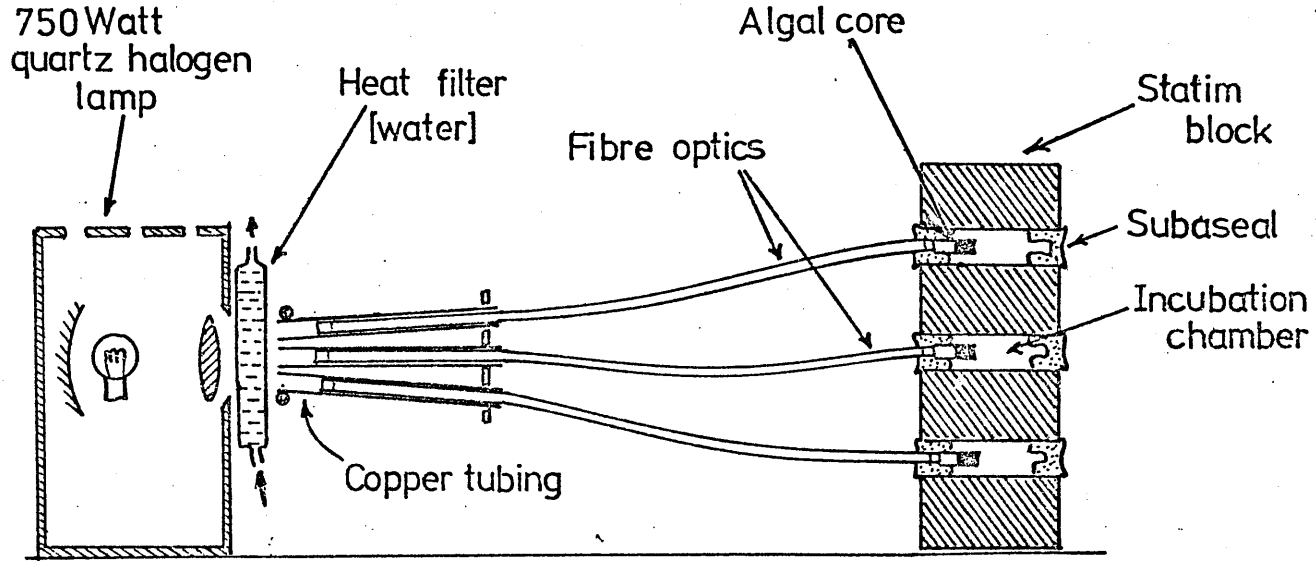


Fig 2·6 Diagram showing the arrangement of apparatus used in fibre optic experiments

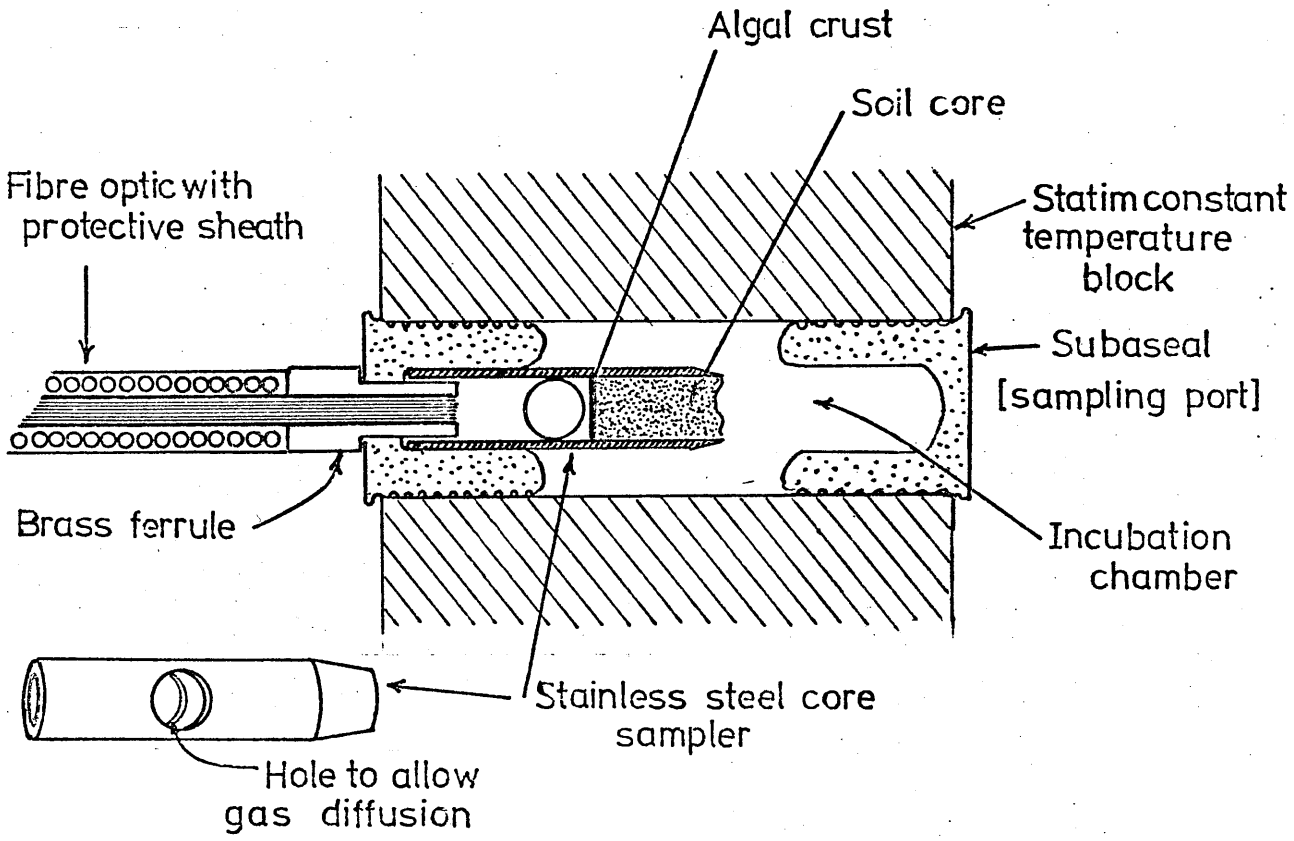


Fig 2·7 Details of incubation chamber and core sampler

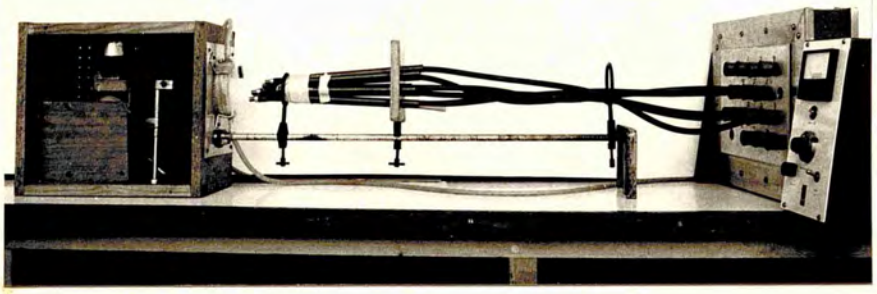
Fig. 2.8 Statim block and fibre optic technique.

(A) Assembled apparatus.

(B) Removal of a core from a sand plate of N.ellipsosporum.

(C) Measurement of light intensity. Fibre optic and photocell.

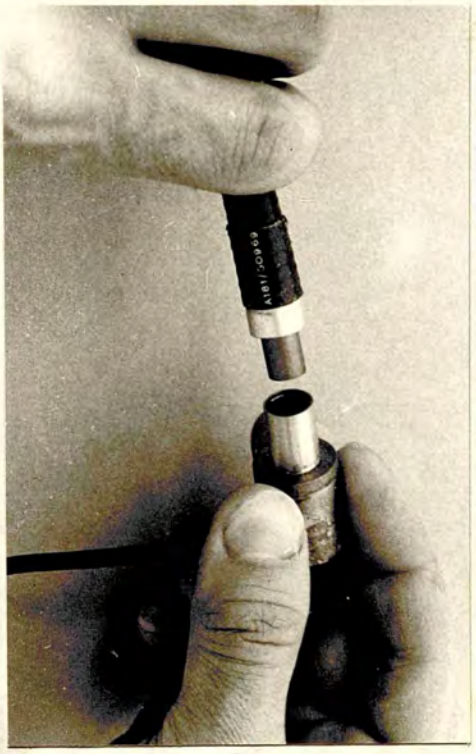
(D) Core samples, subaseal and fibre optic assembled for insertion into the statim block.



(A)

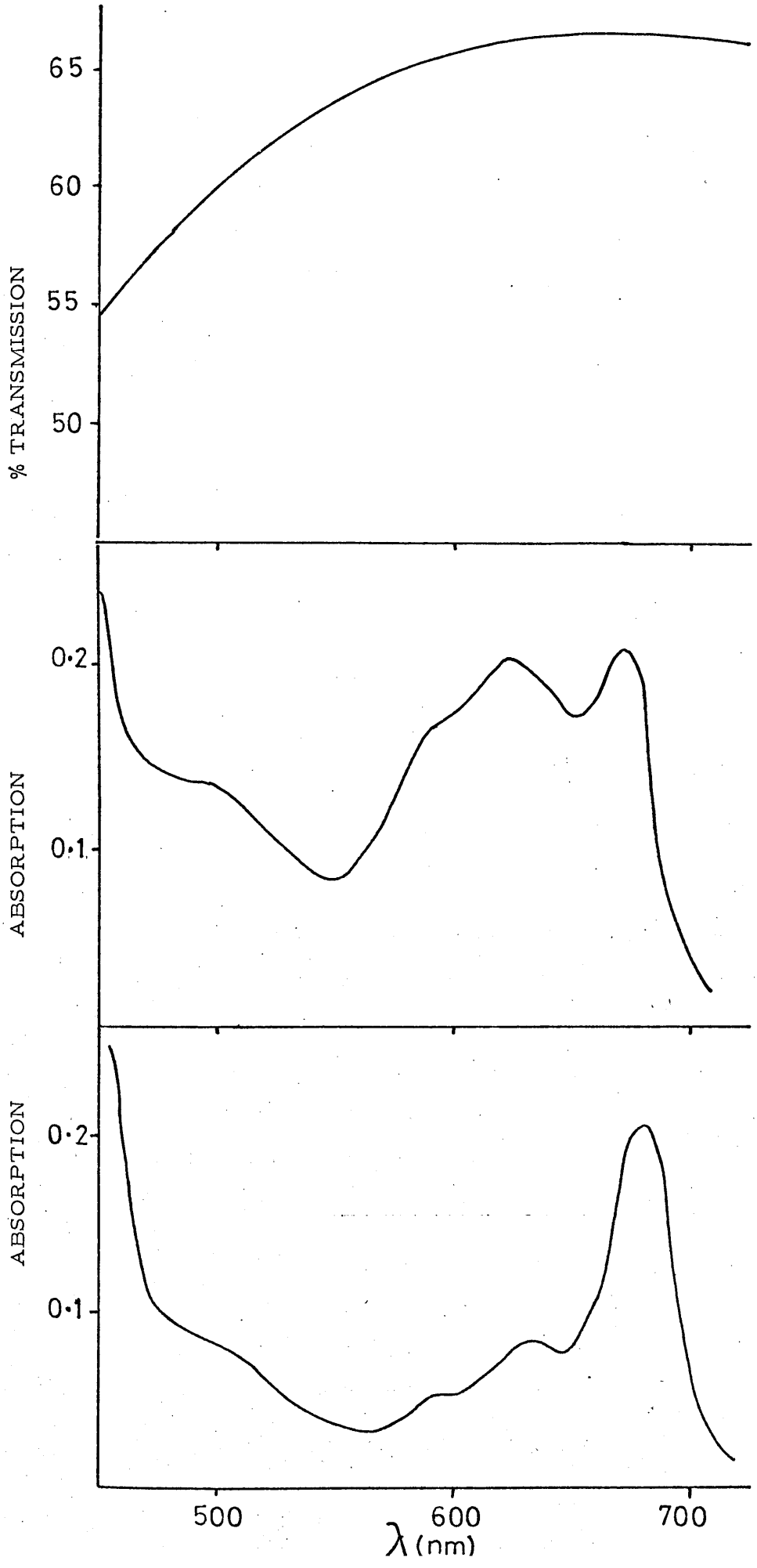
(B)

(C)



(D)

FIG 2.9 Spectral transmission curve of fibre optic and absorption spectra of Anabaena cylindrica



(A) Spectral transmission curve of fibre optic. (Redrawn from fibrox test specification MD 690)

(B) Absorption spectrum of intact filaments of Anabaena cylindrica (Redrawn from Fay 1970)

(C) Absorption spectrum of isolated heterocysts of Anabaena cylindrica (Redrawn from Fay 1970)

Light was provided by a modified microscope lamp furnished with a 750 watt quartz halogen overhead projector bulb and cooling fan. The beam was focussed by a concave mirror and adjustable lens through a heat filter onto the ends of the fibre optics. Water flowing continuously through the heat filter removes sufficient of the I.R. to prevent the fibre optics from becoming excessively hot.

The intensity of illumination passing through the fibre optics was adjusted by moving them nearer to or further away from the light source. The transmitted intensity was measured by plugging the output end of the optic into a specially designed lightmeter (Fig. 2.8,C). The effective area of the photocell was the same size as an algal disc and the same distance from the fibre optic.

The photocell, which was used with neutral density filters, was calibrated by comparison with a EEL Lightmaster lightmeter, which was re-calibrated by the makers every 6 months. A group of fibre optics, held in the light beam by $\frac{1}{2}$" I.D. copper tubes, were individually adjusted to give equal out-put intensities.

Gross changes in intensity were accomplished by moving the copper tubes and light guides to and fro as a unit, or for very low intensities by changing the light source.

The statim block (Scienco-Weston Ltd), which was designed for the purpose, was basically a 15" x 10" x 4" block of copper/aluminium alloy with a high thermal conductivity surrounded by a thermostatically controlled heating element. 25 holes 2cm in diameter were drilled through the block to act as incubation chambers. The temperature of the block could be maintained to within $0.1C^{o}$. For temperatures below ambient coolant was circulated through the vacant chambers.

(b) Method of operation

The light intensity was adjusted to the desired value and the block thermostat set to the appropriate temperature. The temperature in two vacant incubation chambers was monitored with mercury thermometers during experiments.

Discs of test material were taken from the surface of a uniform algal plate by pushing the sharpened end of the 0.84cm diameter stainless steel core sampler into the soil or sand (see Fig. 2.8B). The core was adjusted so that the algal crust was level with the transverse hole through the sampler. This ensured that all the crusts were an equal distance from the end of the fibre optic.

The brass ferrule on the end of the fibre optic was pushed through a drilled subaseal and located in a fixed position in the core sampler and the whole unit pushed into the statim block.

The other end of the incubation chamber was plugged with a subaseal and vented to the outside for a few moments with a hypodermic needle to allow the chamber to equilibrate to atmospheric pressure. Fifteen minutes were allowed for temperature equilibration.

At the start of the experiment 3mls of air were withdrawn from each chamber with a hypodermic syringe and replaced with 3mls of acetylene. The incubation chambers had an approximate volume of 30mls, the exact volume of each chamber was inferred from the concentration of internal standard (either acetylene or propane).

After the desired incubation period, replicate $\frac{1}{2}$ml gas samples were taken and analysed in the usual way.

4. The Estimation of Nitrogen Fixation in the Field

a. Introduction.

The acetylene reduction assay for nitrogenase activity has been applied to a number of field situations, (for example Stewart, Fitzgerald and Burris 1967, Henriksson 1969, Waughman 1971). These assays have normally involved the incubation of small amounts of test material in a sealed bottle under an atmosphere of $Ar/CO_2/O_2$ with 10% acetylene.

Although small, replicate sub-samples may provide a reasonable estimate of overall fixation in a relatively homogenous situation such as lake water, where the "clump" size is small compared with the sample size, the author found that this technique did not work well on the temperate, agricultural soils investigated. The distribution of algae in this habitat is discontinuous with a "clump" size which varies from a few millimetres to over a metre across, and an impossibly large number of cores must be taken to obtain a reliable mean value. Furthermore the physical removal of large numbers of core samples from a test plot over the course of a growing season leads to severe disruption of the soil surface, with a consequent decrease in algal cover.

b. The Incubation Chamber.

The apparatus shown in Fig. 2.10 and Plate 2.12 was designed so that the nitrogenase activity of a relatively large area of soil surface could be assayed *in situ*. It comprises a steel tube 6" in diameter sharpened at the bottom so that it can be driven into the ground. A gas tight incubation chamber is formed by sealing a transparent 1/8" thick perspex lid to the top of the tube. Several sealants were tried, but the best was found to be a steel casement putty such as Arboseal. This material, which makes a reliable gas seal, does not harden, and

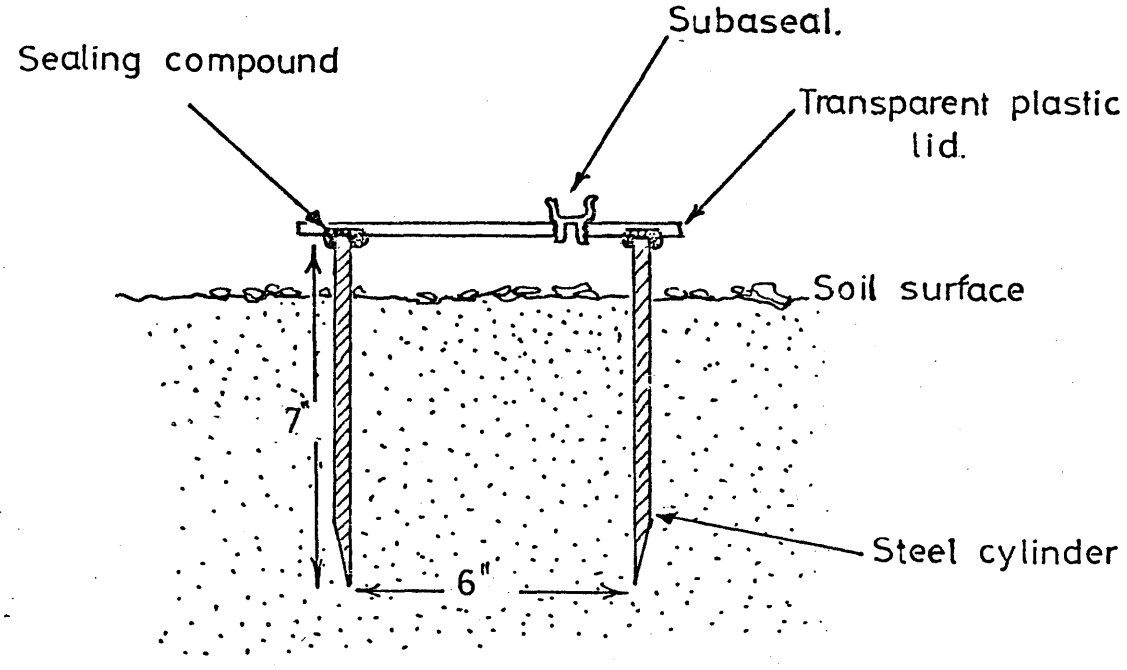


Fig 2·10 Sectional view of field incubation chamber

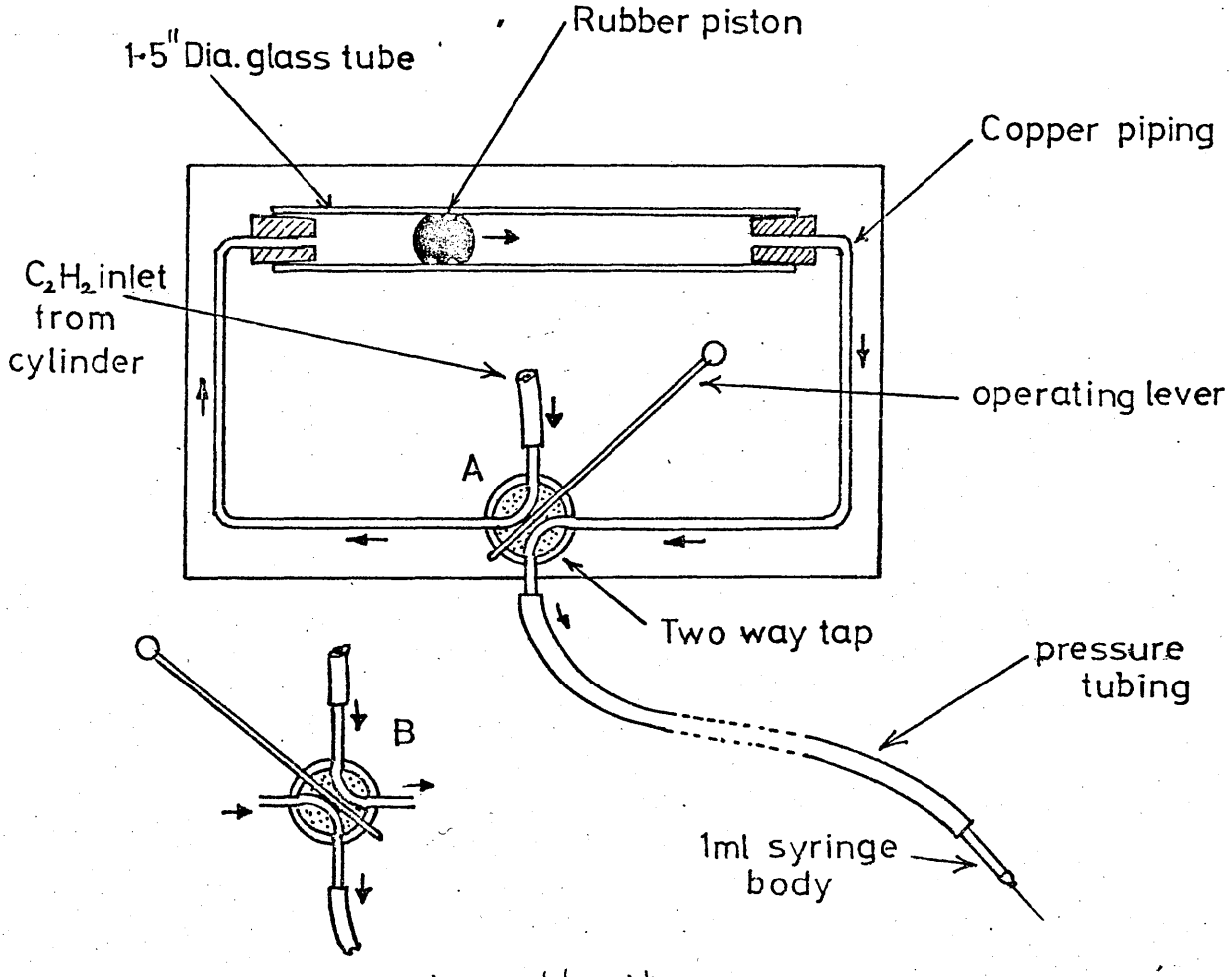


Fig 2·11 Apparatus for ~~acetylating~~ injecting acetylene into incubation chambers.

Fig. 2.12 Field incubation chamber and perspex lid.

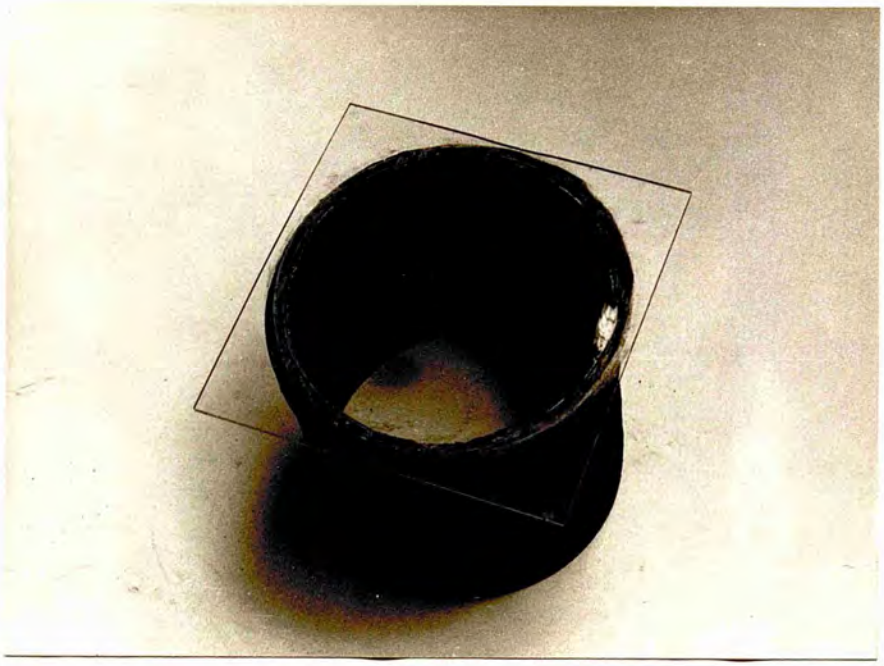
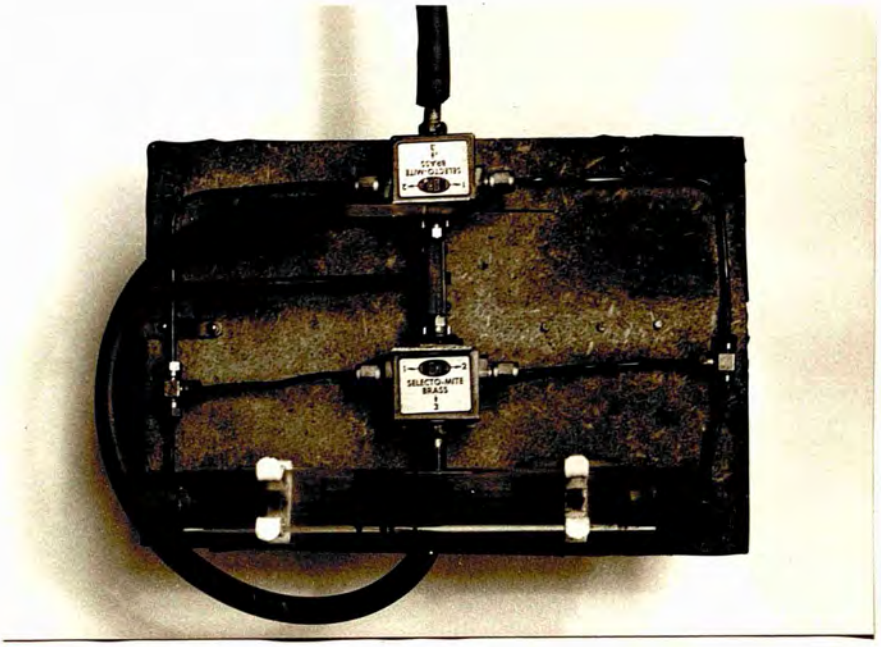


Fig. 2.13 Apparatus used to inject acetylene into the incubation chambers.



sticks to the lids when they are removed so that it can be stored in position and re-used throughout the season. An annular groove, formed in the lid by pressing it onto the heated rim of one of the tubes, was found to be convenient because it prevented the sealant from spreading out during successive incubations.

c. Acetylation

In earlier experiments acetylene was injected into the chambers through the subaseal using a 60ml plastic syringe, but it was found that the introduction of 150mls of gas in this way was so slow as to be impracticable in larger experiments, and the apparatus shown in Fig. 2.11 and Plate 2.13 was constructed. When the operating lever is moved (see inset B. Fig. 2.11) the gas pressure from the acetylene cylinder drives the piston along the glass tube delivering a fixed volume of gas. The volume can be adjusted by either altering the length of the tube or by changing the inlet gas pressure.

In practice the entire apparatus was mounted on a board which could be worn by the operator, and connected to the acetylene cylinder by 50 metres of pressure tubing. The rubber piston constructed from two 50ml plastic syringe plungers, had a tendency to stick in the tube and 'Durol' contraceptive lubricant was found to be effective in preventing this.

d. Gas Losses From the System

A number of field experiments and laboratory simulations were carried out in order to investigate gas losses from the incubation chamber with differing lengths of tube and field conditions.

(1) Depth of tube.

Unless the soil is fully saturated with water, the theoretical volume of the incubation chamber is almost infinite since the gases diffuse down the free space into the soil profile. In practice a rapid decrease

in gas concentration at the sampling port occurs as the gas diffuses into the available free space, but the curve flattens out after about 20 minutes and the diffusion thereafter is relatively slow (see Fig. 2.14) provided that the incubation space is not in direct contact with the exterior via major interstices in the soil.

It is thus important that the cylinder is long enough to pass down through any loosely packed clods which remain after ploughing, into well-compacted soil.

Experiments were carried out with various lengths of cylinder, and it was found that the results were generally satisfactory if the lower edge of pot was 6" below the surface (Fig. 2.15, Curve A). A $4\frac{1}{2}$" depth gave reasonable results when the ground was damp, but the losses increased markedly as the soil dried, (Fig. 2.15, Curve B). The 6" chamber chosen did not show excessive leakage even during prolonged dry periods in the fields.

(2) Correction for gas loss.

Because of the initial rapid non-linear gas loss which occurred (Figs. 2.14 - 2.17) the quantities of ethylene produced in the system are difficult to calculate over the first 20 minutes, and for this reason fixation values were inferred from gas samples taken at 30 and 60 minutes on the almost linear part of the curve.

Both the gas losses between the first and second sampling, and the absolute volume of the system were calculated from the concentration of an internal standard.

(3) Internal standards.

The rate of ethylene loss closely parallels both that of acetylene (Fig. 2.17) and that of propane (Fig. 2.14) but acetylene is a convenient internal standard since a fixed volume of it must, in any case, be injected into the chamber at the start of the incubation and it is readily separated from the other gaseous components during analysis. Objections to its use on the grounds that it is the substrate for the nitrogenase are only theoretical since in none of the experiments was more than 0.5%

FIG 2.14 Propane and ethylene loss from a steel incubation chamber containing soil in the absence of acetylene.

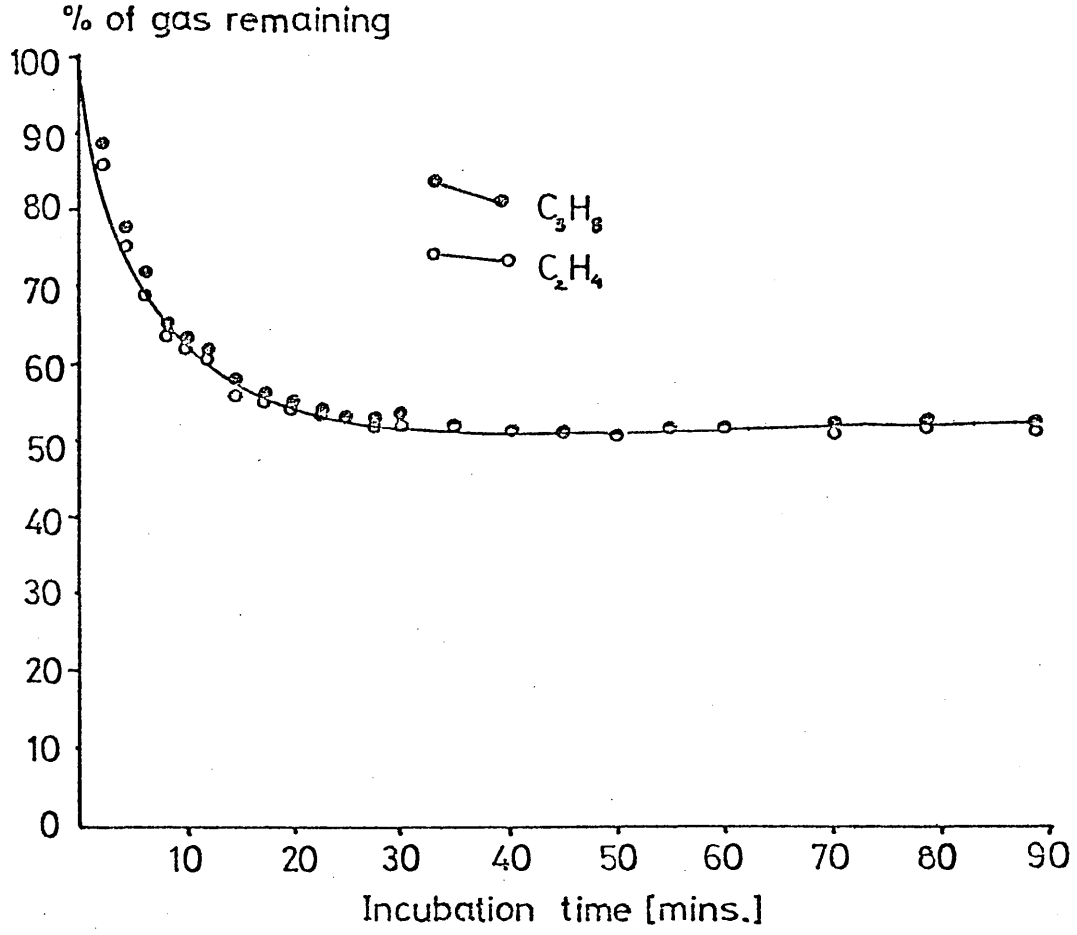


FIG 2.15 The effect of cylinder length on gas loss

A, cylinder edge 6" below surface

B, cylinder edge 3" below surface.

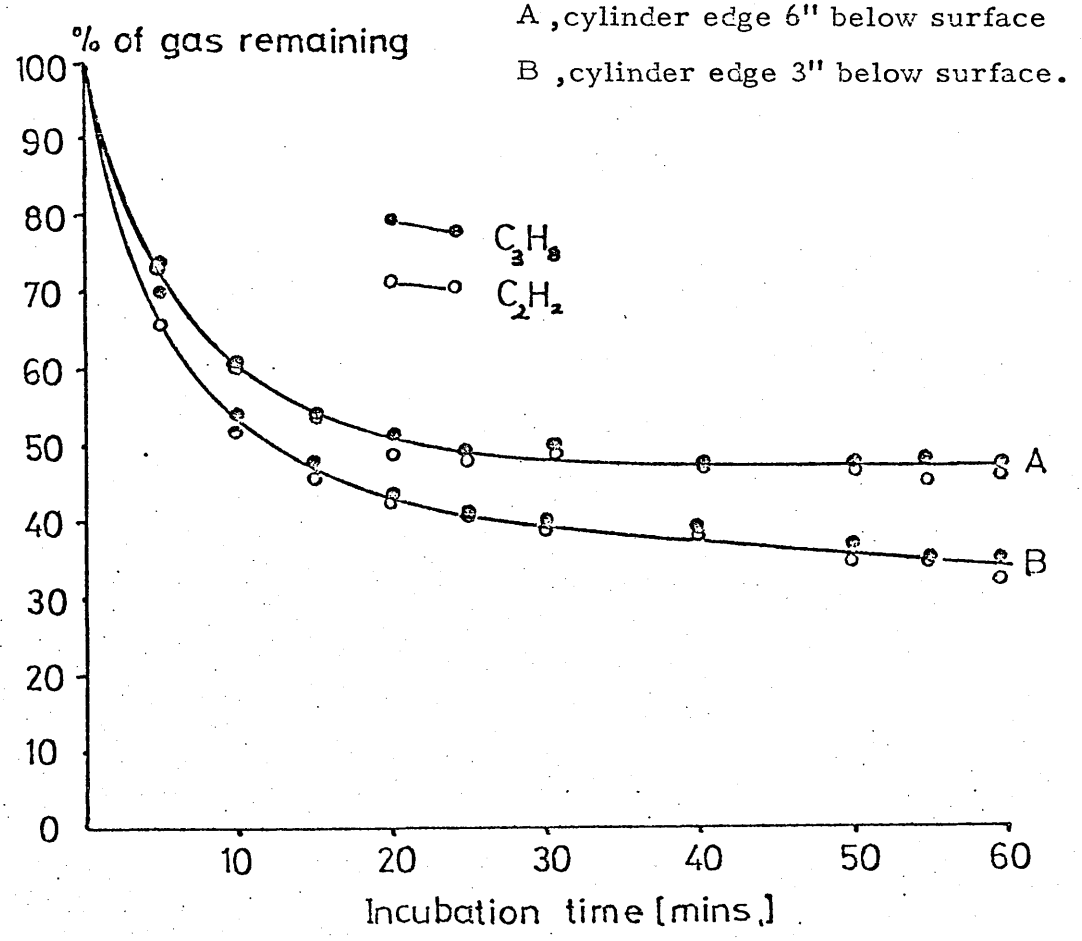


FIG 2.16 Non-biological ethylene production by sterile soil in a steel incubation chamber. Initial conc. of C_2H_2=10 % and C_2H_4= 3 ppm.

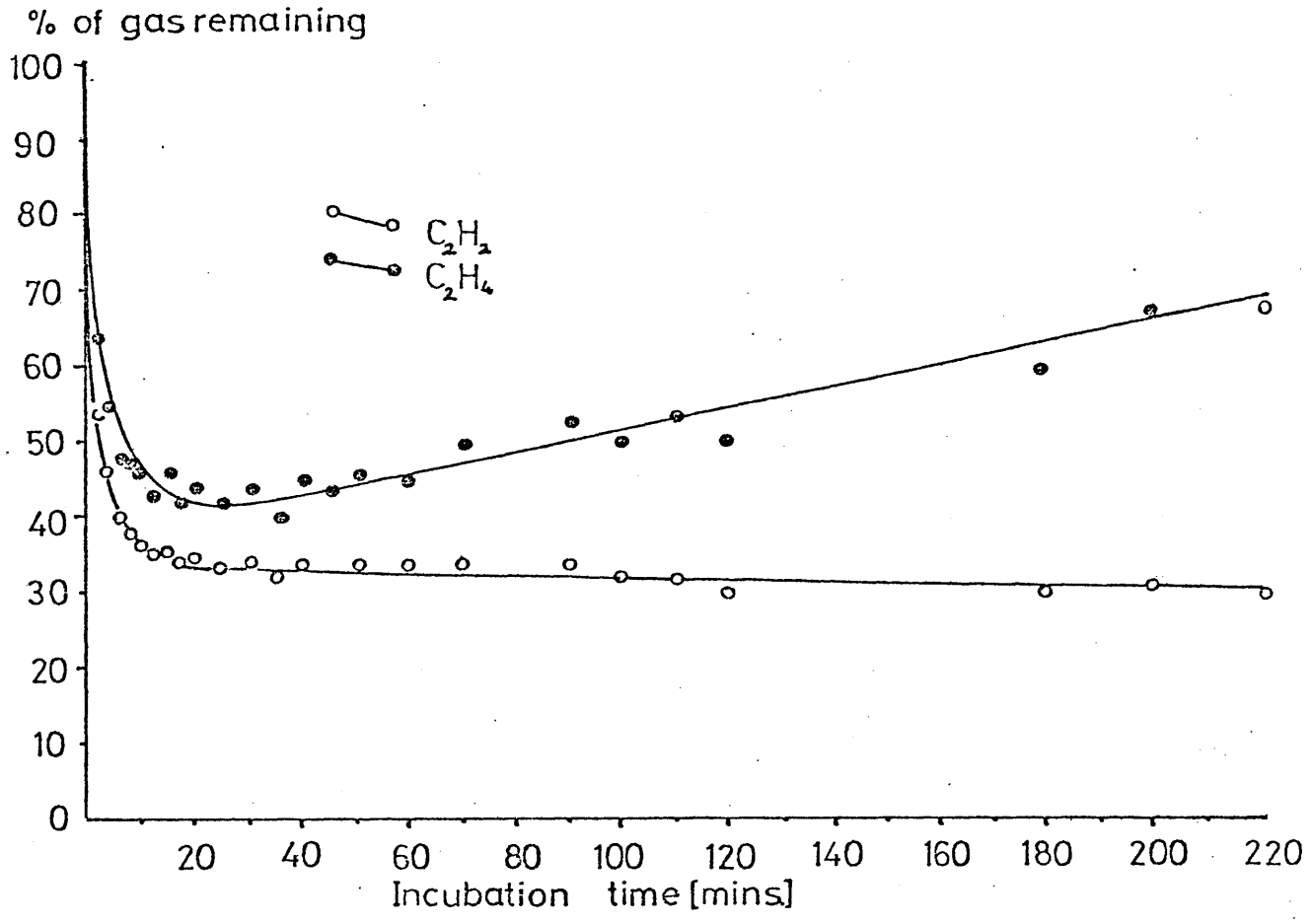
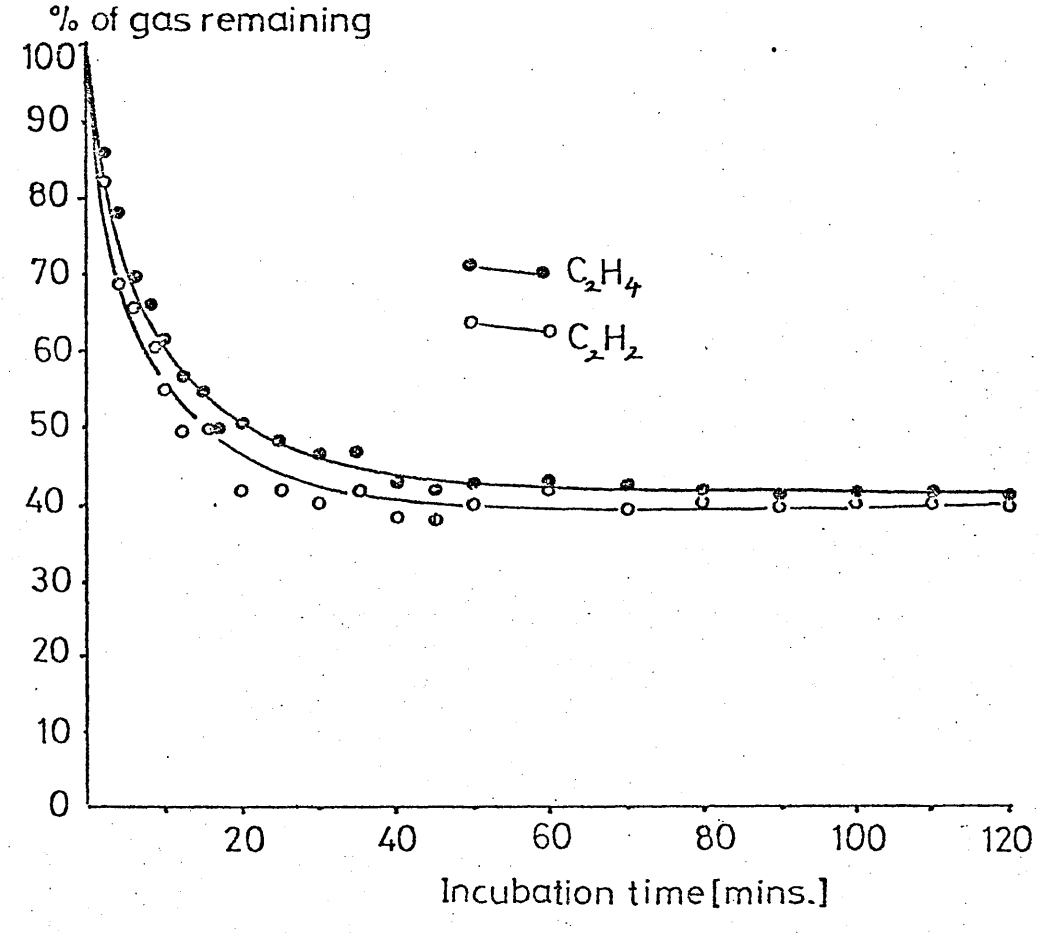


FIG 2.17 Gas losses from a glass incubation chamber containing sterile soil.



of the acetylene reduced.

e. Non Biological Ethylene Production.

It is of interest that the system described acts as a non-biological acetylene reducing system. A typical result is shown Fig. 2.16. The soil and chamber were sterilized by autoclaving at 150 psi for 4 hours before the injection of acetylene. The initial ethylene concentration (3.0 ppm) is that which is present as an impurity in the acetylene. After the usual decrease, the ethylene concentration rises linearly for 200 minutes from 41% to about 68% of the original concentration. This is equivalent to the production of 1.23 mls of ethylene over 200 minutes or 0.066 moles ethylene/cm^2/Hr. Although this quantity is so small that it is of little importance in the field assay procedure (it is equivalent to .00012 KgN/ha/Hr) it is sufficient to be analysed with certainty using the gas chromatogram.

The ethylene is apparently produced by the reduction of acetylene since its concentration does not increase if the acetylene substrate is absent. Fig. 2.14 shows the results obtained when sterile soil is incubated in a steel cylinder with 3 ppm ethylene and propane as an internal standard. This result also implies that the ethylene is not adsorbed and progressively released.

It is also unlikely that the result is caused by imperfect sterilization of the soil or that the reduction is brought about by the sealant, subseal or perspex lid because in identical experiments, using a glass cylinder in place of the steel incubation chamber, no ethylene production occurs (see Fig. 2.17). This would appear to implicate the steel cylinder but this on its own does not reduce acetylene with or without a wetted internal surface.

Two possibilities remain; the sterilization procedure is more effective in a glass cylinder, or a reducing system is formed by the interaction of the steel pot and the soil. The former would seem the less likely

since the glass and metal cylinders were of similar size and glass is a poor conductor of heat compared with steel.

f. Repeated Acetylation.

The algal distribution in the field is such that a considerable number of incubation chambers were required to give a satisfactory mean value. In the field plot experiments involving the effect of algal inoculations on wheat growth and nitrogenase activity, 10 chambers were used on each 7' x 15' plot giving a total of 420 chambers. (Even so the standard deviation values are rather high).
Under these circumstances it became physically impossible to implant and remove the chambers on each monitoring occasion and the chambers were therefore left in situ. The repeated removal and replacement of the chambers is, in any case, undesirable because of the damage to the wheat crop and the general disruption of the soil surface.
Each chamber was acetylated for a period of $1\frac{1}{2}$ hours on one occasion each week throughout the summer. The effect of these repeated acetylations on algal nitrogen fixation was investigated in the laboratory, using algae grown on soil in 4' x 7' galvanised iron tanks. Incubation chambers acetylated for three hours every other day for three weeks fixed at about the same rate at the end of this period in chambers which had not been acetylated.

g. Disadvantages and Precautions.

(1) Changes in the environment around the chamber.
If damage to the crop is to be avoided, the chambers must be driven into the ground before the wheat roots have spread into the areas between the rows. Even if this is done, there is some reduction in wheat growth around the chamber, due to the lateral limitation of root spread.

(2) Changes in the environment within the chamber.

The flora within the chamber was left in-tact as far as possible, but it was necessary to cut the taller plants down to the level of the pot rim so that the lids could be fitted.

Parts of the soil flora and fauna might also be affected by the high acetylene concentration present as well as the ethylene which is known to affect plant growth. These factors, however, produced no obvious effect. Aside from an evident lack of tall plants within the incubation chambers, the flora appeared to be unchanged.

Solubilisation of components in the steel cylinder may have an affect on the iron concentration in the soil, but, as Stewart (1969) observes, iron is seldom a limiting factor and under alkaline conditions it would seem unlikely that the concentration would be excessive.

The area within the rim of the incubation chamber is protected, to some extent, from the drying action of the wind and furthermore compacted slightly so that it becomes less permeable. Although these factors must tend to encourage algal development, visual estimations of algal cover at the end of the season failed to show any significant affect on algal distribution.

SECTION 3 THE EFFECT OF OXYGEN ON NITROGEN FIXATION

A. INTRODUCTION

B. EXPERIMENTAL TECHNIQUE.

C. THE EFFECT OF OXYGEN CONCENTRATION ON THE RATE OF DARK AND LIGHT FIXATION.

D. THE EFFECT OF TEMPERATURE ON NITROGENASE ACTIVITY IN THE PRESENCE AND ABSENCE OF OXYGEN.

E. EFFECT OF TEMPERATURE ON LIGHT PROMOTED FIXATION AT ATMOSPHERIC OXYGEN CONCENTRATIONS.

F. DISCUSSION.

G. SUMMARY.

3. THE EFFECT OF OXYGEN ON NITROGEN FIXATION

A. INTRODUCTION

Nitrogen fixation by blue-green algae is exceptional in its relative insensitivity to free oxygen. (Stewart and Pearson 1970).

The nitrogenase of facultative anaerobes such as Klebsiella pneumoniae, Bacillus macerans and Bacillus polymyxa operates only under anaerobic conditions. These organisms will grow aerobically only in the presence of combined nitrogen (Witz, Detroy and Wilson 1967, Hamilton and Wilson 1955). Nitrogen fixation by the aerobe Azobacter is most efficient at low partial pressures of oxygen.

Cell-free preparations of algal nitrogenase are immediately and irreversibly inhibited by oxygen (Burris, 1966, 1969, Hardy and Burns 1968, Fay and Cox 1967) and it is thus the micro-environment surrounding the nitrogenase which protects it from oxygen in the intact cell.

Experimental evidence strongly suggests that nitrogen fixation is localized within the heterocyst (Fay, Stewart, Walsby and Fogg 1968, Stewart, Haystead and Pearson 1969). These specialized cells, which occur at intervals along the vegetative chain of all filamentous algae known to fix nitrogen under aerobic conditions, lack the pigments of photosystem II and are therefore unable to carry out non cyclic electron flow and evolve O_2 (Fay, Stewart, Walsby and Fogg 1968, ~~Fogg and Than-Tun 1960~~). The respiratory rate within the heterocyst is high (Fay and Walsby 1966) and the reduction of triphenyltetrazolium chloride and photographic emulsion indicates that a strongly reducing environment exists within the cell (Stewart, Haystead and Pearson 1969).

There is a close relationship between N fixation and photosynthesis which provides energy, carbon skeletons and reducing power (Fogg and Than-Tun 1960). These authors note that the heterocyst, lacking photosystem II, could provide ATP by cyclic photophosphorylation but would require an exogenous source of reducing power which might be provided by the intimately connected adjacent vegetative cells.

A pool of reductant would appear to exist since DCMU or CMU (p-chlorophenyl-1, 1-dimethylurea)which inhibits photosystem II, has no immediate effect on ^{15}N uptake (Cox and Fay 1969, Bothe 1970). Under conditions which stimulate photorespiration (low pCo_2 and high pO_2) N-ase activity in A.cylindrica, estimated by using the acetylene reduction technique, does become DCMU sensitive because the two processes appear to complete indirectly for the same reductant pool (Lex et al 1972). The competition is not for ATP since photorespiration is not an ATP consuming process (DeDuve and Baudhuim 1966) and it is unlikely to be due to a shortage of carbon skeletons since these are not required to accept the products of acetylene reduction.

Changes in light intensity produce an apparently immediate change in nitrogenase activity and it would seem likely that the ATP generated by cyclic electron flow in photosystem I provides the energy necessary for the reduction of N_2 (Cox and Fay 1969, Fogg and Than-Tun 1960). A.variabilis has been shown to produce ATP by oxidative phosphosphorulation (Carr, Hood and Pearce 1969, Leach and Carr 1969) and the data presented in this section suggests that at atmospheric oxygen concentrations, at least half of the ATP requirements is met by oxidative phosphorylation. The findings of Lyne and Stewart (1973) published after these experiments had been carried out are in agreement with this conclusion.

B. EXPERIMENTAL TECHNIQUE

Batch cultures of Anabaena cylindrica and Nostoc ellipsosporum were grown in the nitrogen-free medium of Allen and Arnon at 23°C and 3200 Lux.

For the solid surface experiments uniform crusts of these species were grown on soil at a pH of 7.5 under dayglow fluoreseent tubes at 3400 Lux and 23°C.

Uniform aliquots of liquid culture were obtained using a method similar to that of Day, and Hopkins (1967). The algal suspansion was first filtered through glass beads (3mm diameter) and then stirred

vigorously to maintain a homogenous suspension while 5 ml aliquots were removed with a pipette and transferred to 120 ml medical flats. (Aliquots taken in this way are reproduceable to within about 1%). Reproduceable samples from the solid surface were obtained by cutting out discs of the algal crust with a cork borer.

The gas mixtures were prepared using a BOC special gas mix of Ar/CO_2 (99.96% : 0.04%) and O_2 in various proportions. The apparatus used for this is shown in Fig. 3.1 overleaf. The oxygen concentrations actually produced were measured using the apparatus shown in Appendix 2

The volume of gas entering the bladder is shown by displacement in the graduated aspirator after the pressure in the bladder has been brought to atmospheric using the manometer. The football bladder was used in preference to direct displacement to prevent the gases dissolving in the water.

The medical flats were capped with subaseals and the gas mixtures were introduced by successive evacuation and replacement. The pressure was reduced to about 60 mm Hg on each occasion so that after 4 changes only 0.003% of the original bottle contents remained.

Six replicate samples were used for each treatment. 10 mls of gas was removed from each bottle and replaced with acetylene so that the oxygen concentration in each case was reduced by 9%. Using the acetylene reduction technique for assaying N-ase activity, it is thus impossible to obtain values for $100\%0_2$.

The samples were incubated at a suitable light intensity for 30 mins. and then $\frac{1}{2}$ ml samples were taken and analysed for ethylene in the usual way.

C THE EFFECT OF OXYGEN CONCENTRATION ON THE RATE OF DARK AND LIGHT FIXATION.

The results are shown graphically in Figs. 3.2 to 3.5. The various

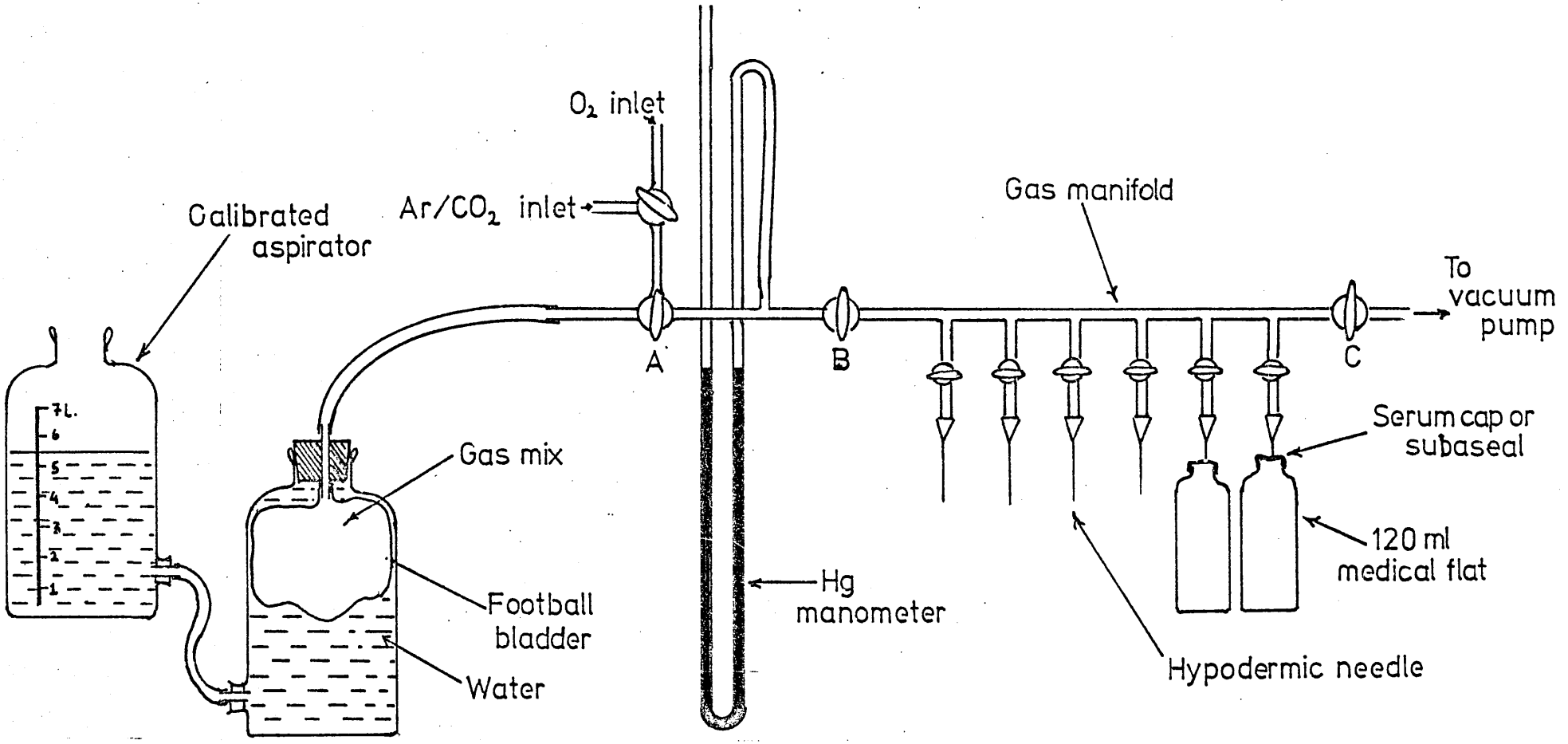


Fig 3·1 Apparatus used to produce Argon/Carbon dioxide/Oxygen gas mixes

figures are not quantitatively comparable because it was not possible to use the same material in all experiments.

The inhibition of acetylene reduction by O_2 is apparently immediate with a time course which remains linear for at least 1 hour (Fig. 3.2).

Qualitative comparisons show that the oxygen concentration giving maximum fixation in the dark was relatively constant at about 30% irrespective of species (N.ellipsosporum or A. cylindrica) in both liquid culture and on soil (Figs. 3.3 to 3.5) and in all cases the rate of dark fixation in the absence of oxygen was negligible.

The oxygen concentration giving maximum fixation in the light varied between 0 and 25% depending on the intensity of illumination, but at light intensities below 3000 Lux nitrogenase activity increased with increasing 0_2 concentration up to about 20% 0_2 (Figs. 3.3 B and C, 3.4 B and C).

The increase in fixation associated with illumination ("Light minus dark" fixation shown as hatched lines in Figs. 3.3 and 3.4) is more sensitive to 0_2 at high light intensities than at low light intensities. At 5900 Lux the nitrogenase activity falls by more than 50% as the 0_2 concentration increases from 0 to 20%, whereas at 400 Lux the value drops hardly at all.

At atmospheric oxygen concentrations a substantial proportion of the light fixation continues in the dark. The dark fixation rate represented about 50% of that at 5900 Lux and 60 to 70% of that at 3400 Lux.

The results obtained with liquid cultures of the two algal species are basically similar to those obtained with soil grown material, but they do differ in that they are apparently less sensitive to increased 0_2 concentrations (see Fig. 3.5). The nitrogenase activity in liquid cultures at 5000 Lux drops to half of its maximum value at an 0_2 concentration of about 70%. A similar decrease is produced with solid surface cultures with only 50% 0_2.

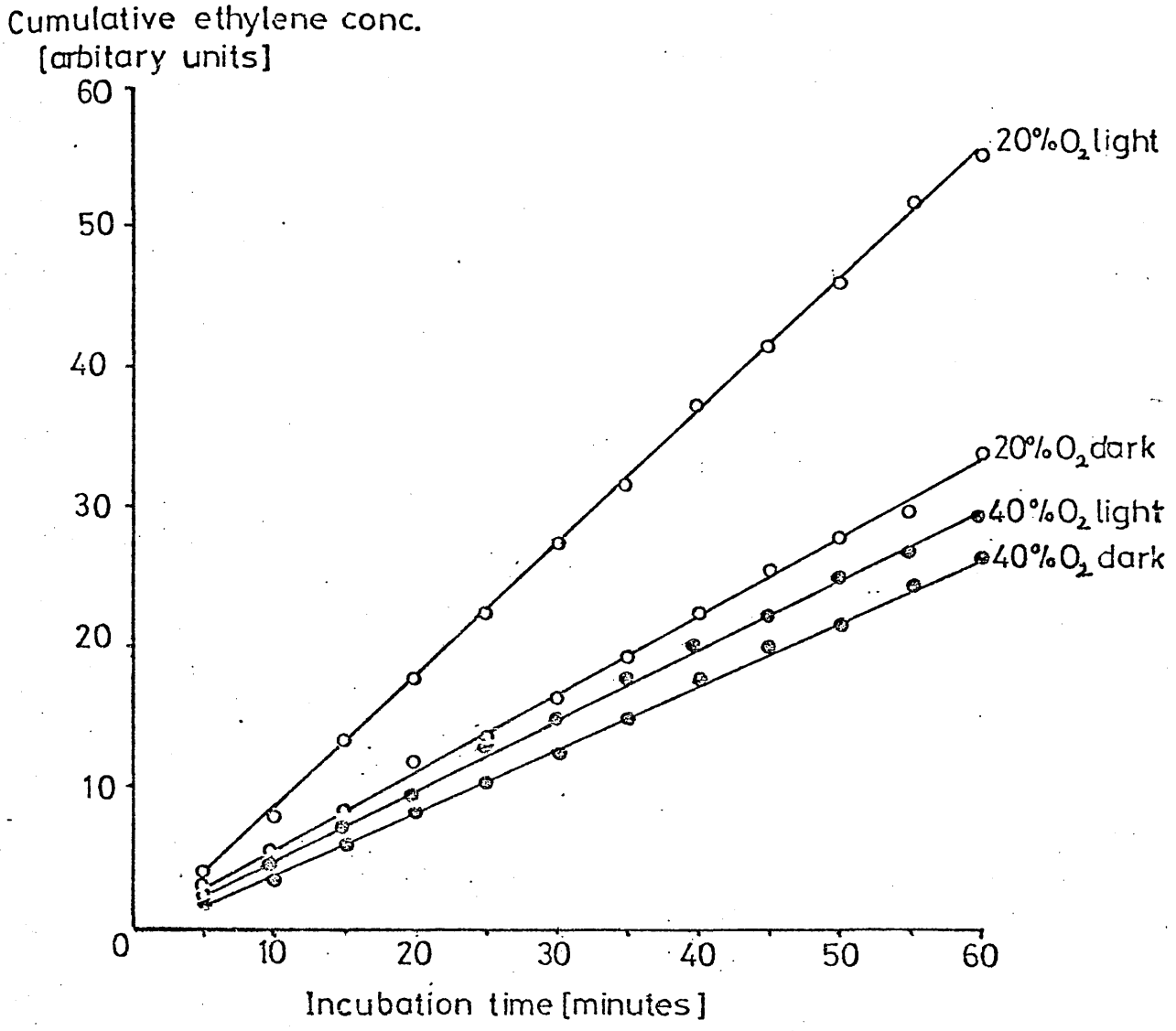
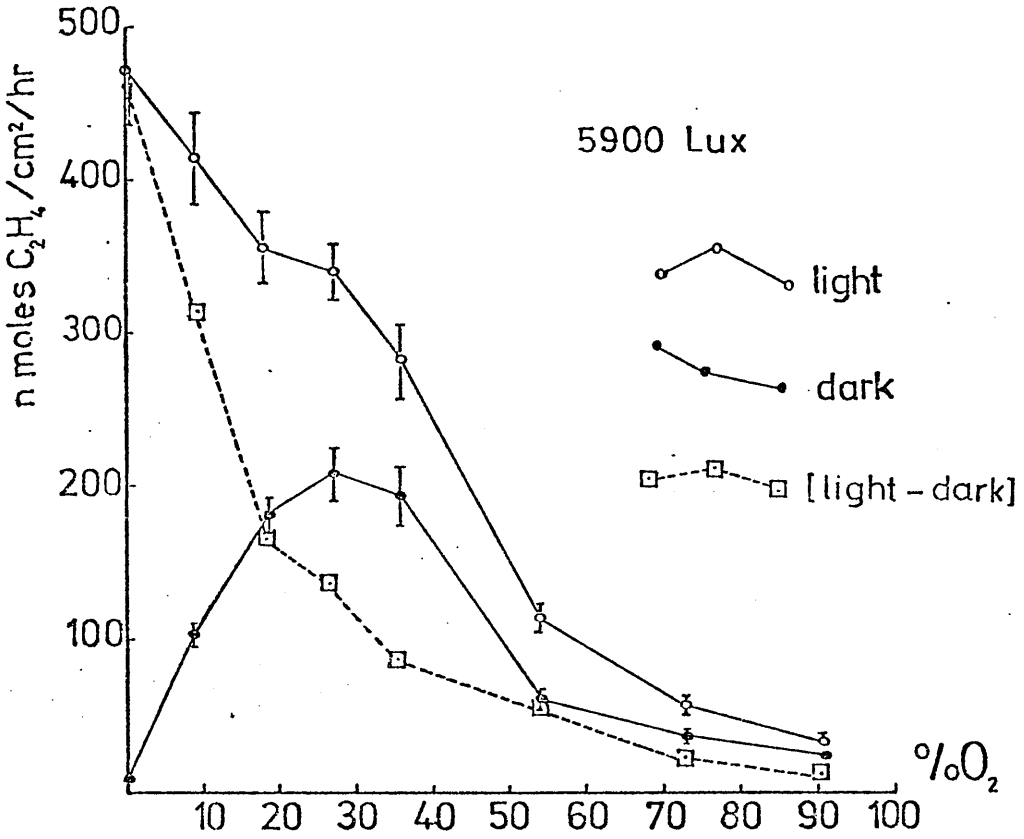
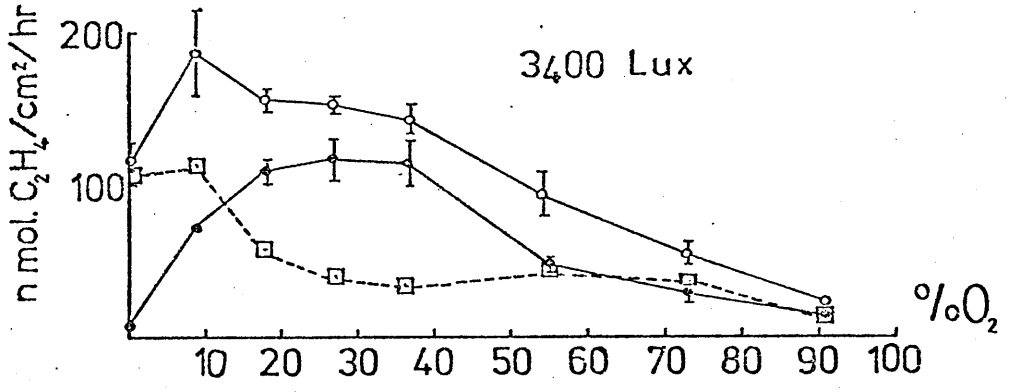


Fig 3·2 Time course for ethylene production in the light and in the dark at two O_2 concentrations [2700 ft candles]

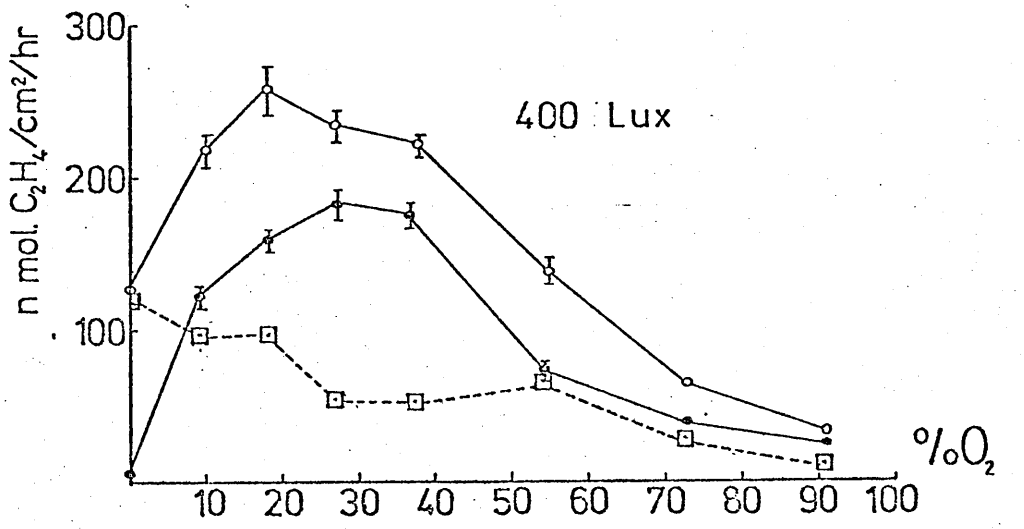
Fig3·3 Effect of Oxygen concentration on nitrogenase activity at three light intensities. (1) Anabaena cylindrica on soil [23°C]



[A]



[B]



[C]

Fig 3·4 Effect of Oxygen concentration on nitrogenase activity at three light intensities. (2) Nostoc ellipsosporum on soil [23°C]

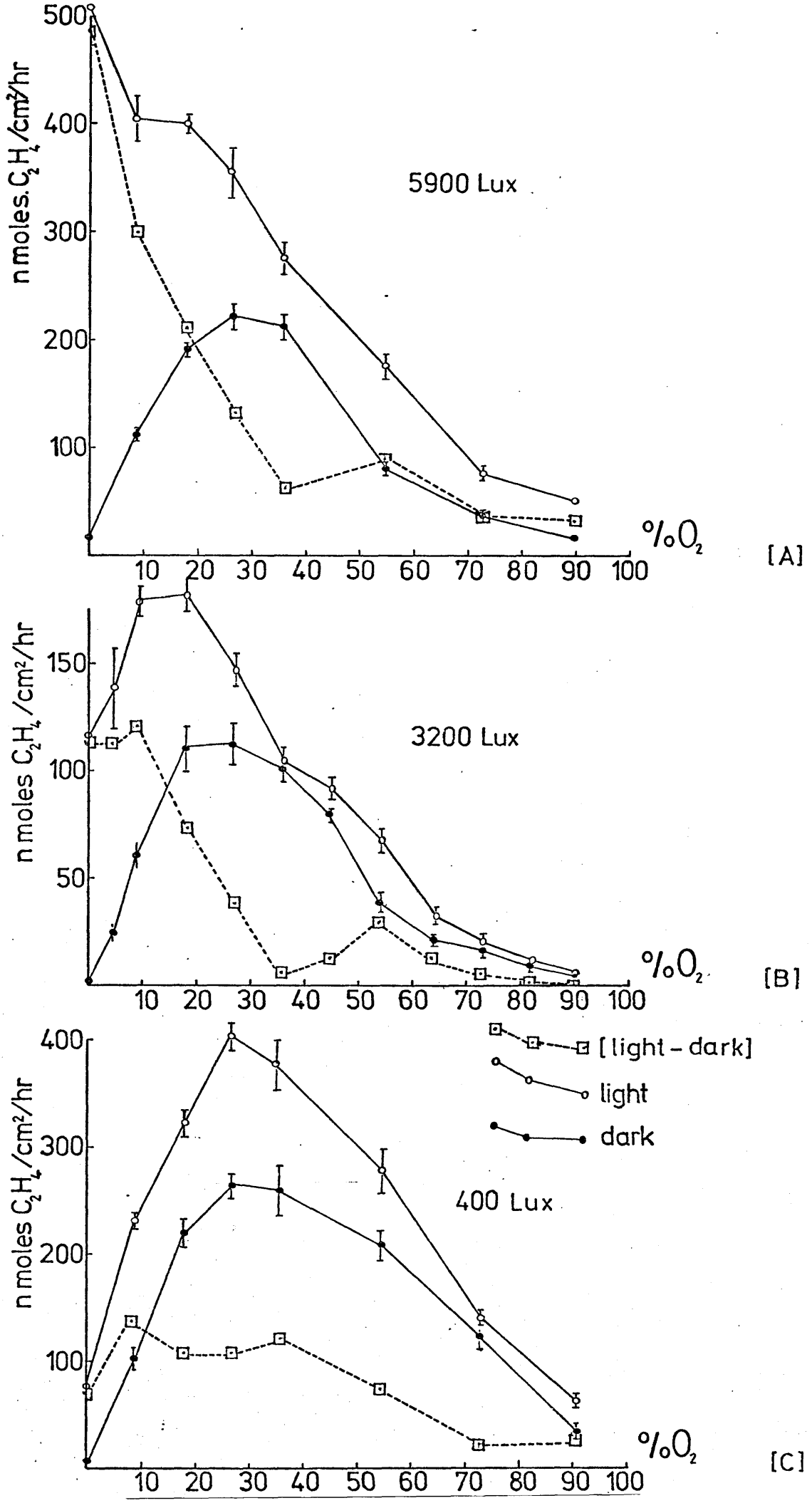
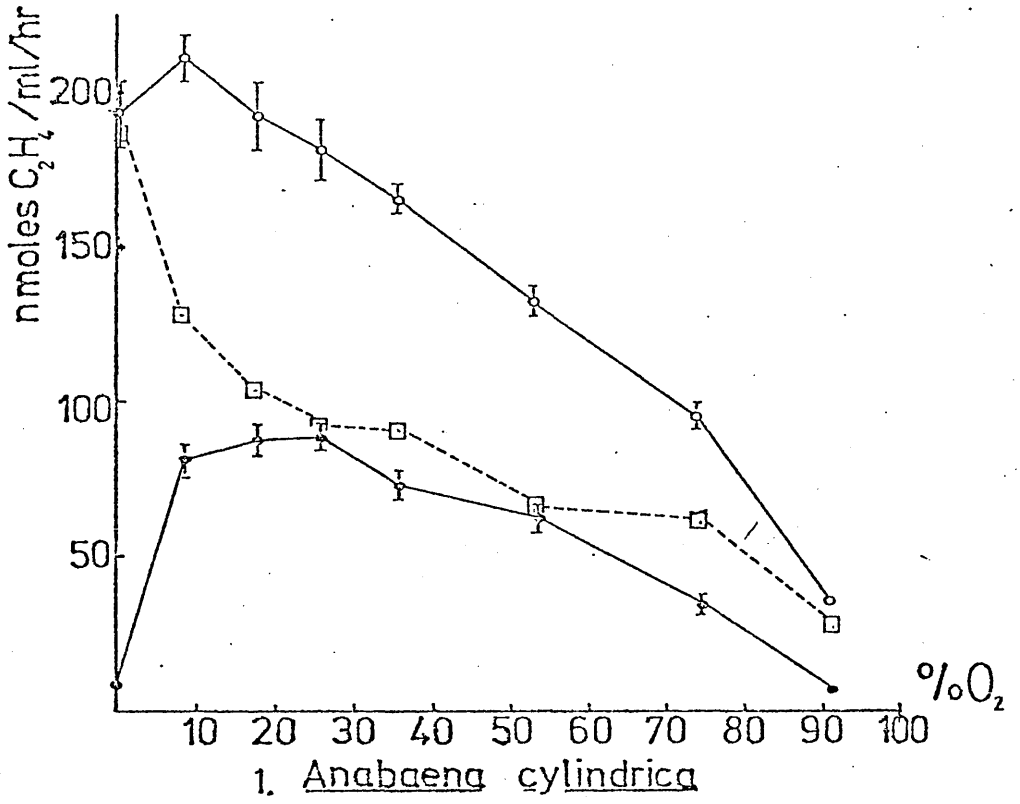
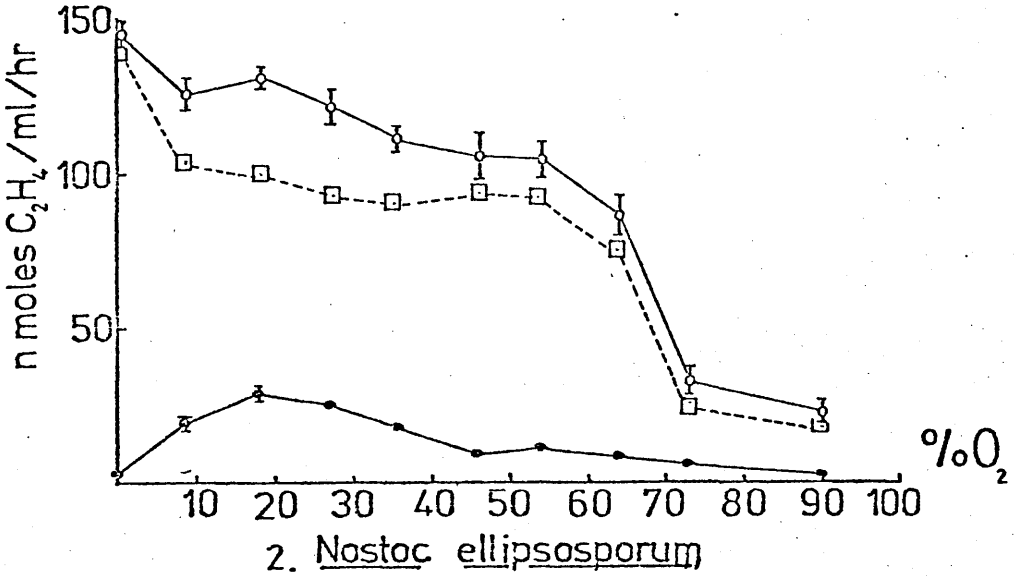


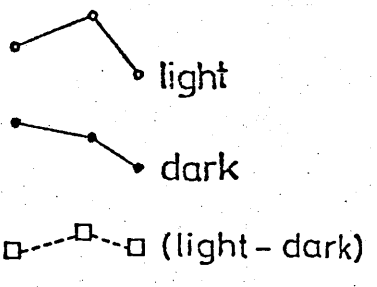
Fig 3·5 Effect of Oxygen concentration on nitrogenase activity in liquid cultures of A.cylindrica and N. ellipsorum at 5000 Lux and 23°C.



1. Anabaena cylindrica



2. Nostoc ellipsosporum



Oxygen concentrations in both types of experiment were measured by analysing samples from the gas phase. Despite the fact that 5 ml samples were used in 125 ml medical flats and shaken for 10 minutes before acetylation, it is possible that the insensitivity of liquid cultures to 0_2 is attributable to an imperfect equilibrium between the phases.

D THE EFFECT OF TEMPERATURE ON NITROGENASE ACTIVITY IN THE PRESENCE AND ABSENCE OF OXYGEN.

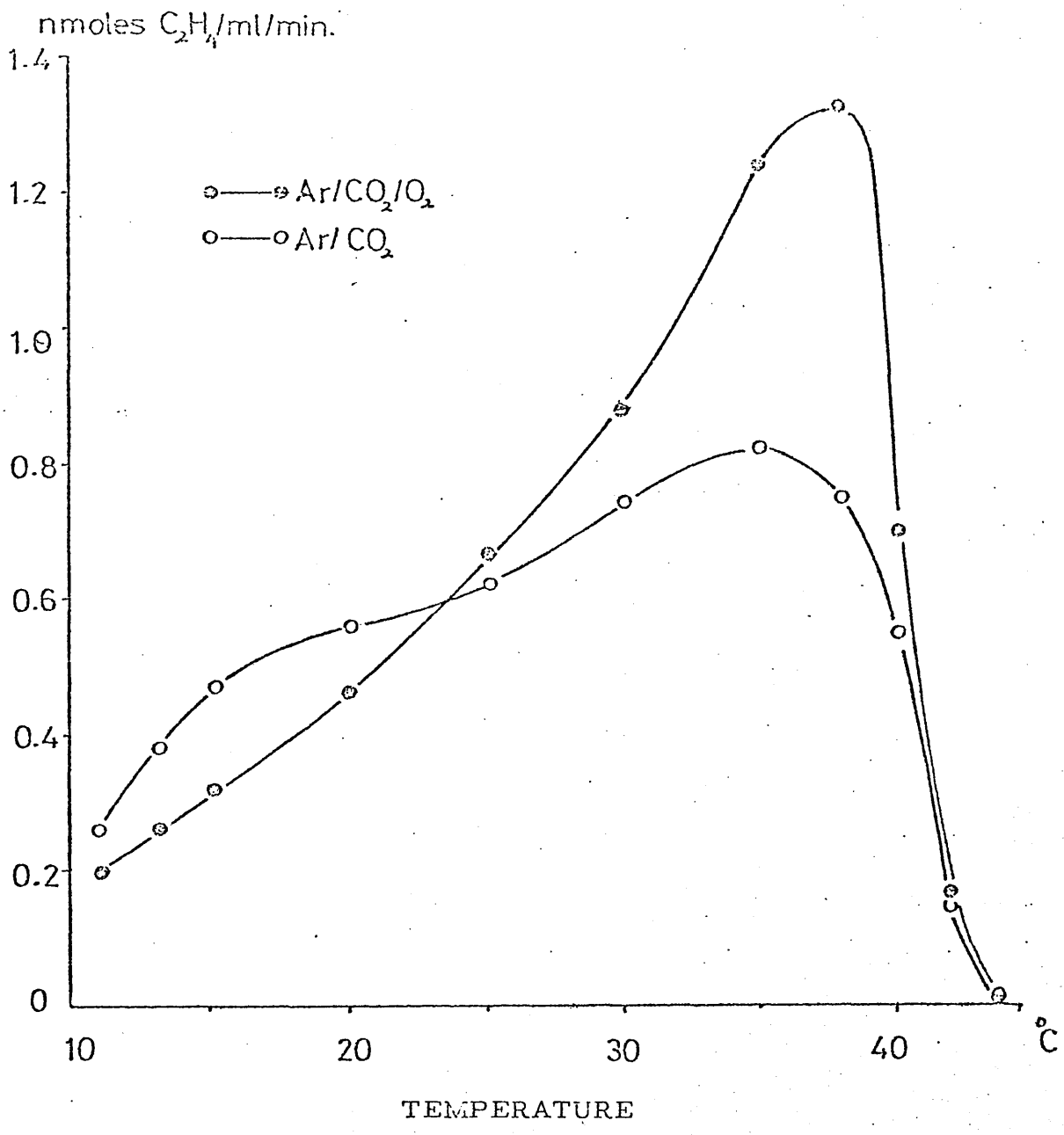
The fact that the rate of dark fixation in the absence of 0_2 is negligible suggests that the ATP requirements in the light under such conditions are met entirely by photophosphorylation. Thus at low light intensities, where the light reactions of photosynthesis are rate limitting, the nitrogenase activity under anaerobic conditions might be expected to be independant of temperature. A number of experiments were carried out in order to test this hypothesis.

Uniform 5 ml aliquots of N.ellipsosporum were incubated in 120 ml medical flats at various temperatures in a water bath at 1100 Lux. The bottles contained, initially, a gas phase of either $0_2/Ar/C0_2$ (20: 79.5: 0.5) or Ar/CO_2 (99.5 : 0.5). Acetylene was injected into the bottles ~~They were acetylated~~ as soon as they had reached the desired temperature and the gas phase was sampled after 30 minutes and analysed in the usual way.

The results of one such experiment at 1300 Lux are shown in Fig. 3.6 overleaf. The values plotted are the means of two samples. The replicates varied by less than 5% at all temperatures up to 38°C.

Although the nitrogenase activity in the absence of 0_2 is not independant of temperature, it is far less temperature sensitive than the rate in the presence of 0_2. As the temperature increased from 11 to 38°C, the photosynthetically driven fixation increased by a factor of 2.6 (0.26 n moles C_2H_4/ml/min. to 0.76 n moles C_2H_4/ml/min.) whereas in the presence of 0_2 the rate increased by a factor of 6.5 (0.2 n moles C_2H_4/ml/min. to 1.31 n moles C_2H_4/ml/min.).

FIG 3.6 Effect of temperature on nitrogenase activity in the presence and absence of oxygen.



The shoulder on the anaerobic curve at about 17°C would appear to be the point at which some factor required for nitrogenase activity becomes limiting. The rate under aerobic condition is subject to no such gradient change suggesting that the limiting factor is one which might be otherwise produced in the presence of 0_2, probably ATP produced by oxidative phosphorylation.

E. EFFECT OF TEMPERATURE ON LIGHT PROMOTED FIXATION AT ATMOSPHERIC OXYGEN CONCENTRATIONS.

The production of ATP by oxidative phosphorylation is a temperature dependant process whereas the production of ATP by photophosphorylation at low light intensities, where the light energy is limiting, might be expected to be independant of temperature.

A number of experiments were carried out using the statim block and fibre optic technique to investigate this hypothesis at light intensities between 100 and 800 Lux.

In most of these experiments the rate of light fixation followed that of dark fixation (that is; light fixation minus dark fixation = constant) only up to about 20°C. Above this temperature the relationship breaks down as the apparent contribution from dark fixation increases. Fig. 3.7 shows a typical result.

In a number of experiments of this type, with very low light intensities, the rather surprising result of the type shown in Fig. 3.8 was obtained, where light actually repressed nitrogenase activity. It has not been possible to reproduce this result consistently and it was at first thought to be an artifact. Similar results have, however, been obtained using several different techniques, both by myself and by other workers in the laboratory. The repression was noted in 8 out of 13 experiments. The results of one such experiment are shown below.

Five replicate bottles, each containing 4 algal discs were incubated at 150 Lux and 33°C with an atmosphere of air and 10% acetylene.

	Mean C_2H_4 Production (n moles $C_2H_4/CM^2/Hr$)	Standard Deviation
Dark	68.8	6.75
Light	42,8	7.5

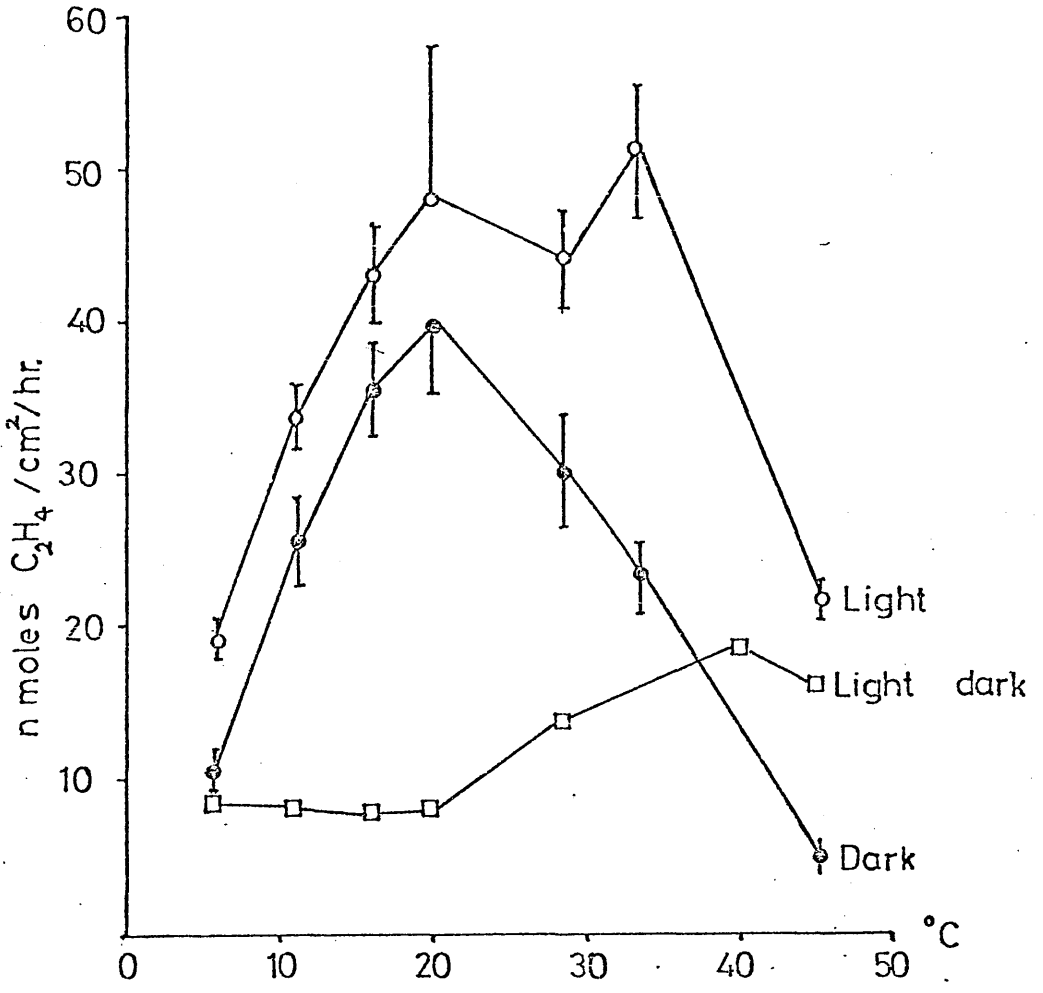


Fig 3·7 Effect of temperature on nitrogenase activety at 400 Lux

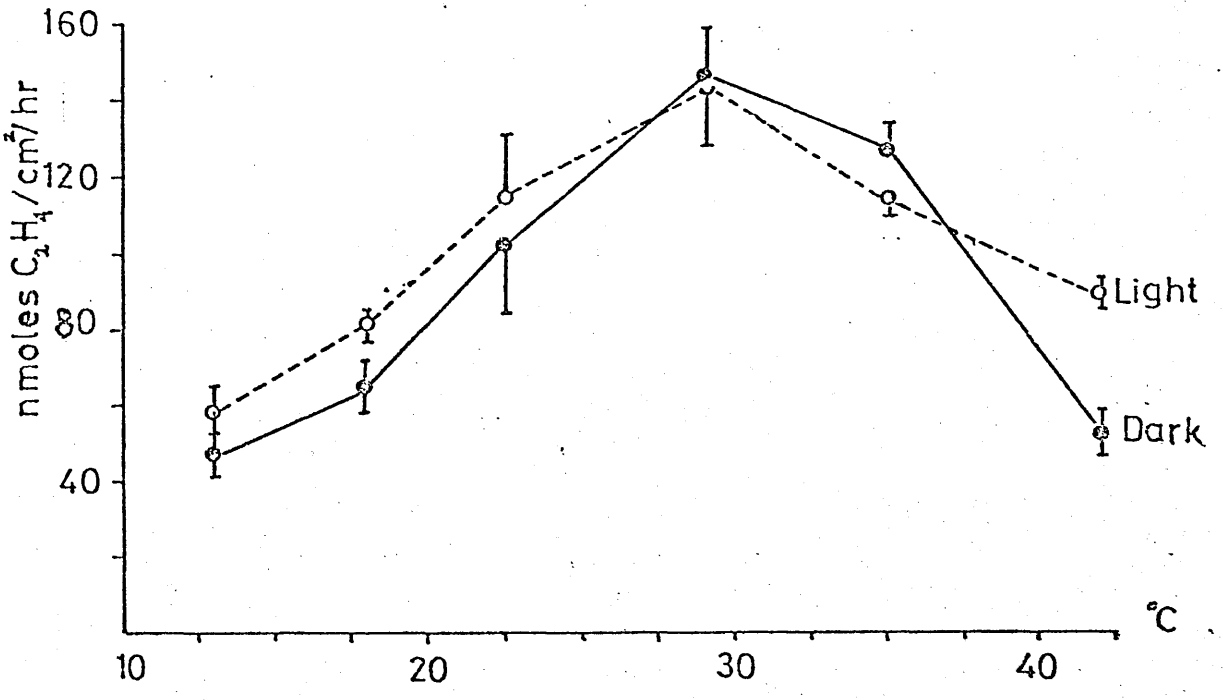


Fig 3·8 Effect of temperature on nitrogenase activety at 200 Lux

The 't' test shows that the means are significantly different at the 1% level.

The author can present no proven reason for this effect. Fay and Cox (1966) noted a similar occurrence in cell free preparations of A.cylindrica and suggested that it may be due to the photo-oxidation of some factor required in the reduced state by the nitrogenase. It is possible that the effect is due to stimulated photorespiration at atmospheric O_2 and CO_2 concentrations competing with nitrogenase activity for reductant. The effect was noted in all instances at temperatures between 30°C and 40°C.

F. DISCUSSION

The data obtained suggests that an oxygen dependant dark reaction contributes to nitrogen fixation even when the cells are growing in the light.

An oxygen dependant dark reaction evidently exists since both A. cylindrica and N.ellipsosporum will fix nitrogen in the dark provided oxygen is present. The fact that this fixation continues for some time without the addition of organic energy sources (see section 4) suggests that a substantial potential reductant/energy pool is available.

Evidence for the continuation of the reaction in the light is twofold:

First the data shows that, although oxygen is a recognised inhibitor of nitrogenase activity, acetylene reduction in de-oxygenated cultures incubated at light intensities below 3000 Lux increased with rising 0_2 concentration up to about 20% 0_2.

Secondly the nitrogenase activity in illuminated cultures of N.ellipsosporum in the presence of oxygen increased steadily with temperature up to about 38°C but in the absence of oxygen, a partial limitation in nitrogenase activity occurs, presumably caused by a lack of some factor required by the nitrogenase which can be provided in the presence of 0_2.

The fact that changes in 0_2 concentration produced an apparently immediate change in nitrogenase activity, suggests that the limiting factor

is ATP rather than reductant since evidence suggests that a pool of reductant exists which becomes limiting only under exceptional circumstances (Lex et al 1972) or after prolonged periods (Stewart and Pearson 1970).

The oxygen concentration giving optimum nitrogenase activity depends on the intensity of illumination. Although, as Lex et al (1972) point out, the increased oxygen sensitivity at high light intensities could be due to stimulated photorespiration competing with nitrogenase for reductant, the relative contribution from oxidative phosphorylation and photophosphorylation would also seem to be contributary.

The total fixation (shown in Figs. 3.3 and 3.4) appaars to be a summation of the light contribution which has no oxygen requirements, and the respiratory ("dark") contribution, which has an optimum value at about 30% 0_2. At high light intensities the respiratory contribution is small (although large enough to produce the shoulder at about 25% 0_2 on curves 3.3 (A) and 3.4 (A)) but as the intensity of illumination falls its contribution to the total becomes larger, so that the optimum 0_2 concentration tends towards 30% (Figs. 3.3.(C) and 3.4 (C)).

Under aerobic conditions the increase in fixation associated with illumination may be solely due to photosynthetically produced ATP supplementing that produced by oxidative phosphorylation. Under anaerobic conditions, when oxidative phosphorylation is unable to operate, the rate of dark fixation is extremely small. The substantial increase which occurs when such cultures are subjected to high levels of illumination, may be due to the use of photosynthetically produced oxygen for respiratory ATP production as well as the direct provision of ATP by photosynthesis. That is, the ATP requirement at high levels of illumination, even under anaerobic conditions, may be partially fulfilled by oxidative phosphorylation.

The algae in the soil crusts used for the experiments were in intimate contact with the gas phase in the incubation chamber which showed a negligible increase in oxygen concentration over the 30 min. incubation period. Even so the oxygen produced by photosystem II

may be used for the production of energy from endogenous substrates without ever leaving the vegetative cells. The ATP produced could then be transferred to the heterocyst. Stewart (1973) considers that the ATP used to drive the nitrogenase in the dark, in aerobic cultures, is provided in this way.

If this is indeed the case, it would lead to another explanation for the observation that the apparent contribution from illumination is more 0_2 sensitive at high light intensities than at low light intensities. In addition to the stimulated photorespiration noted by Lex et al (1971) it may be that, at high light intensities, the photosynthetically produced oxygen is already meeting the requirements of oxidative phosphorylation and the addition of further oxygen only serves to inhibit the nitrogenase.

It might also suggest that the sensitivity of in vivo systems, even under anaerobic conditions, to respiratory inhibitors such as KCN and chloropromazine (Cox 1966) is via oxidative phosphorylation rather than the reductant generating system.

The continuation of acetylene reduction in the dark is of obvious relevance to estimations of the total daily fixation in the field.

An investigation of over-night fixation in the field is described in the following section.

G. SUMMARY

Studies of acetylene reduction by soil grown crusts and liquid cultures of A. cylindrica and N. ellipsosporum suggest that a dark reaction contributes to nitrogen fixation even when the cells are growing in the light. The evidence for this is twofold.

(1) Acetylene reduction by aerobic cultures in the light increased steadily with temperature whereas , in the absence of 0_2, a partial limitation was noted.

(2) The nitrogenase activity in de-oxygenated cultures at light intensities below 3000 Lux increased with increasing 0_2 concentration up to about 20% O_2.

The fact that the increase was apparently immediate suggests that the limiting factor is ATP rather than reductant since other evidence suggests that a reductant pool exists (Lex et al 1972).

Under limiting light intensities the contribution from light fixation to the total remained constant only up to about 20°C, and under some conditions of low illumination and high temperature, light actually appeared to repress acetylene reduction.

The 0_2 concentration giving optimum nitrogenase activity depended on the intensity of illumination. This dependency may be accounted for by the relative contributions of 'dark' and light fixation processes to the overall fixation rate. Under conditions of high illumination the contribution from ATP produced by oxidative phosphorylation, which has an optimum 0_2 concentration of 30%, is small whereas, at high lith intensities, its contribution is sufficiently large to bias the optimum 0_2 requirement of the overall process towards 30% 0_2.

SECTION 4 INVESTIGATION OF OVERNIGHT FIXATION IN THE FIELD

A. INTRODUCTION.

B. EXPERIMENTAL DESIGN.

C. RESULTS.

D. DISCUSSION.

E. SUMMARY.

4. INVESTIGATION OF OVERNIGHT FIXATION IN THE FIELD

A. INTRODUCTION

Cultures of N.ellipsosporum grown aerobically in the laboratory with a 16 hour daily illumination period continued to reduce acetylene in the dark at a rate which decreased little over a 9 hour period. (see Fig. 4.1). Such a continuation of fixation in the field might have a considerable effect on estimates of daily fixation, and an experiment was designed to evaluate this effect in the field.

The fixation associated with an area of good algal cover was monitored overnight.

B. EXPERIMENTAL DESIGN

The area chosen, Broadbalk, plot 3, non herbicide, is one of Rothamsted's classic plots which have been sown to wheat since 1840 without the addition of any nitrogenous fertilizer.

The experiment was carried out in the middle week of August, during a period of fine, dry weather (midday air temperature = 29°C).

32 test chambers were driven into the ground and well watered for 48 hours prior to the experiment.

The fixation was measured at midday, the lids were removed from the chambers, and the experiment was resumed at 6 O'clock.

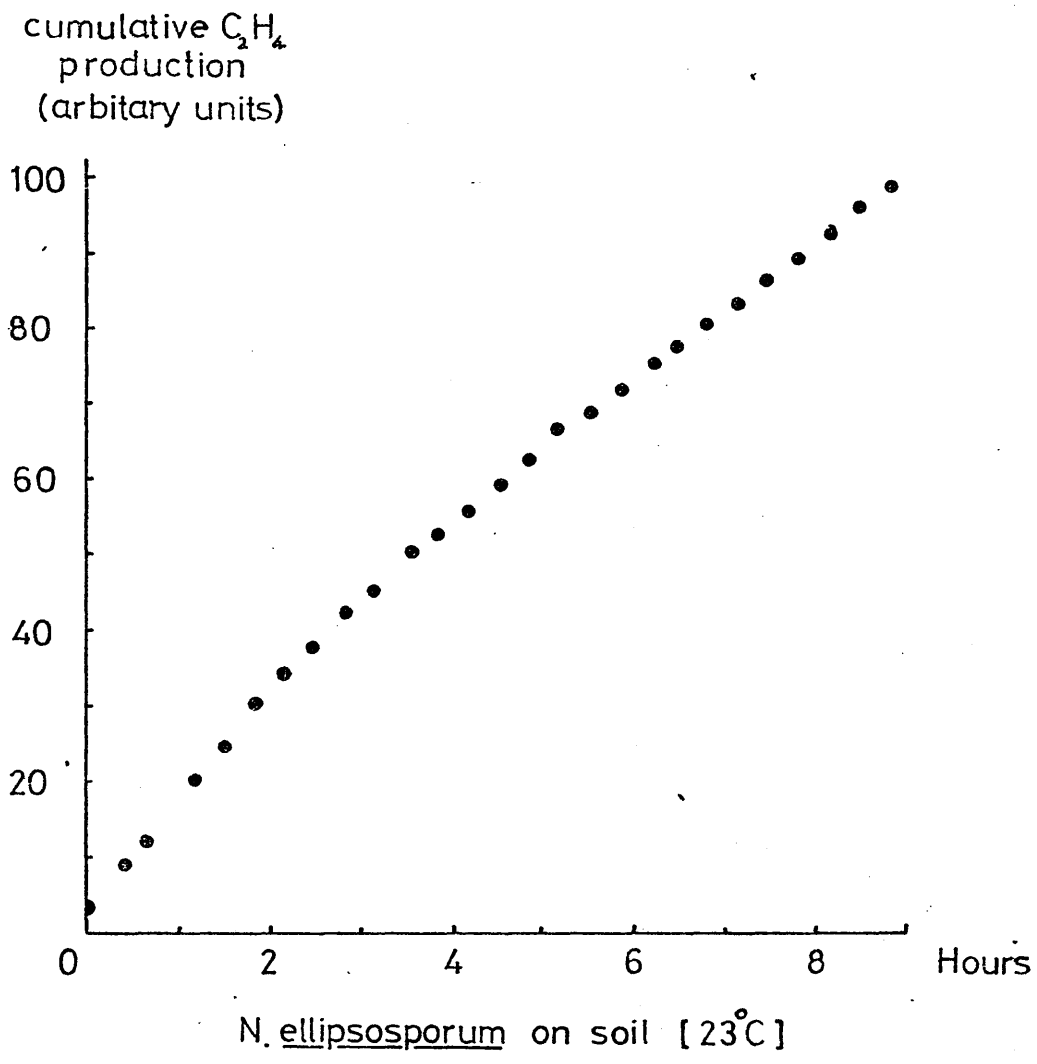
The chambers were used in replicate pairs, each pair being used to estimate fixation over a one hour period.

Acetylene was introduced into pairs of chambers on the hour every hour between 6.00 p.m. and 10.00 a.m the following morning. Gas samples were taken 30, 60 and 90 minutes after the addition of acetylene, and the pair of chambers was then discarded.

The samples were analysed in the usual way and the rate of N-ase activity estimated.

The variation in absolute fixation between test chambers is considerable because of variations in algal cover and in order to obtain comparable

Fig 4·1 Continuation of acetylene reduction in the dark



data the N-ase activity for each pot is expressed as a percentage of the midday value.

C. RESULTS

The results are shown in Fig. 4.2 overleaf.

The points on the graph joined by continuous lines represent the successive mean readings from particular pairs of chambers. Although there is some overlap in readings between 6.00 a.m. and 11.00 a.m., the expected increase in rate between successive readings can be observed.

The minimum recorded N-ase activity (4.30 a.m.) is 20% of the midday reading while the average night time fixation, based on a 16 hour day (8 hour dark period), is about 33% of the midday reading. Thus a significant part of the total fixation occurs at night.

D. DISCUSSION

Qualitative estimates of daily and seasonal nitrogen fixation have been made assuming a 16 hour day with no fixation occurring during the 8 hours of darkness. Although this assumption is evidently false, the error incurred does not appear to be very large, because the additional night time fixation is offset by a decrease in fixation at dawn and dusk. In comparisons between the two estimates on 14 occasions, the 16 hour light/8 hour dark method gave results that were between 4% lower and 11% higher than the 'continuous fixation' method.

Graph 4.3 shown overleaf was prepared so that nightime fixation rates could be estimated from the values measured in the field during the day.

The proportion of daytime fixation which continues at night is dependant on two factors.

(1) The light intensity during the day. The proportion of fixation continuing in the dark will be less if the daytime fixation is high.

(2) The decrease in temperature at nightfall.

The rate of dark fixation is related to temperature in an approximately linear manner over small temperature changes.

Fig 4·2 OVERNIGHT FIXATION (Broadbalk)

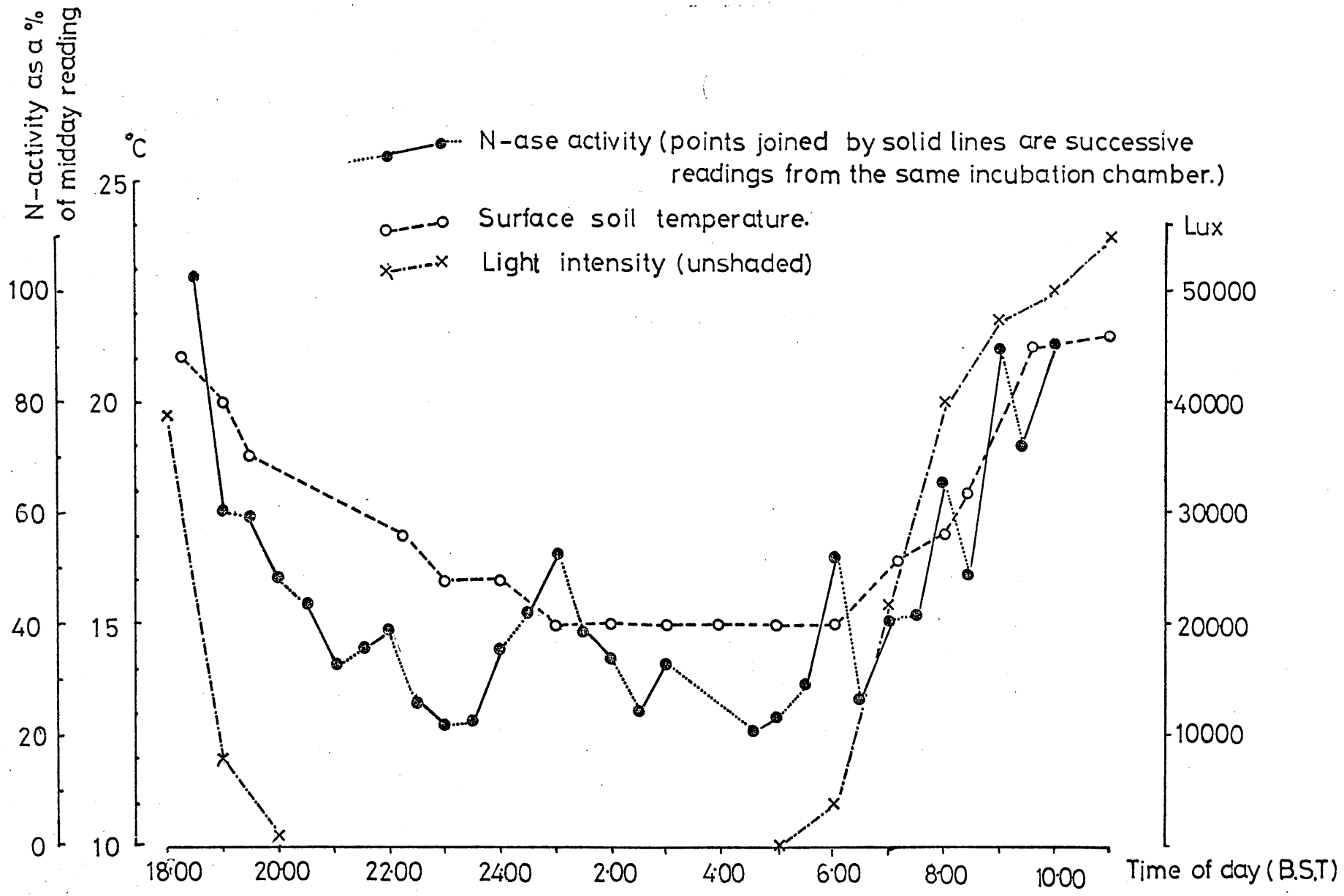
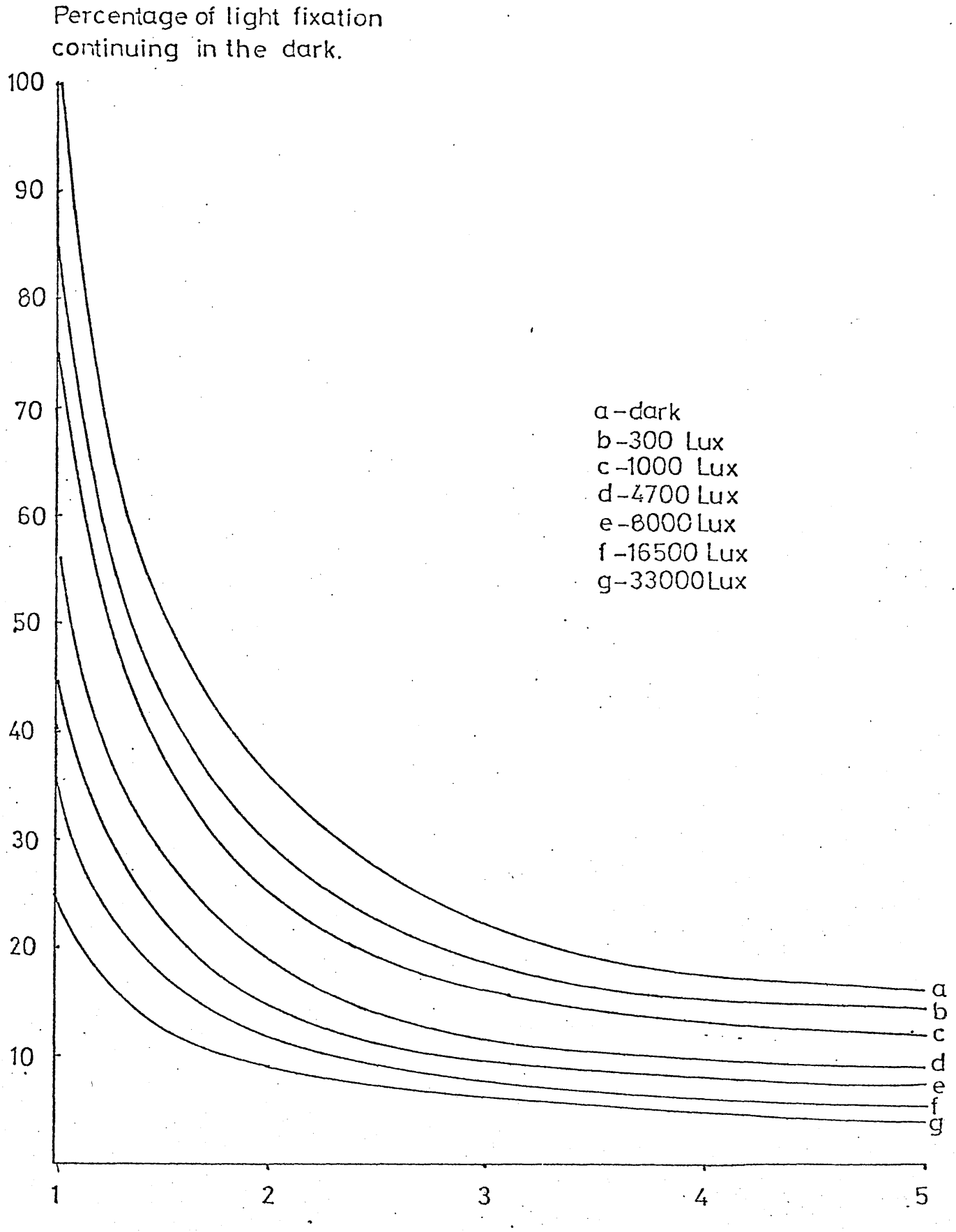


FIG 4.3 ESTIMATED PROPORTION OF LIGHT FIXATION CONTINUING IN THE DARK AT VARIOUS LIGHT INTENSITIES.



The graph is composite of results from a number of other experiments carried out by myself, in both the laboratory and in the field, concerning the affect of light intensity on fixation and the effect of temperature on the dark fixation rate. The effect of these two factors is cumulative, for example when a 6200 Lux light source is turned off the fixation drops to 51% of its previous value. If the temperature now drops by 50% (temperature day/temperature night = 2.0) the fixation is reduced further to 34% of its previous value. Thus on a day when the light intensity is 6200 Lux and the night time temperature is half of the daytime temperature

the night time fixation rate = Daytime fixation rate x $\frac{51}{100}$ x $\frac{34}{100}$

= Daytime fixation rate x 15/100

The values predicted from the graph appear to be accurate to within about $\pm$ 15% at temperatures between 10°C and 30°C.

The plots chosen for the overnight experiment were fairly open. The light intensity measured at midday under the wheat stand averaged 29000 Lux (Full sunlight was measured at 61000 Lux). The value predicted from the graph with this light intensity and a day/night soil temperature ratio of 1.6 (24°C/15°C) is about 17% of daytime fixation.

Light intensity measurements under wheat with various fertilizer and herbicide treatments show some intensities as low as 400 Lux and under a similar temperature regime the predicted dark fixation rate would be as high as 40% of the light value.

The results obtained in these experiments must be treated with caution since, unlike N_2 fixation, acetylene reduction does not require carbon skeletons to take up the reduced products. It is thus possible that although the algae can meet the requirements for acetylene reduction for long periods, carbon skeletons could become limiting for the reduction of N_2.

A simple laboratory investigation of ^{15}N fixation over an extended dark period would be of interest.

E. SUMMARY

The overnight nitrogenase activity in the field was estimated using the in situ incubation chamber technique during a period of fine weather in August.

The fixation appeared to continue throughout the night at a rate which dropped to about 20% of the midday value. Other experimental findings suggest that the proportion of daytime fixation which continues at night depends on the daytime light intensity and the drop in temperature at dusk.

SECTION 5. FACTORS AFFECTING NITROGEN RELEASE

A. INTRODUCTION

B. THE EFFECT OF ACETYLENE ON MEMBRANE LEAKAGE.

C. THE RELEASE OF NITROGENOUS SUBSTANCES UNDER NORMAL GROWTH CONDITIONS.

D. THE EFFECT OF FROST ON ALGAL CRUSTS.

E. THE EFFECT OF DESICCATION ON NITROGENASE ACTIVITY AND N RELEASE.

F. THE EFFECT OF RAINFALL DAMAGE ON ALGAL CRUSTS.

G. THE EFFECT OF PATHOGENIC INFECTION ON ALGAL CRUSTS.

H. SUMMARY.

5. FACTORS AFFECTING NITROGEN RELEASE

A. INTRODUCTION

The atmospheric N_2 fixed by the algal cells over the growing season is ultimately released by the death of the cells. Nitrogenous products are also released during active growth due to cellular leakage.

Fogg (1952) investigated the leakage of nitrogenous substances by A.cylindrica in relation to a number of cultural factors. He found that between 10 and 30% of the total N fixed appeared in the culture medium, and that the percentage release was highest in young cultures. The percentage was increased in older cultures by deficiencies of F_e, K and to a lesser extent Mo. Neither light intensity nor the addition of glucose to the culture medium appeared to have a significant effect. The 10-30% released products consisted of; NH_3 - N (0-2%), Amide-N (6-21%) and Amino-N (5.5 - 7.7%).

Taha and Refai (1962) note that 30% of the total N fixed by Nostoc commune is released into the culture medium. The nitrogenous products composing this 30% appear in the following proportions; NH_3 - N (28.5%), NO_3^- - N (37%), Amide-N (11%) and Amino-N (20.12%).

Watanabe (1951) investigated nitrogenous release by several algal species and obtained the following values for the percentage of the total N fixed appearing in the culture medium: Tolypothrix tenuis 15.6% Calothrix brevissima 73%, Anabaenopsis sp 26% and Nostoc sp. 24%. The investigations described in this section are primarily concerned with factors occurring in the field which might induce the release of nitrogenous substances. The soil on which the experimental material was grown was not sterilized, so that the prevailing microflora was similar to that which occurs in the field. In these conditions substances released by the algae are rapidly degraded. Experiments with un-sterilized soil normally yield ammonium salts as the final degradation product.

B. THE EFFECT OF ACETYLENE ON MEMBRANE LEAKAGE

In a number of experiments described in this section such as those involving the progressive desiccation of an algal crust, the same material was used for both the nitrogenase assay and measurement of soluble N. This assay involved the incubation of samples under 10% C_2H_2 for periods of up to 1 hour, and it seemed possible that C_2H_2 with its high lipid solubility might itself affect membrane permeability. In samples which have been inadvertently left under acetylene for 48 hours, phycocyanin and chlorophyll are visible in solution. Experiments were carried out in the laboratory to evaluate this effect.

Cultures of N.ellipsosporum and A. cylindrica growing on sand were used so that the total N could be determined using the Kjeldahl method.

1.2cm^2 cores were cut from the surface of the cultures and incubated under 10% C_2H_2 in universal bottles at 23^{o}C and 2000 Lux for the required time.

The results are shown in Fig. 5.1A and B. The values for NO_3^- - N, NH_4^+ - N and C_2H_4 production are means of 8 replicate bottles. The values for total soluble N are the means of 3 Kjeldahl digests.

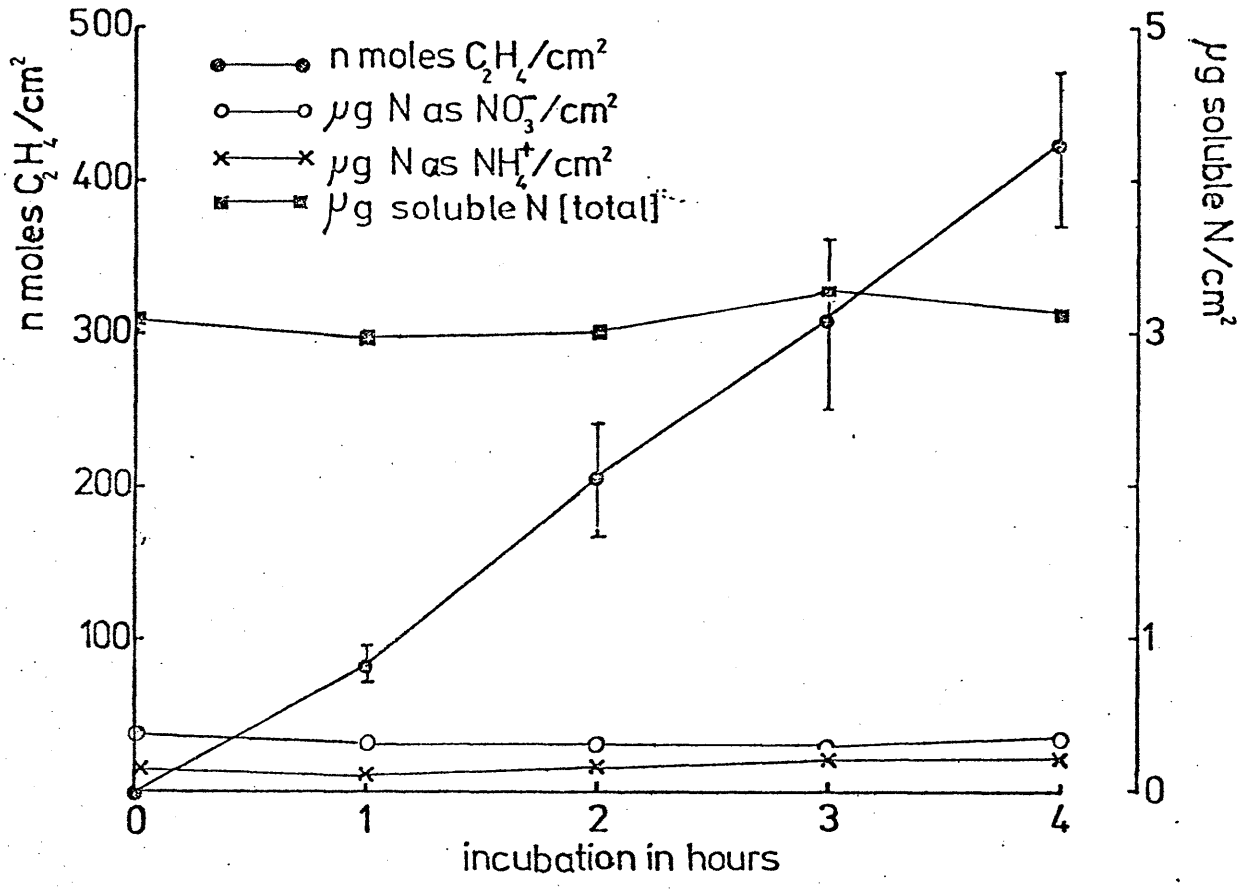
The ethylene production proceeds more or less linearly over 5 hours. There is no significant change in extracellular N over this period.

C. THE RELEASE OF NITROGENOUS SUBSTANCES UNDER NORMAL GROWTH CONDITIONS.

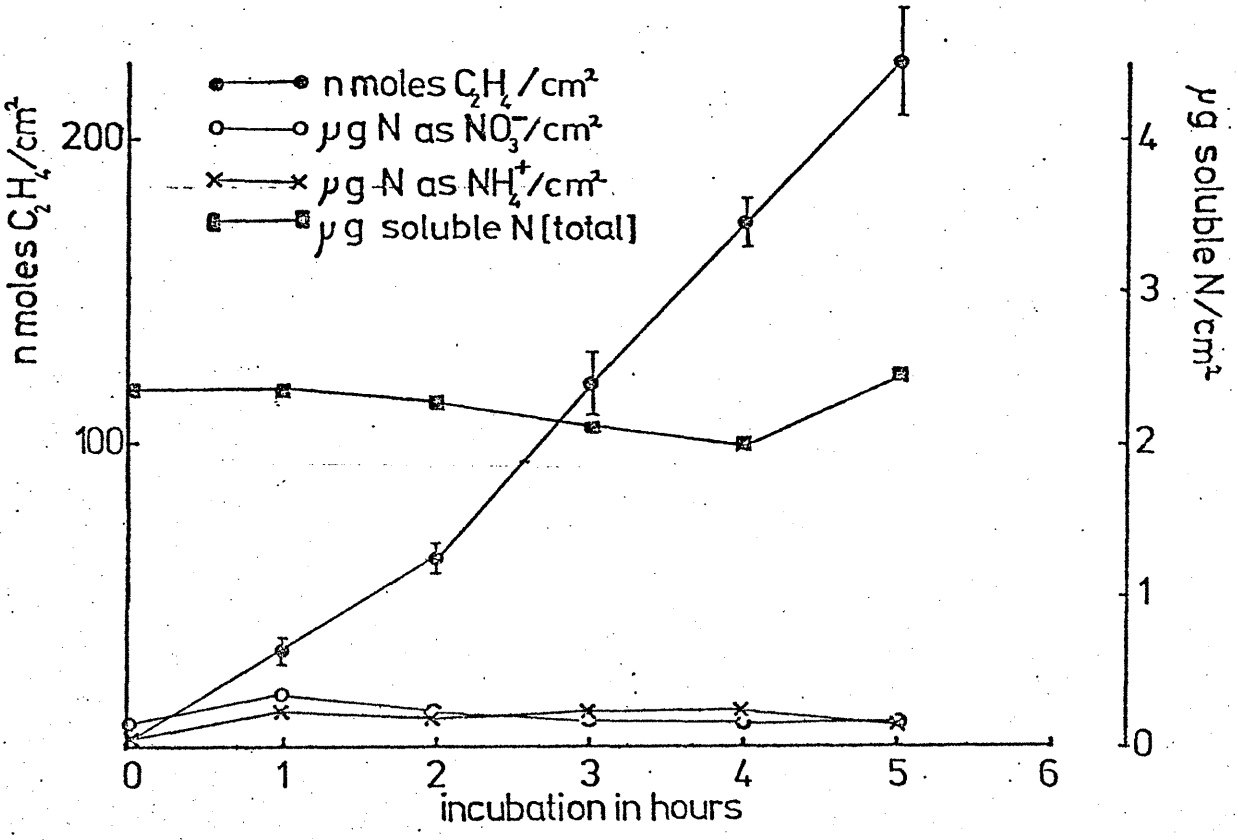
A series of experiments were carried out using N.ellipsosporum in liquid cultures on sand, and on soil.

For sand and water cultures the total N was estimated by Kjeldahl digestions. The ammonium salts produced by the digestion were assayed using an NH_4^+ ion selective electrode. Estimation of total algal N is not possible with soil grown material because of the high organic nitrogen content of this medium. Estimates of cellular leakage were therefore based on the measurement of NO_3^- and NH_4^+ concentrations using ion

FIG 5.1 EFFECT OF 10% ACETYLENE ON MEMBRANE LEAKAGE



A) Nostoc ellipsosporum on sand at 23°C and 1800 lux (Total N=0.282mg/cm



B) Anabaena cylindrica on sand at 23 C and 1800 lux (Total N=0.195mg/cm

selective electrodes.

The results obtained in liquid cultures are similar to those noted by other workers. Between 9 and 35% of the total N fixed appeared in the culture medium and NH_4^+ and NO_3^- represented only a small proportion of this (Fig. 5.2).

The results obtained from experiments on solid surfaces are substantially different. In all experiments using healthily growing material on solid surfaces, the proportion of extracellular N was low. Fig. 5.3 shows the results from a typical experiment carried out on sand. The high initial reading for percentage extracellular N is due to soluble nitrogen carried over with the liquid innoculum which was used to start the plate. The absolute quantity of extracellular nitrogen in the core samples remains relatively constant as the crust grows, but the proportion of the total which this represents falls as the total becomes larger, to a value which is usually below 2%. There are probably three reasons for this.

Firstly in liquid cultures disruption of the algal filaments may occur to a small extent because of stirring. No such disruption occurs on solid surfaces.

Secondly the algal filaments on a solid surface are in intimate contact with one another so that the volume of intercellular medium is small. Under these conditions, the release of a small quantity of material would cause considerable local concentrations opposing further release. This effect is the reverse of that noted in young cultures where a large volume of medium is available to each cell.

Thirdly it is possible that the intimate contact between neighbouring filaments and between the filaments and the substratum, might affect the nutritional status of the algae.

D. THE EFFECT OF FROST ON ALGAL CRUSTS

The nitrogen fixation estimated in the field decreased rapidly towards the end of September and the algal crust, which was apparent on many

Fig 5.2 Changes in extracellular nitrogen in sand grown cultures of Nostoc ellipsosporum

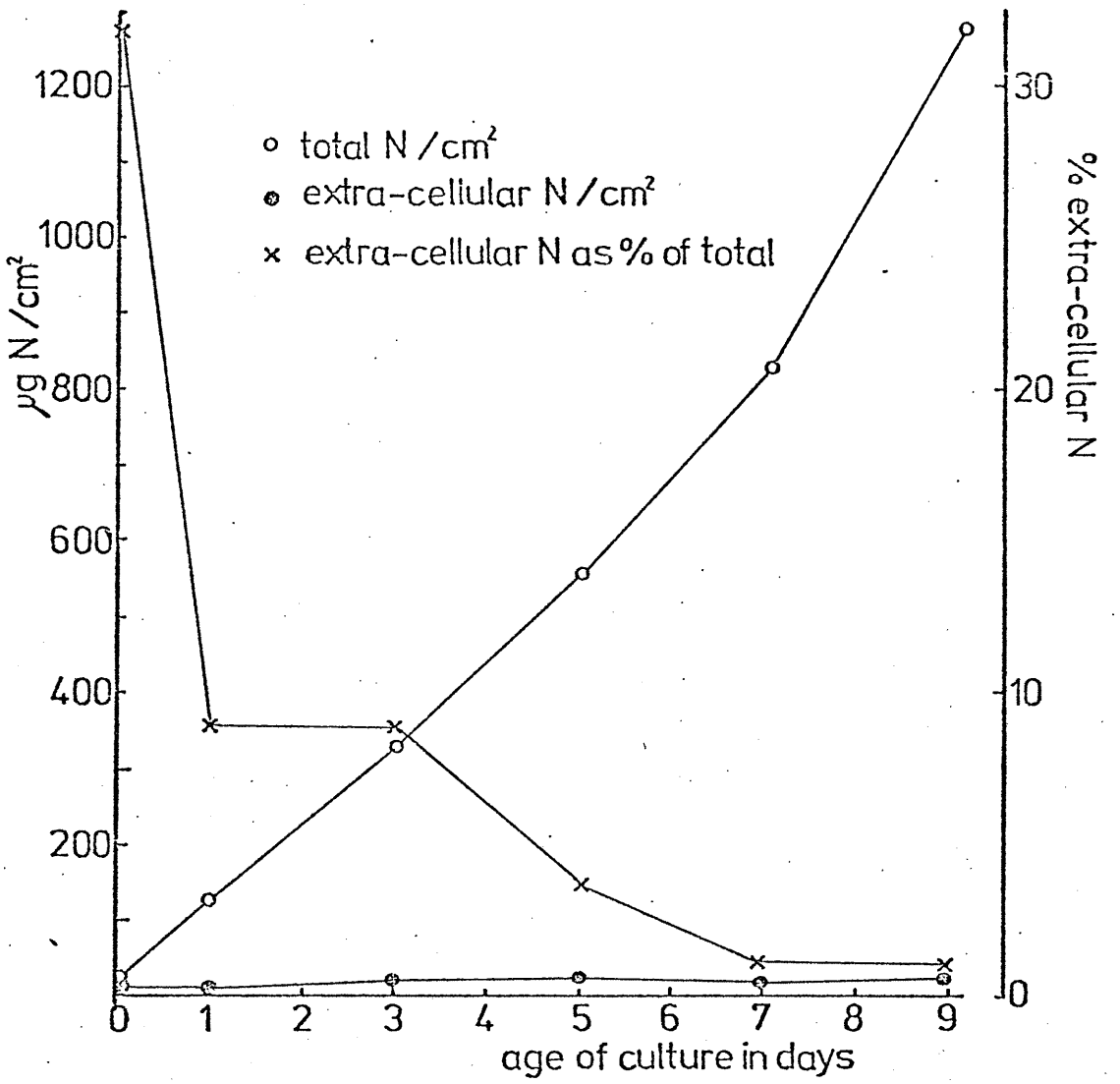
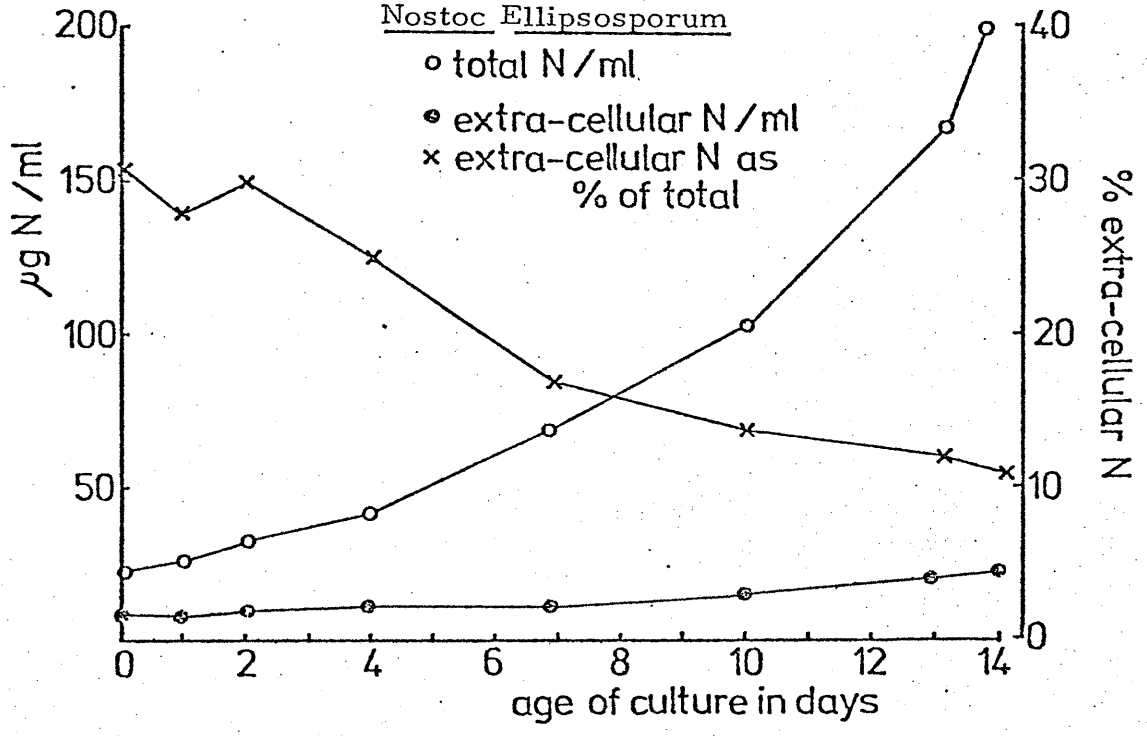


Fig 5.3 Changes in extracellular nitrogen in liquid cultures of Nostoc Ellipsosporum



of the low N plots after harvesting, disappeared even in areas which escaped ploughing. This decrease in algal cover is presumably attributable to a number of factors including daylength and temperature. It is possible that early frost might be an important factor causing cell death and nitrogen release.

A series of simple laboratory experiments were carried out in order to investigate the effect of freezing on algal crusts.

Uniform 8cm discs of N.ellipsosporum were cut from a soil tray together with about 1 cm depth of the underlying soil and placed in plastic Petri dishes. Accurate thermometers (graduated in 1/10°C intervals) were pushed into the surface of the crusts through holes in the side of the dishes and these units were transferred to suitable refrigerators.

The temperature decrease was noted and the samples were removed when the desired temperature had been reached and returned to the growth cabinets. The first samples were taken only after the crust had returned to room temperature.

Particular care was taken at 0°C where the soil water can exist in both a solid and a liquid state. The transition is readily observable since the cooling curve for the samples showed the normal plateaux at 0°C associated with the latent heat of fusion of water.

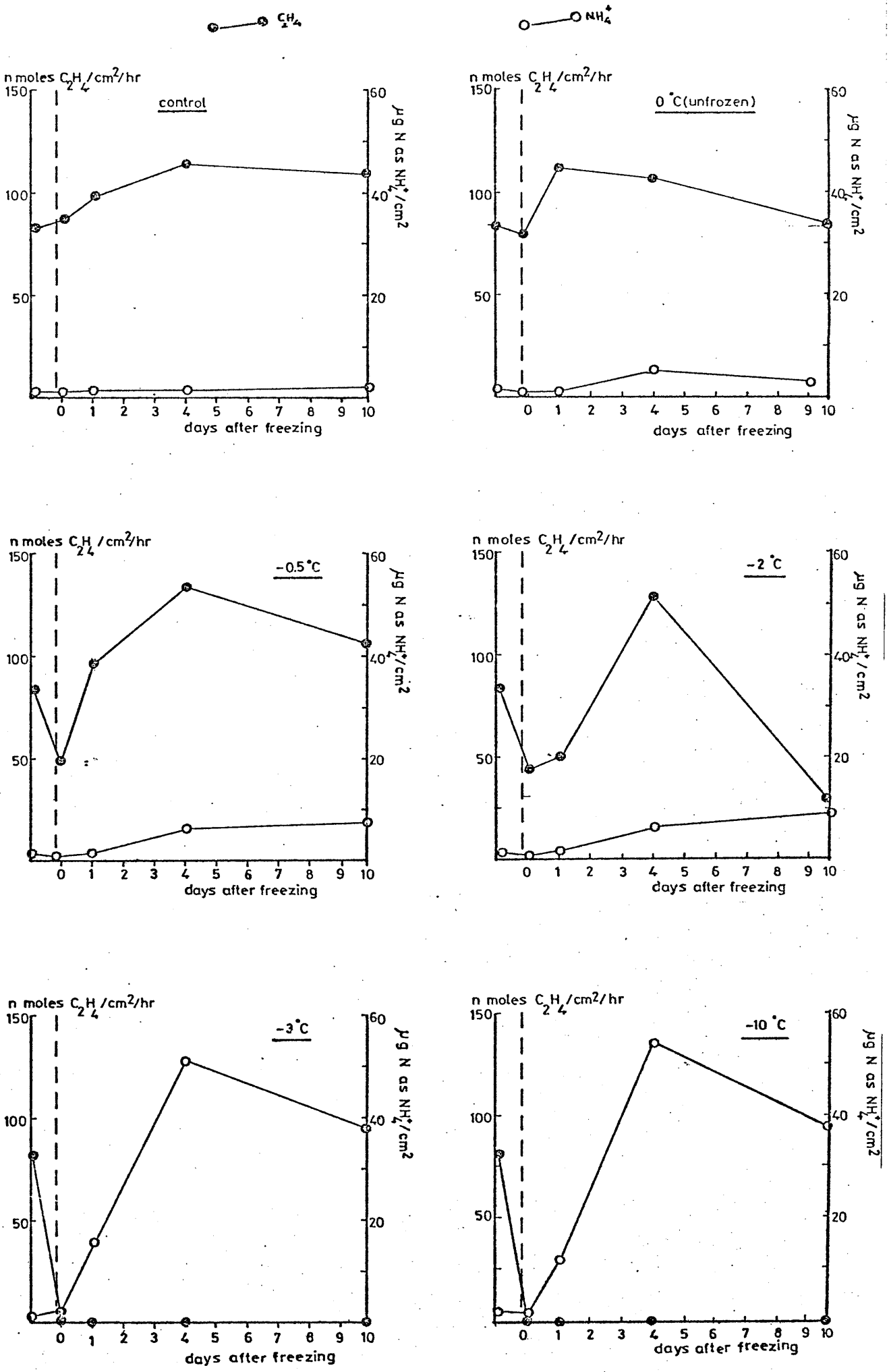
The 0°C sample shown in the graph was removed from the refrigerator at the onset of the plateaux before freezing had occurred. The -0.5°C sample was removed when the temperature started to fall again after complete freezing.

Care was also taken to prevent the samples resting on cold surfaces inside the refrigerator and freezing from the bottom upwards.

The results are shown in Fig. 5.4 overleaf.

The depression in activity noted immediately after freezing increases towards -3°C but at temperatures down to -2°C the full activity is recovered after 1-3 days. Although no cellular damage is apparent under the microscope, there is an increase in extracellular

Fig 5.4 EFFECT OF FREEZING ON SOIL GROWN CRUSTS OF NOSTOC ELLIPSOSPORUM



N evidenced by a progressive increase in the concentration of free NH_4^+, suggesting that the permeability of the cell membrane is affected.

C_2H_2 reduction in samples frozen to between -0.5°C and -2°C increases to a rate which exceeds the control in 3-4 days and then decreases. The increase may be attributable to an initial intracellular N deficiency caused by membrane leakage, offset, after a few days, by an NH_4^+ concentration which opposes nitrogenase synthesis and heterocyst formation.

At -3°C all nitrogenase activity is lost and the cells do not recover. Microscopic examination shows that all the vegetative cells have ruptured leaving only the akinetes and heterocysts intact. The effect of this is clearly seen in the entire crusts which lose their dark green appearance and become yellowed (See Fig. 5.5). The disruption of the cells is presumably caused by the freezing of the protoplast at a temperature between -2 and -3°C.

The fact that the NH_4^+ concentration increases with time up to the fourth day after cellular lysis suggests that NH_4^+ is not the primary product of cellular leakage but is produced by the reduction of the initially released nitrogenous substances in the soil.

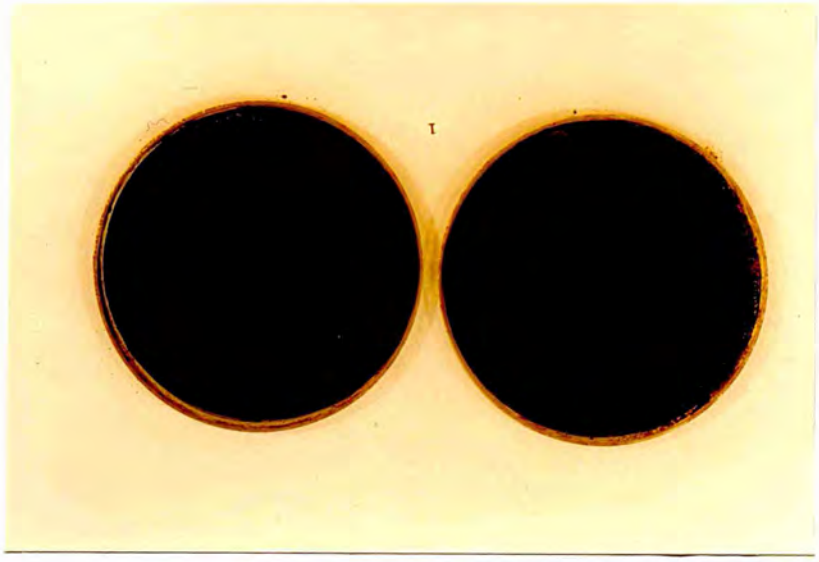
As the ruptured cells are broken down, the NH_4^+ concentration rises to a relatively high level. The 50.4μgN/cm^2 as NH_4^+ noted in the -3°C sample, for example; corresponds to 2930 ppm in soil water of the top 1 cm compared with about 5 ppm in the control.

Soil samples without algae frozen to -10°C showed no significant increase in free NH_4^+.

In all the plates frozen to temperatures below -3°C (including some frozen to -18°C) small dark green colonies reappear after 4-6 days. Microscopic examination shows that these are not N.ellipsosporum but a Lyngbya sp. which is favoured by the high nitrogen concentration.

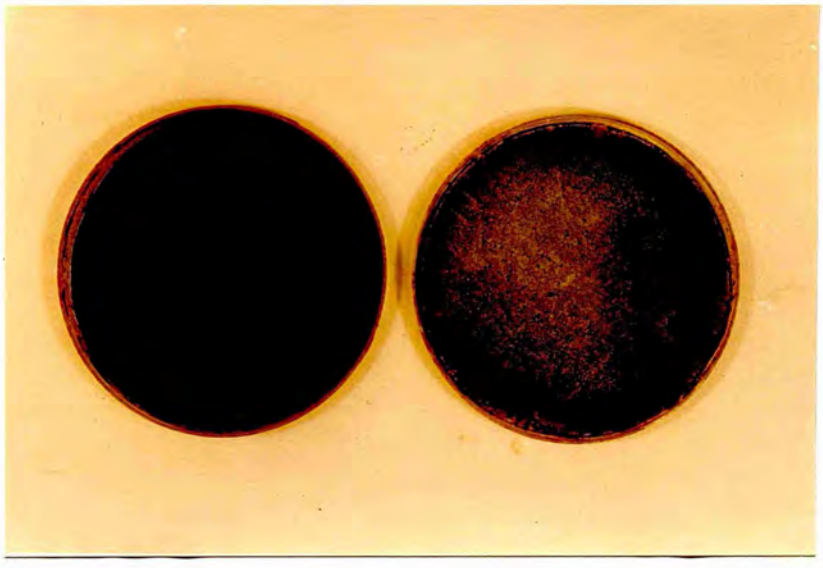
Occasional N.ellipsosporum akinetes can be seen developing to form filaments even in plates frozen to -18°C, but these remain few and

Fig. 5.5 The effect of freezing in soil grown crusts of Nostoc ellipsosporum



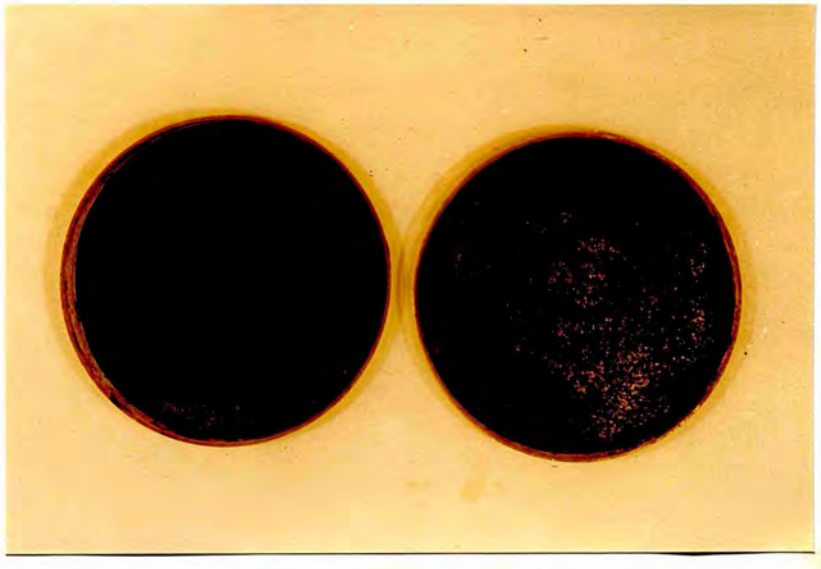
(A)

Control (Right) and experimental sample before freezing.



(B)

Control (~~Right~~ Left.) and experimental sample immediately after freezing. White areas are due to frost.



Control (~~Right~~ Left) and experimental sample 4 hours after freezing. The experimental sample is yellowed and microscopic examination showed extensive cellular lysis.

are eventually overgrown by the Lyngbya.

Freezing the samples to -3°C over periods of between 30 minutes and 6 hours had no effect on the result.

Watanabe (1959) notes a recovery of over 90% in cells of Tolypothrix tenuis immediately after freeze drying at -80°C in the presence of human serum albumen. Extremely rapid cooling of this type prevents the formation of crystals within the cell, but is of little relevance to conditions occurring in the field.

E. THE EFFECT OF DESICCATION ON NITROGENASE ACTIVITY AND N RELEASE

Field observations suggest that soil moisture is a major factor in controlling the distribution, growth, and nitrogenase activity of blue green algae. A simple laboratory experiment was designed to investigate this effect.

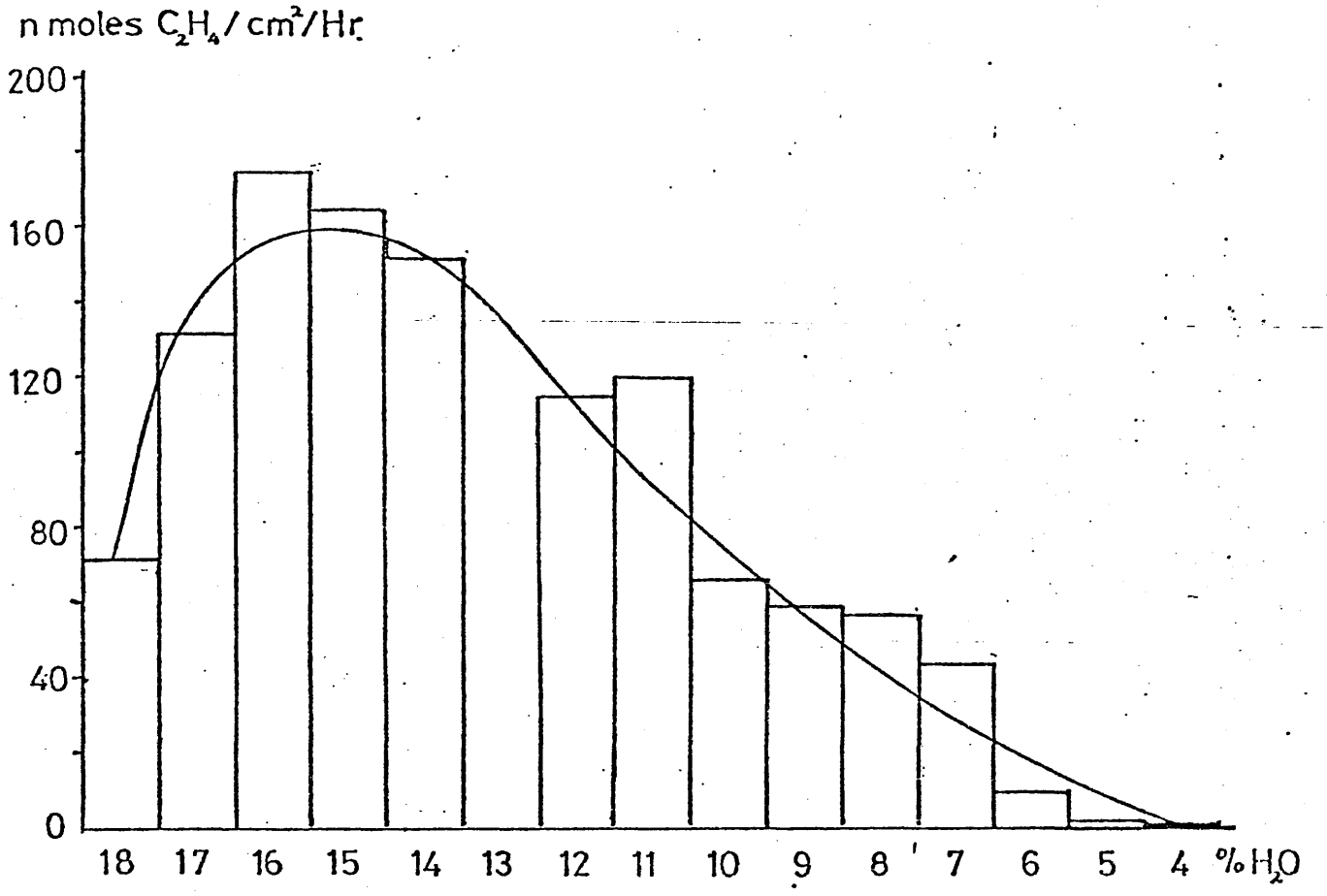
A uniform crust of N. ellipsosporum was grown on about 2" of soil in a 12 x 18" tray.

At the beginning of the experiment the glass cover was removed and the soil was allowed to dry slowly over 24 hours under continuous illumination. Three replicate samples, each containing 3 x 1.2cm^2 cores, were taken each hour and incubated under 10% acetylene at 23°C and 3000 Lux for 30 minutes. Gas samples were taken and analysed in the usual way and the soil cores were dry weighted at 105°C. Sub samples were taken for NH_4^+ and NO_3^- analyses.

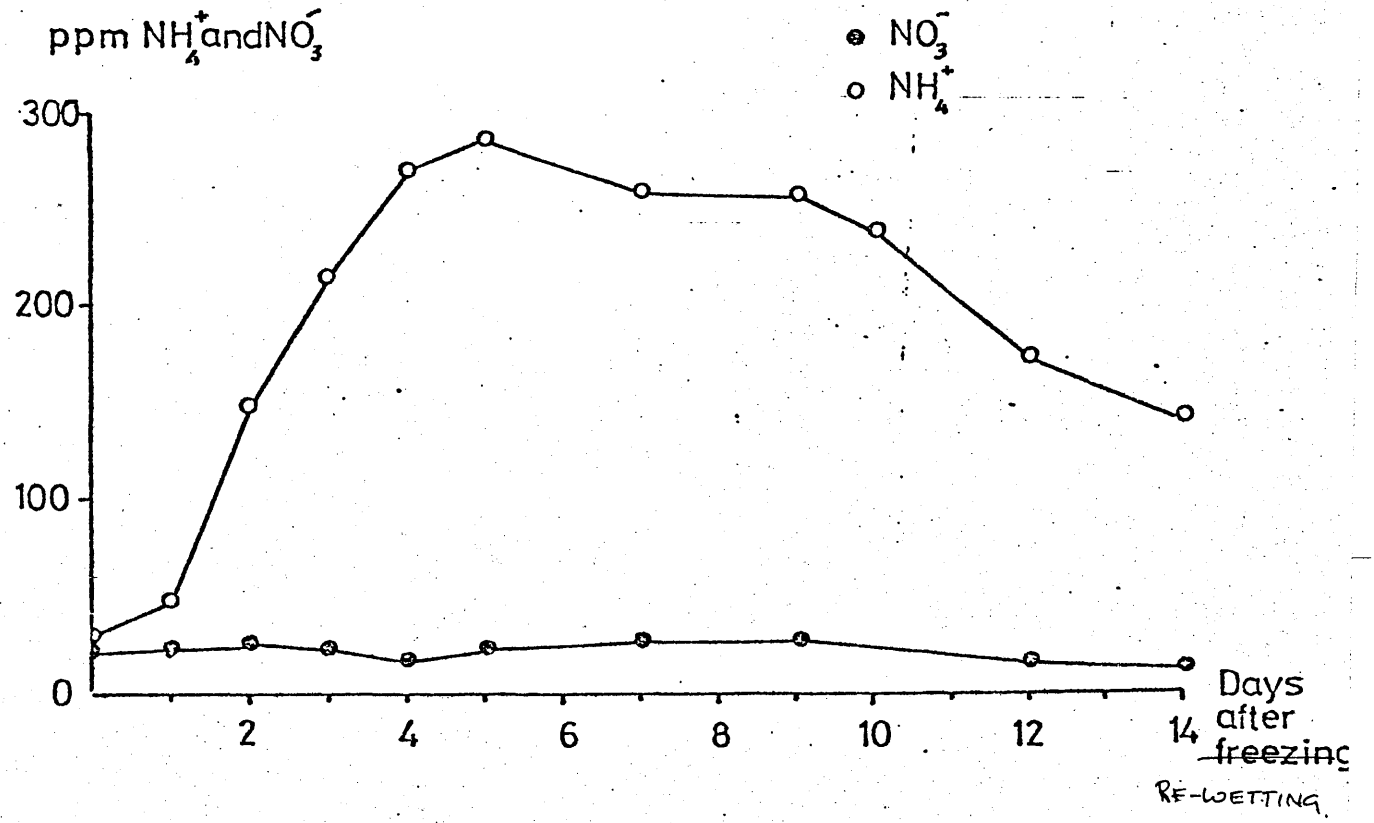
The results are shown in Fig. 5.6A. Each plot represents the mean of all results falling into a 1% moisture interval between 4% and 18% moisture There was no increase in the levels of free NH_4^+ or NO_3^- until the material was re-wetted after the experiment.

The rate of ethylene reduction increases to a maximum at 16% moisture. It would seem probable that this increase is associated with an increased diffusion rate through the surface water layer overlying the algae. With an 18% moisture content the algal surface appears shiny

FIG 5.6, A. The effect of progressive desiccation on the nitrogenase activity of soil grown crusts of Nostoc ellipsosporum



B. Increasing ammonia concentration after rewetting desiccated crust.



because of this water, but at 16% the surface assumes a dull appearance.

The rate of N-ase activity declines slowly from 16% moisture and ceases entirely at 4%. The soil appears dry and crumbles in the fingers when the moisture content falls below about 9%.

Fixation has been recorded in the field with moisture contents varying between 21% and 6%.

The culture tray was re-wetted after 48 hours and the free NH_4^+, NO_3^- and nitrogenase activity monitored for 14 days (see Fig. 5.6B).

There is no recovery of N-ase activity but a substantial increase in free NH_4^+ was noted with a maximum value occurring on the 5th day after re-wetting. The original concentration of 28 ppm increased to 285 ppm.

Microscopic examination showed that the vegetative cells had lysed completely leaving only heterocysts and occasional akinetes (see Fig. 5.7). A similar effect was observed even when the period of desiccation was extended to 7 days.

The results after the surface is re-wetted are very similar to those noted in the freezing experiment. The cellular lysis and NH_4^+ surge was followed in 6-7 days by re-colonization of the surface by Lyngbya (Fig. 5.7D) and an associated decrease in NH_4^+ (Fig. 5.6B).

The recovery from partial desiccation (down to about 8% water) is rapid, without a concommitant release of NH_4^+. The optimum and minimum moisture content of N-ase activity depends to a considerable extent on the physical nature of the soil and the results so far described only apply to a fine sandy soil.

Several similar experiments were carried out on sand and the algae appear to stop fixing when the moisture content in the core samples falls below about 20%. These experiments suffer from the disadvantage that the moisture content in the sand does not necessarily follow that of the algal crust. Because of the open texture of sand the capillary rise is small compared with that in soil, and the water column breaks in localized areas

Fig. 5.7 The effect of desiccation on a soil grown crust of Nostoc ellipsosporum.

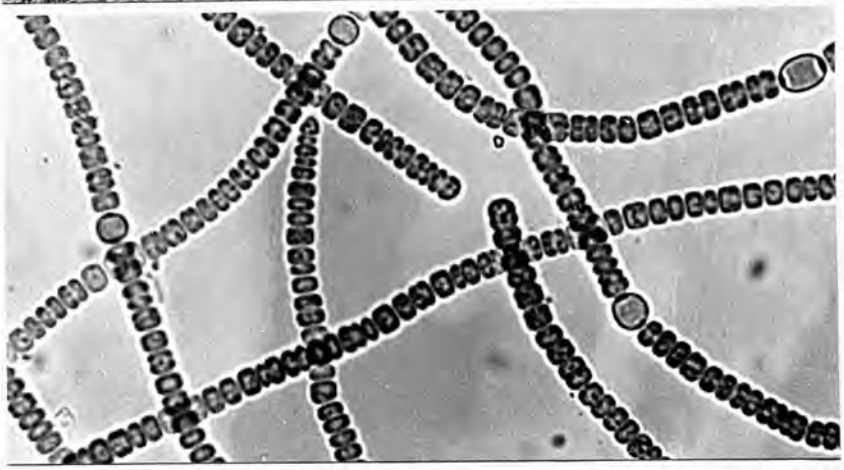
(A) Portions of the crust removed from the culture tray before desiccation (left) and 2 days after desiccation and re-wetting (right)

(B) Healthily growing N.ellipsosporum taken from the tray before drying (x 1300).

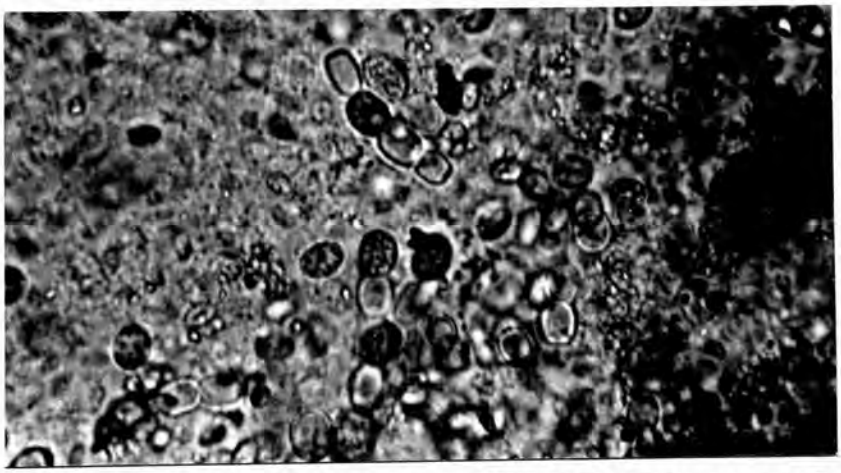
(C) Re-wetted material 2 days after drying. The vegetative cells have lysed leaving considerable amounts of cellular debris together with akinetes and heterocysts (x 1300).

(D) Re-wetted material 8 days after drying. Fine filaments of Lyngbya can be seen recolonizing the surface (x 1300).

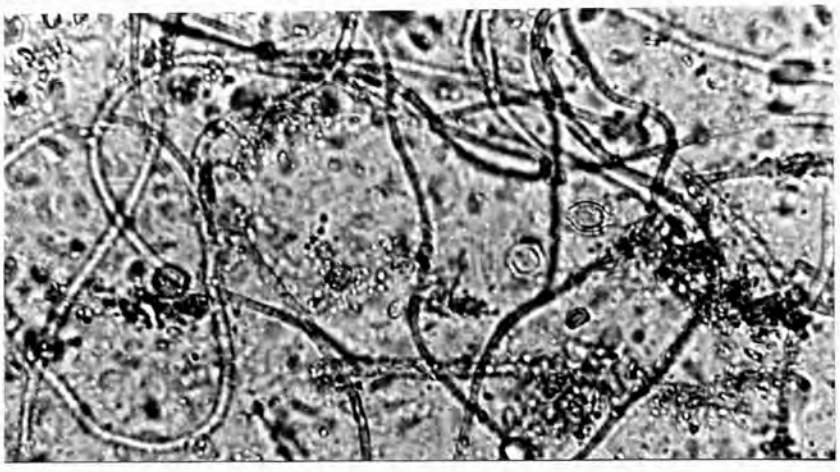
A



B



C



D

when the moisture content drops below 25%. The gelatinous algal crust then dries rapidly and curls away from the surface. The dried algal material in these experiments produced an NH_4^+ surge similar to that in soil when the samples were re-wetted, but the sand did not give rise to colonies of Lyngbya.

Although the N.ellipsosporum used is a strain which was originally isolated from Broadbalk, it had been maintained for a period of two years under conditions of continuous uniform moisture on agar slopes in the laboratory and finally grown in liquid culture for several months before inoculation onto the sand tray. Repeated asexual division under these circumstances may have led to physiological changes which have reduced the tolerance of the strain to desiccation.

The experimental findings described are, however, supported by field observations and experiments (See section 6).

F. THE EFFECT OF RAINFALL DAMAGE ON ALGAL CRUSTS

Observations in the field indicate that blue-green algae are seldom found on a loose substratum, but rather colonize areas where the soil is firm and compacted.

These areas include flattened lanes between the wheat rows, the sides of large clods of soil, and local depressions such as those left by foot-prints or tractor tyres.

During heavy rainfall the soil particles in the uncompacted areas move over each other producing a mild grinding action possibly damaging and/or burying the algal filaments.

In order to evaluate the mechanical effect of rainfall as an environmental factor controlling algal distribution and possible nitrogen release, several simple experiments were carried out with N.ellipsosporum. These experiments were of two types.

1. Algal material grown in the laboratory on uncompacted soil was subjected to heavy watering for a short period.
2. Algal material was shaken vigorously with a dense suspension of quartz sand using a wrist action shaker for periods of up to 4 hours.

Neither type of experiment produced a significant effect.

The algae buried by watering grew back to the surface in a few days and even after shaking with sand for 4 hours the extracellular nitrogen increased very little. Microscopic examination showed that the mean filament length had decreased, but the algae grew readily when it was re-inoculated on soil.

Laboratory and field experiments, discussed earlier, show that desiccation is a critical factor controlling algal distribution in the field, and it would seem probable that the lack of algal cover on loose soil is attributable to this factor. In areas where the soil is uncompacted the water column is readily broken allowing rapid surface desiccation even when the underlying soil is damp. Algal development in the depressed areas may be enhanced by puddle formation after rainfall.

G. THE EFFECT OF PATHOGENIC INFECTION ON ALGAL CRUSTS

The pathogenic destruction of soil grown algal cultures has been observed in the laboratory on several occasions. The effect of one such infection on nitrogenase activity, NO_3^+ and NH_4^+ concentration in a soil grown culture of N.ellipsosporum is shown in Fig. 5.8.

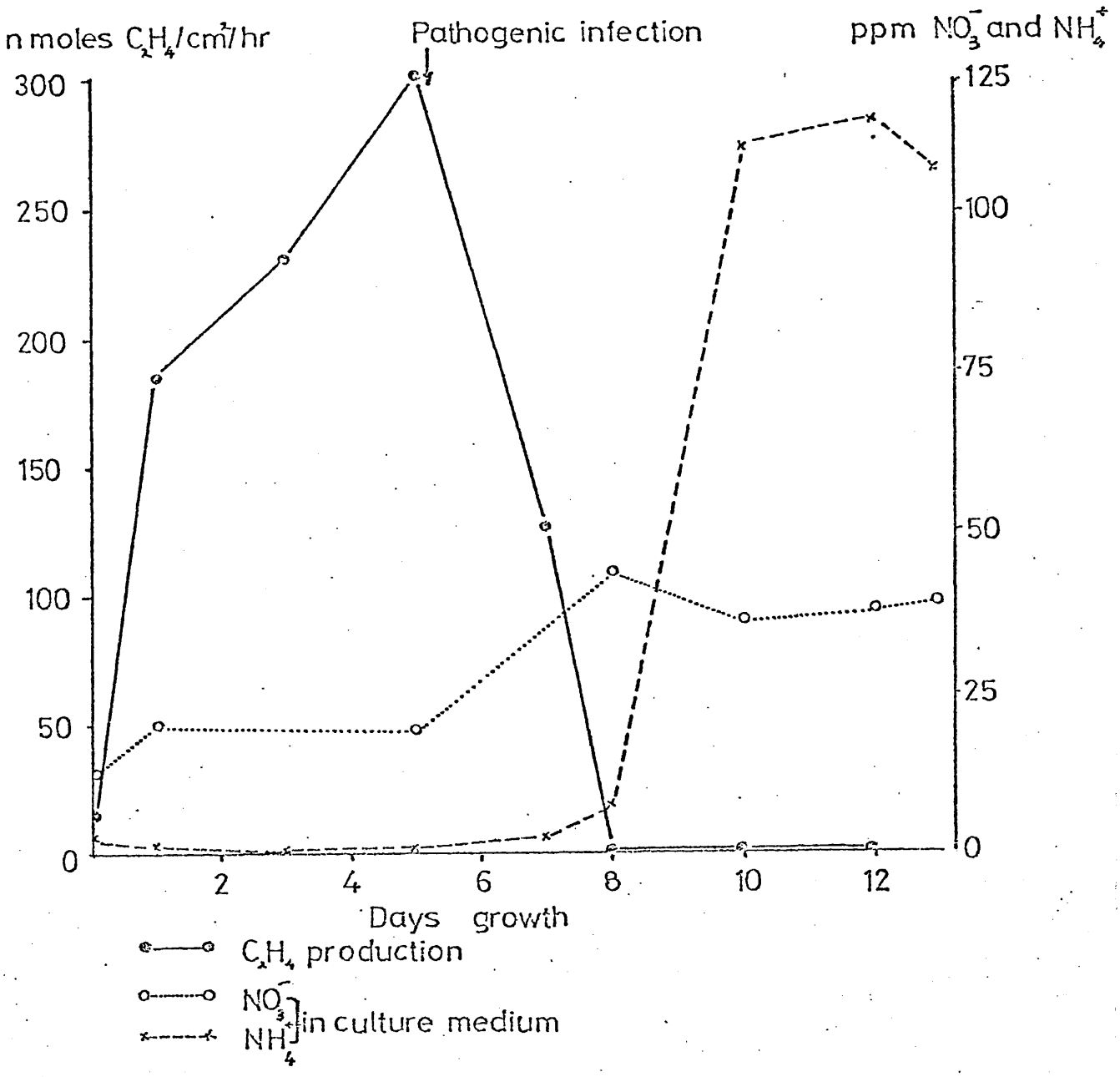
The sharp decrease in nitrogenase activity after the sixth day of growth is associated with a visible yellowing of the soil plate which appeared to spread out from a single locus. The fixation ceased entirely on the eigth day and microscopic examination showed that most of the vegetative cells had lysed.

The ammonium concentration did not rise appreciably until two days later when the released nitrogenous compounds had been degraded.

The pathogenic organism was not identified. Microscopic examination of the infected material revealed the presence of large numbers of motile rod-like bacteria but it is not known if these are the primary cause of lysis, or whether they are secondary, merely utilizing the available organic compounds.

Pathogens appear to be favoured by overly wet plates and the

FIG 5.8 The effect of pathogenic infection on acetylene reduction and nitrogen release.



infection can be spread from plate to plate by the transfer of infected material.

H. SUMMARY

The release of nitrogenous substances by cultures of N.ellipsosporum under various environmental conditions was investigated.

The acetylene reduction assay which was used in some of the experiments did not appear to induce membrane leakage in liquid cultures of either N.ellipsosporum or A.cylindrica.

Under normal growth conditions in the laboratory liquid cultures released into the medium between 9 and 30% of the total nitrogen whereas the release by sand grown cultures was smaller with less than 2% of the total nitrogen appearing in the culture medium.

The vegetative cells of soil grown cultures which were temporarily frozen to -3°C underwent extensive cellular lysis resulting in an immediate and permanent cessation of nitrogenase activity, followed in two to three days by a surge in ammonia concentration. Cooling to temperatures of between 0 and -2°C caused some nitrogen leakage and a temporary drop in nitrogenase activity, but this recovered after one to four days to give rates of acetylene reduction which exceeded those of the controls.

A similar cellular lysis, cessation of nitrogenase activity, and ammonia surge occurred when soil samples bearing algal crusts were allowed to dry to below a 4% moisture content over a period of 24 hours or 7 days. Acetylene reduction was optimal at 16% H_2O and the recovery of activity by samples which had been only partially desiccated (about 8% H_2O) was rapid.

Although occasional filaments of N.ellipsosporum could be seen germinating from the akinetes which remained after freezing or drying, the surface of the plates was, in fact, recolonized after about 7 days by Lyngbya which may have been favoured by the high soluble nitrogen concentration.

The grinding and burying action of the soil which appears to occur on

loosely compacted areas in the field during heavy rainfall was simulated in the laboratory. Extracellular nitrogen did not increase significantly when cultures were shaken vigorously with sand, and the algal cells buried by heavy watering rapidly recolonised the surface.

Nitrogen release by laboratory grown sand cultures which were lysed by pathogenic infection was observed.

SECTION 6. THE RECOVERY OF NITROGENASE ACTIVITY AFTER A PERIOD OF DRY WEATHER.

A. INTRODUCTION.

B. RESULTS.

C. DISCUSSION.

D. SUMMARY.

6. THE RECOVERY OF NITROGENASE ACTIVITY AFTER A PERIOD OF DRY WEATHER

A. INTRODUCTION

An experiment was carried out in July 1974 to investigate the recovery of fixation after a 3 week period with very little rain. An area of Broadbalk plot 3 (Herbicide treated) about a meter square was selected for the conspicuous presence of algal cover. Samples were taken for dry weighting and as controls, and the area was thoroughly watered. 6 replicate 1.5 cm^2 core samples, selected for the visual presence of algae, were taken from this area at approximately hourly intervals and incubated in universal bottles with 10% acetylene at 18^{o}C and 3000 Lux.

Because the samples could have become active during the incubation, the gas phase in each bottle was sampled at intervals to give a series of overlapping time courses.

B. RESULTS

The soil moisture before watering varied from between 4.5% and 4.8% in the open areas to about 6.0% beneath stands of Equisetum. After watering, the moisture content increased to about 15% over the entire area. The nitrogenase activity of dry control samples taken before the plot was watered was negligible. The ethylene production by samples placed under acetylene 15 minutes after wetting is shown in Fig. 6.1. Although the nitrogenase activities vary considerably from bottle to bottle all become active after about 200 minutes.

The samples assayed 75 and 138 minutes after the plot was wetted, give similar results and in order that the lag periods could be compared, the mean fixation rates for each batch of samples was calculated. These values are compared graphically in Fig. 6.2.

The curves have been lifted off the lower X axis so that the points can be clearly separated and adjusted so that points lying above each other are coincident in time since wetting. Acetylene was added to the three batches of samples at "A" on their respective X axes. All the samples taken from the plot become active about 200 minutes after wetting irrespective of when

FIG 6.1 Recovery of nitrogenase activity by dessicated core samples

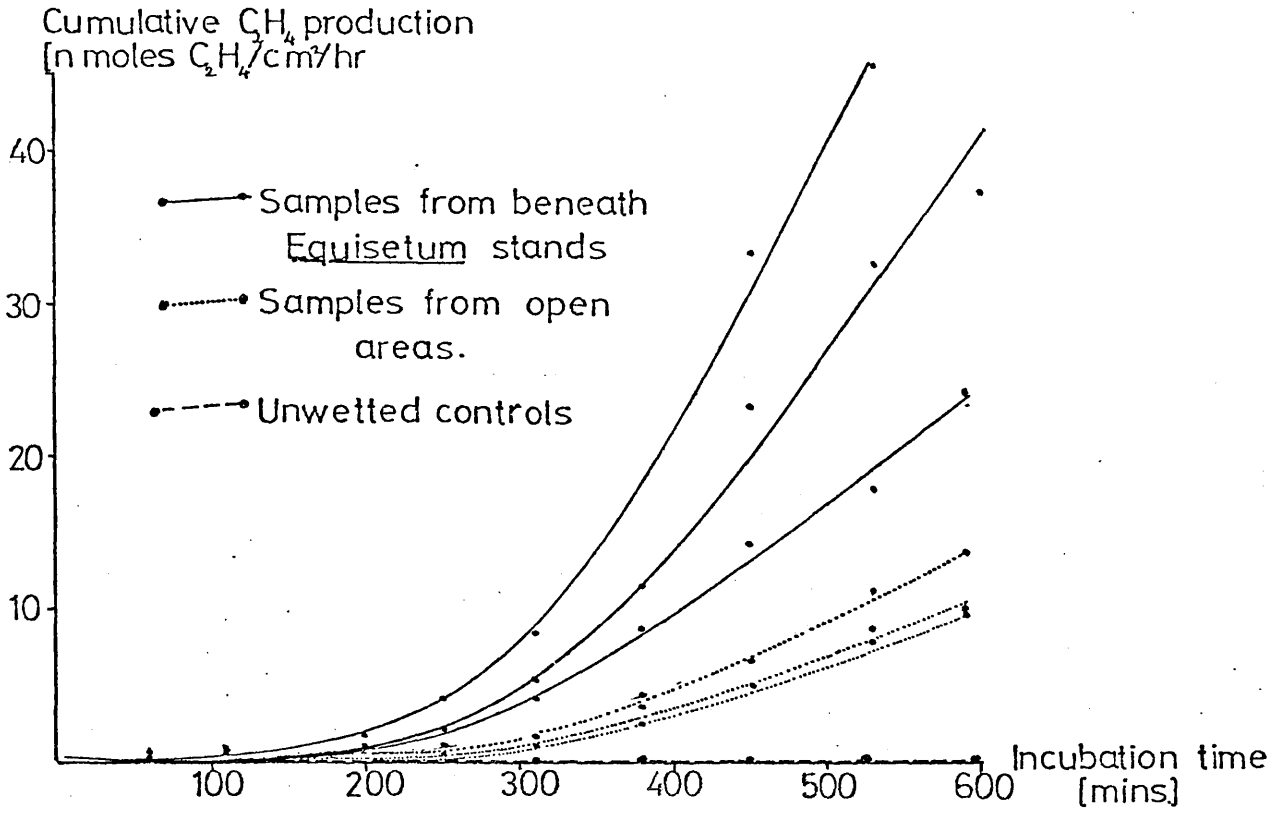
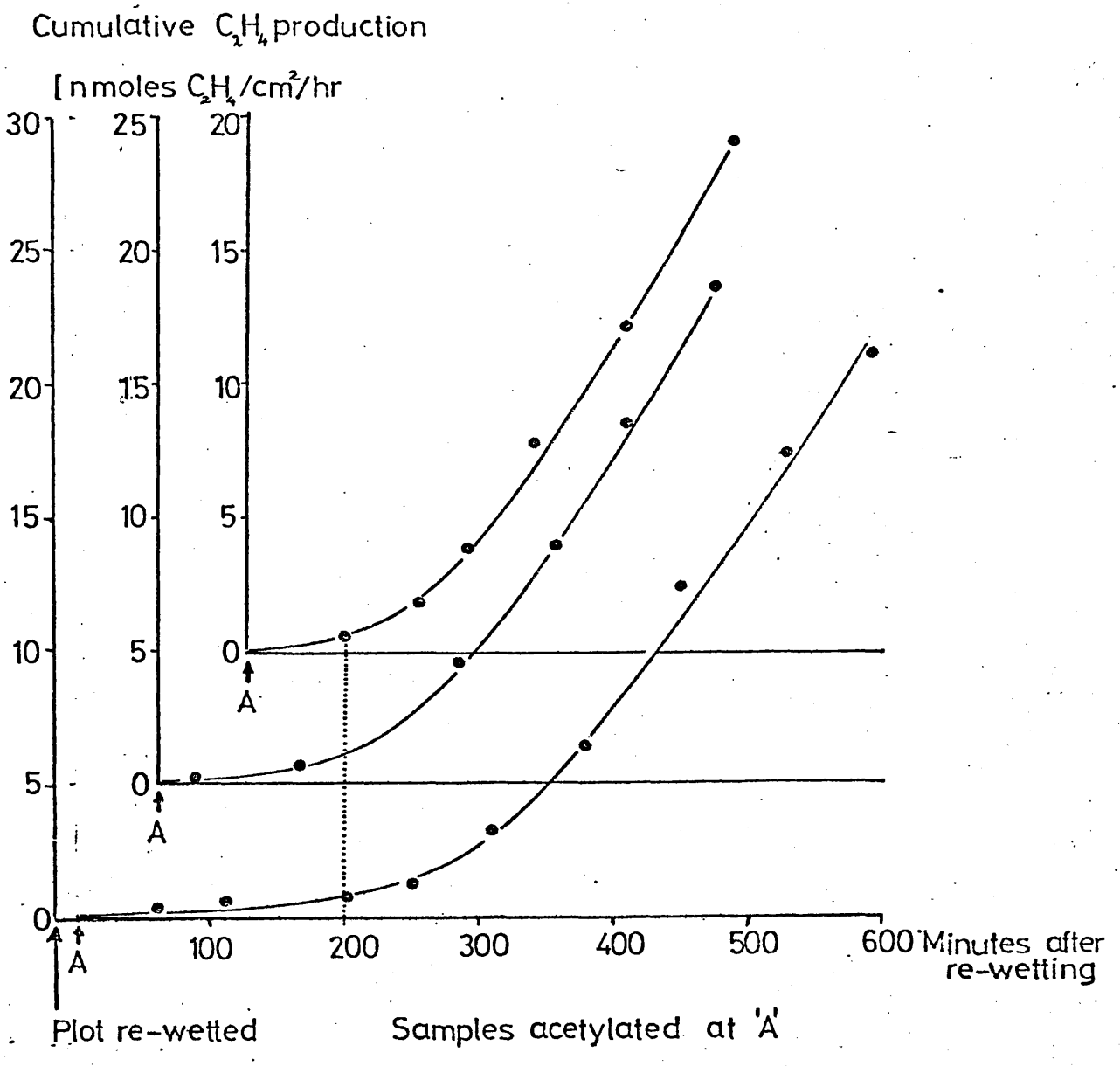


FIG 6.2 (See text for explanation)



the acetylene was added, and achieved an approximately steady rate after 450 minutes irrespective of the magnitude of recovery (see also Fig. 6.1).

Fig. 6.3 shows the change in rate of fixation with time in samples from beneath the Equisetum and samples from open areas. The values have been derived from the mean gradients shown in Fig. 6.1. Although the less desiccated samples from beneath the weeds show a greater recovery of activity, both curves flatten to an approximately steady rate after 450 minutes of 8.1 and 2.3 moles $C_2H_4/cm^2/hr$.

The weed covered and open areas were sampled again the following day and gave fixation rates of 9.03 and 2.26 moles $C_2H_4/cm^2/hr$ respectively.

C. DISCUSSION

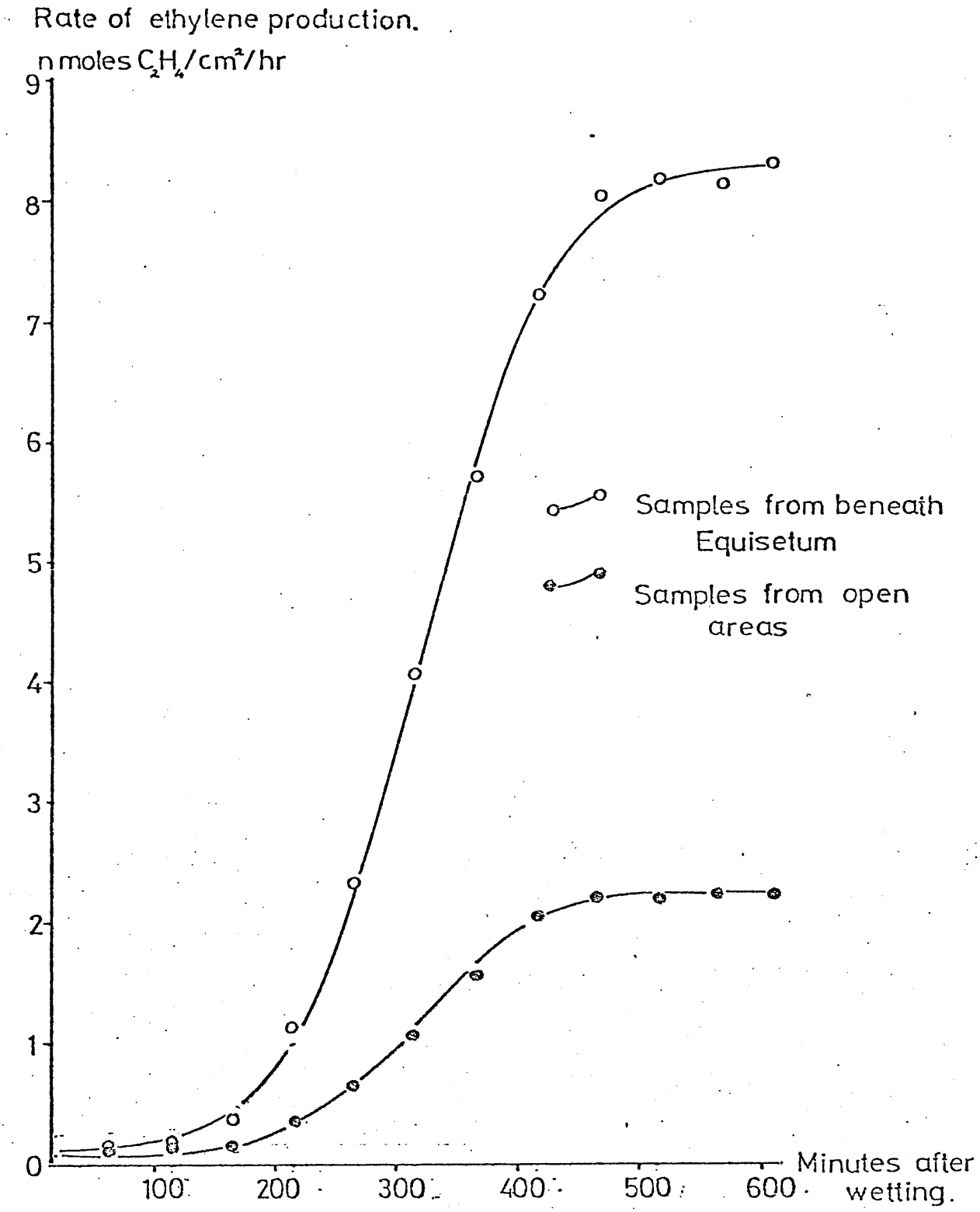
These results, together with the observed lag between rainfall and recovery of fixation and the laboratory evidence for the lysis of the vegetative cells of the indigenous strain of N.ellipsosporum lead to the hypothesis that a large proportion of the vegetative cells comprising the naturally occurring blue-green algal population of Broadbalk are destroyed during periods of drought. It would appear that cell systems which are able to recover do so within 450 minutes and that increases thereafter, to the next maximum in nitrogenase activity, are associated with increases in cell numbers by growth.

Although the area covered by a visible algal crust was greater beneath the weeds than in the open areas the density of cover, where the crust was present, appeared to be similar in the two locations.

The different fixation rates given by samples from the two areas may be solely due to differences in initial algal development, but they may also reflect the degree of de-activation by desiccation in the two areas.

The fact that the time taken for recovery is the same irrespective of the magnitude of fixation attained may thus suggest that the individual cells are not 'more or less' innactivated by desiccation, but are rather destroyed or not destroyed, so that the degree of recovery is dependant

FIG 6.3 Recovery of fixation by desiccated algal field samples.



on the proportion of cells remaining in a viable state.

The mean nitrogenase activity recorded on plot 3 (Herbicide) during the previous maximum was 10.3 nmoles $C_2H_4/cm^2/hr$. Samples from beneath the Equisetum, with soil moisture contents of between 5.2 and 6.1% regained about 90% of this activity compared with about 20% for the visually similar samples from the open areas with moisture contents of between 4.5 and 4.8%. Assuming that the original algal cover was similar, these values suggest that about 10% of cells from the more moist samples and 90% of the cells in the drier samples have been destroyed by desiccation.

In the field, in open areas and under wheat which has been herbicide-treated to remove much of the ground cover, the surface desiccates rapidly even in short dry periods and this must contribute substantially to the lack of algal cover in these areas.

Even in well protected areas under good wheat stands the surface desiccates during long dry periods towards the end of the growing season, and this must result in the dumping of considerable quantities of N in the next period of rain. Under these circumstances it is probable that the crop is too old to benefit from the available N.

D. SUMMARY

An area of obvious algal cover in the field was re-wetted after a period of dry weather and the recovery of nitrogenase activity investigated.

Samples with a slightly higher moisture content taken from beneath stands of Equisetum showed a greater recovery than samples from drier areas, but all samples became active about 200 minutes after re-wetting irrespective of the magnitude of recover.

SECTION 7. SEASONAL MONITORING OF NITROGENASE ACTIVITY ON BROADBALK.

A. INTRODUCTION.

B. METHOD.

C. RESULTS.

(1) Estimates of Annual Fixation.

(2) The Effect of N,P,K,Na and Mg.

(3) The Effect of Herbicide Treatment.

(4) Algal Cover and Species Composition.

D. SUMMARY.

7. SEASONAL MONITORING OF NITROGENASE ACTIVITY ON BROADBALK

A. INTRODUCTION

Broadbalk is one of the classic fields at Rothamsted Experimental Station which has been cultivated under recorded conditions since 1843. The lower end of the field is divided into plots which have been sown to winter wheat continuously for 130 years (see Fig. 7.1). Each plot receives a separate fertilizer treatment which has remained substantially the same over this period. The arrangement of the plots and the fertilizer treatments which they receive, together with the crop yields, are shown in Fig. 7.2.

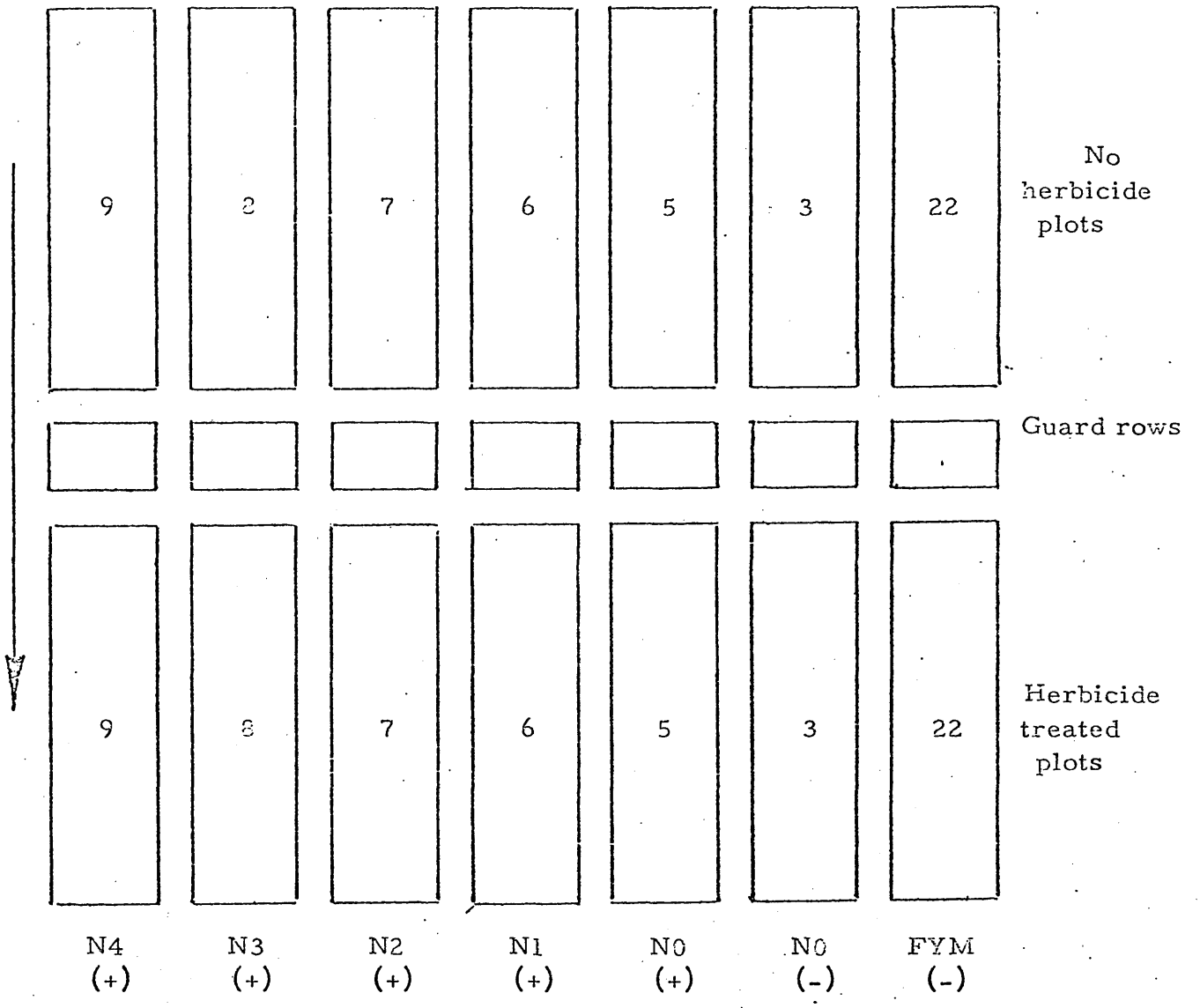
The topsoil is a silt loam overlying clay-with-flints and chalk which provides good natural drainage (Avery and Bullock 1968). The pH is adjusted to about 7.5 by the periodic addition of lime. This has been particularly necessary on plots receiving high levels of nitrogen because the ammonium sulphate used up to 1967 produced a marked drop in pH. From 1968 onwards nitrogen fertilizer was applied as nitro-chalk.

Jenkinson (1971) investigated the nitrogen economy of Broadbalk and estimated that rainfall accounts for 1.4 Kg N/ha/annum, dry sorption of ammonia a maximum of 13 Kg/ha/annum and organic nitrogen in rain, dust and bird droppings 1.5 Kg/ha/annum. Allowing for these gains the nitrogen balance sheets for the continuous wheat show unexpected gains of nitrogen in plots not receiving fertilizer nitrogen. Over the past 90 years Plots 3 and 5 appear to have fixed N_2 at a rate of 35 Kg/ha/annum.

Rhizosphere fixation was examined by Day et al (1973) using the acetylene reducing assay on soil cores from which the top 1 cm had been removed. The fixation rates obtained were low and the author's concluded that bacterial nitrogen fixation in the top 16 cm of soil could account for only 2-3 KgN/ha/annum, and that most of the nitrogen fixed could be attributed to the blue-green algal crust which is clearly visible on the surface at certain times of year.

Fig 7.1 Broadbalk continuous wheat experiment

FIG 7.2 PART OF BROADBALK CONTINUOUS WHEAT EXPERIMENT



N1,N2,N3,N4 = Nitro-chalk at 48, 96, 144, 196 Kg N/ha.
FYM = Farm yard manure
(+) and (-) = With and without P, K, Na, Mg

Recent crop yields (tonnes of grain per ha.) from herbicide treated plots *

Plot No.	1970	1971	1972
9	5.5	5.3	5.1
8	5.8	5.7	5.0
7	5.1	6.0	5.3
6	4.1	4.5	4.7
5	2.6	2.5	2.1
3	2.1	2.1	1.6
22	5.3	6.5	6.3

* Taken from Rothamsted Experimental Station Annual Report for 1971.

The work described in this section was carried out in an attempt to evaluate the contribution of these algae to the nitrogen economy of several plots on Broadbalk. The nitrogenase activity beneath the wheat crop in herbicide treated plots 3,5,6,9 and 22 was monitored on a regular basis over a two year period from 1971 to 1973, and the non-herbicide treated plots 3,5,6 and 9 were monitored in 1971. Occasionally samplings were carried out on plots 7 and 8.

B. METHOD

Estimates of fixation were inferred from the nitrogenase activity of small core samples (0.84cm diameter by 2.5mm deep) which were incubated in universal bottles in daylight at air temperature. Six replicates were normally taken from each plot at weekly or fortnightly intervals from May until the crop was harvested in September, and a total of about 2000 cores were assayed each season.

In early samplings cores were selected for either the visual presence or absence of algal cover. On certain plots on some occasions during the season, it was extremely difficult to find positively identifiable areas of blue-green algae and some samples, which did not appear in the field to have any algal cover, fixed relatively well. Subsequent microscopic examination of these samples showed that blue-green algae were present and the visual selection procedure was later abandoned in favour of random sampling.

The large in situ incubation chambers used in the field experiments described earlier could not be used on Broadbalk because of the possibility of crop damage. In the third year permission was obtained to use the incubation chambers in the guard rows of the main plots. Comparison of the data obtained using the two methods on three occasions showed that the fixation values inferred from the incubation chambers are 5-15% lower than those obtained from the small core samples.

A comparative estimate of the visual cover of green and blue-green algae on the various plots was attempted using a random quadrat method at the end of the 1972 season. Nylon fishing line was passed through holes in the

edge of a 10cm square quadrat to give a 1cm grid so that the percentage of algal cover within each 100 sq.cms. could be estimated. Green and blue-green algae were separated by their appearance and the results checked under the microscope in doubtful samples. (see table 7.5).

C. RESULTS

(1) Estimates of annual fixation

The estimates of annual fixation are shown in Table 7.1 and 7.2. They have been made using the theoretical ratio $3C_2H_4 = 1N_2$ and integrating the areas under the graphs shown in Figs. 7.5 to 7.7 between May and September, assuming a 16 hour daily fixation period. The standard deviations are derived from the summed variances of the weekly fixation values. The high standard deviation values for plots 9 and 22 are believed to reflect differences in algal distribution (see later).

Table 7.1 Estimated annual nitrogen fixation on Broadbalk 1971

Plot No.	Treatments	Estimated annual fixation KgN_2/ha			
		Herbicide treated	s.d.	No herbicide	s.d.
22	Farmyard manure	10.66	5.13	11.22	4.04
9	N=4,P,K,Na,Mg	1.44	3.83	2.38	3.40
6	N=1,P,K,Na,Mg	24.54	2.36	28.22	6.50
5	N=0,P,K,Na,Mg	22.88	6.02	23.45	4.85
3	N=0	13.35	2.45	18.93	4.31

Table 7.2 Estimated annual fixation on Broadbalk 1972

Plot No.	Treatment	Estimated annual fixation (KgN_2/ha) Herbicide treated	Standard deviation
22	Farmyard manure	2.35	0.88
9	N=4,P,K,Na,Mg	0.81	0.59
6	N=1,P,K,Na,Mg	9.57	2.61
5	N=0,P,K,Na,Mg	12.82	4.46
3	N=0	2.82	1.39

The 1971 figures are considerably higher than those for 1972 because of the wetter summer in the first year. Total rainfall of 232mm between May and September in 1971 was associated with a mean fixation rate on all herbicide treated plots of 14.6 KgN_2/ha. A total of 145 mm of rain fell over a similar period in 1972 giving a mean fixation rate by herbicide treated plots of 5.57 KgN_2/ha.

The fixation rates recorded on all plots over the period of monitoring are clearly related to rainfall although subject to individual modifications according to cover from the standing crop and nitrogen status.

The average overall fixation rate for all plots on various dates is compared graphically with rainfall in Fig. 7.3 and 7.4.

The fixation rates are correlated, not with the rainfall at the time of sampling, but rather with the rainfall in the previous fortnight at the 10% probability level in 1971 but less well in 1972. Although the rainfall averaged about 10mm a week from May onward in 1972, the fixation did not increase until the middle of July. This lag may be partly attributable to soil temperature, but the growth of the wheat canopy is also important in preventing the soil from drying out between successive showers. Laboratory experiments show that severe desiccation of the surface (soil moisture content 4%) can cause lysis of vegetative cells whilst less severe decreases considerably reduced nitrogenase activity and growth. (See section 5, the effect of desiccation on algal crusts). The fact that the existing algal population has been reduced to some extent by dry periods is shown by the lag between rainfall and increasing nitrogenase activity in August 1971 (see Fig 7.3). If the algae, which were fixing well in the previous month, were merely inactivated by desiccation the response to rainfall might be expected to be somewhat more rapid (see section 6, Recovery of nitrogenase activity after a period of dry weather). The field was sampled on the 28th July, two days after the 15.5mm of rainfall shown for the week 24-31st July in Fig. 7.3. All plots were thoroughly wetted with a soil moisture content in excess of 15% (see Fig 7.8) but despite this the mean fixation rates represented less than 25% of the

FIG 7.3 Seasonal nitrogen fixation ; Broadbalk 1971

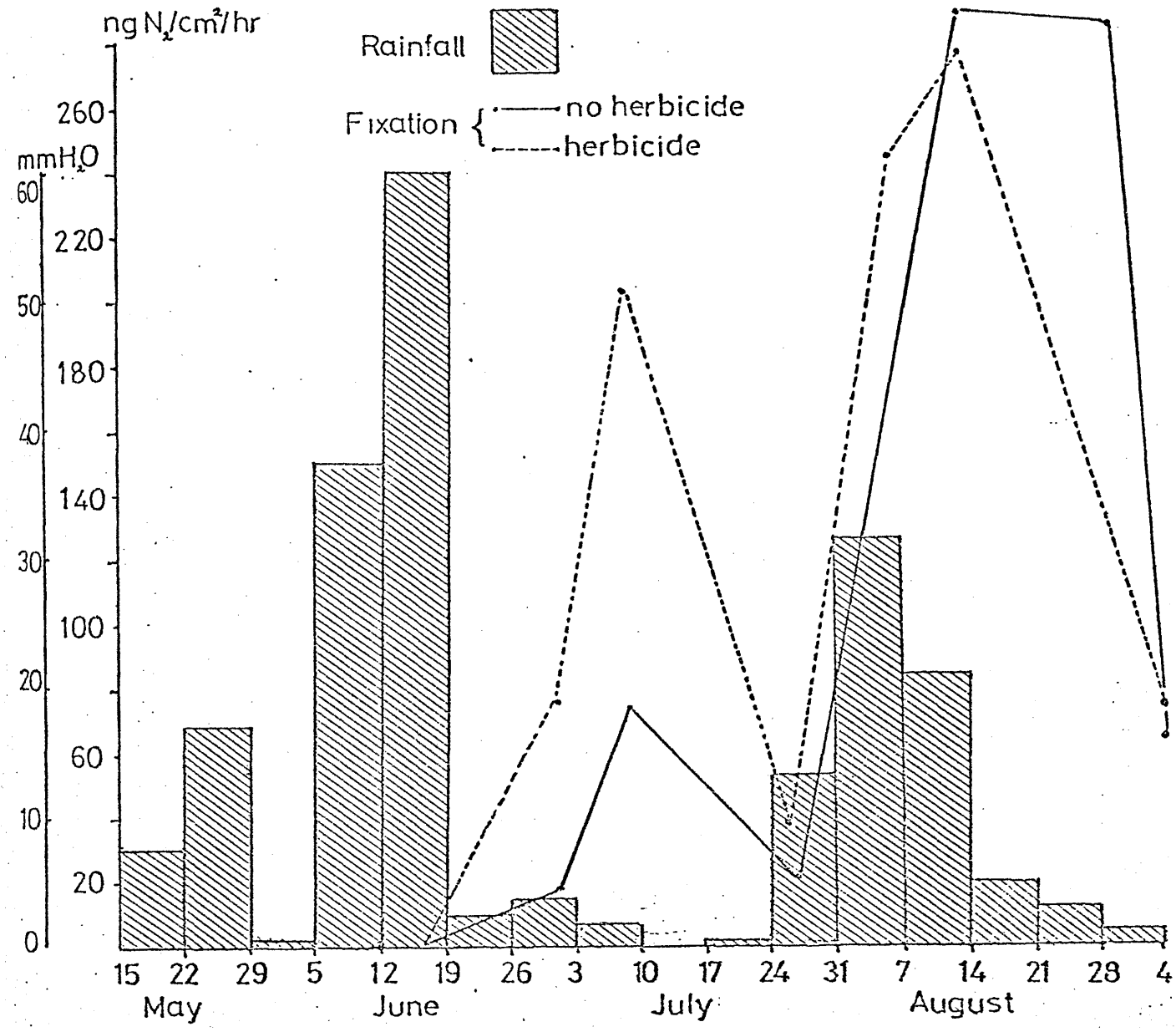
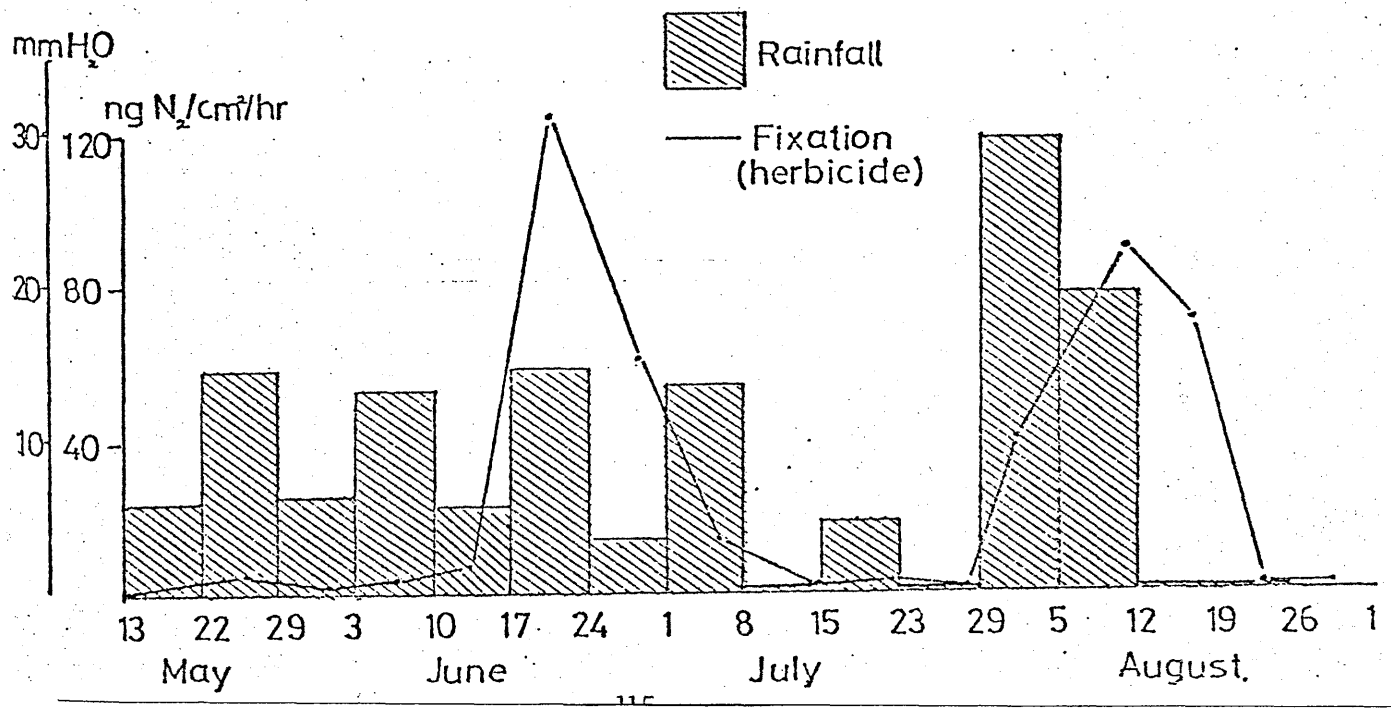


FIG 7.4 Seasonal nitrogen fixation ; Broadbalk 1972



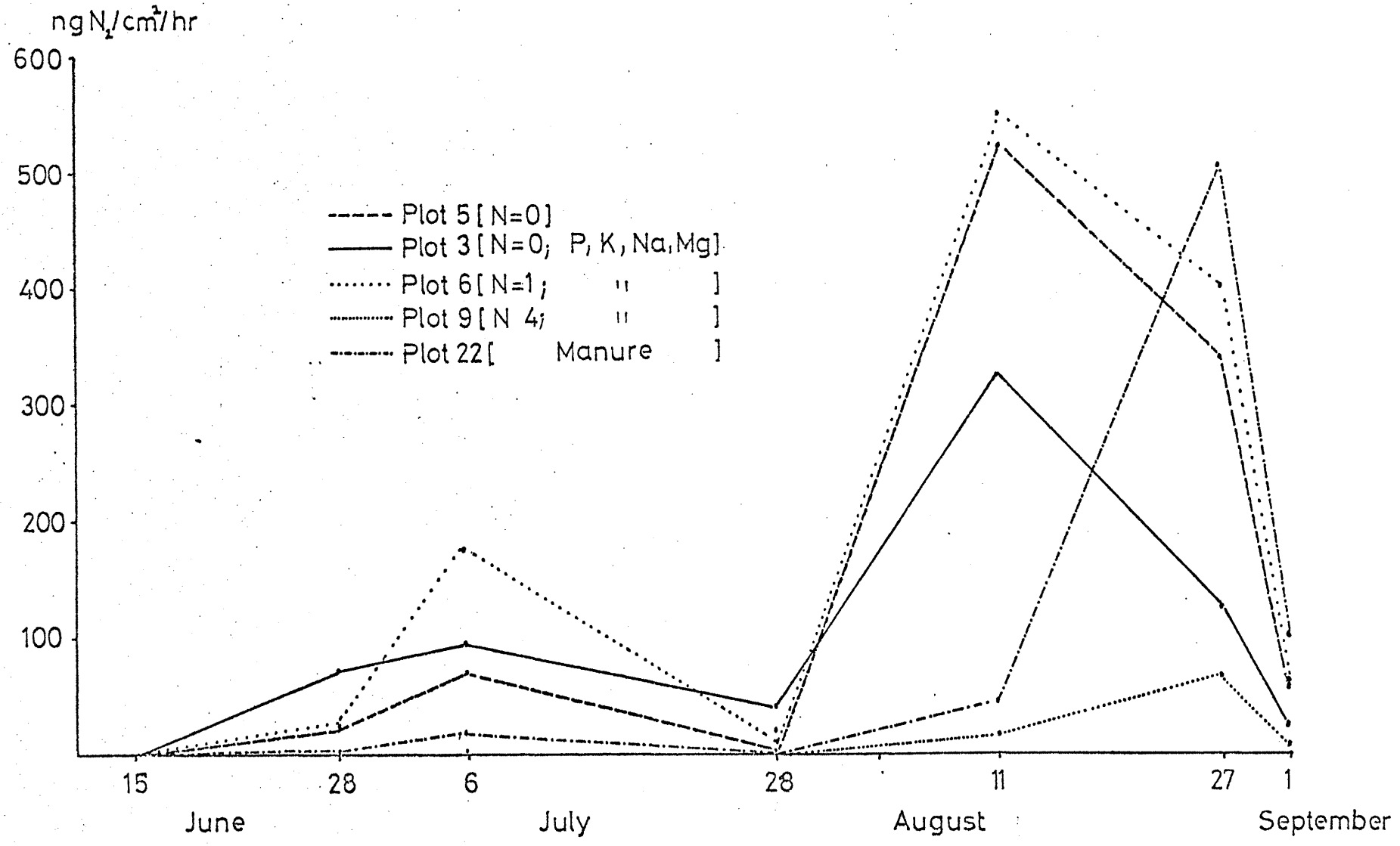


FIG 7.5 Seasonal nitrogen fixation on Broadbalk 1971 ; Herbicide treated plots.

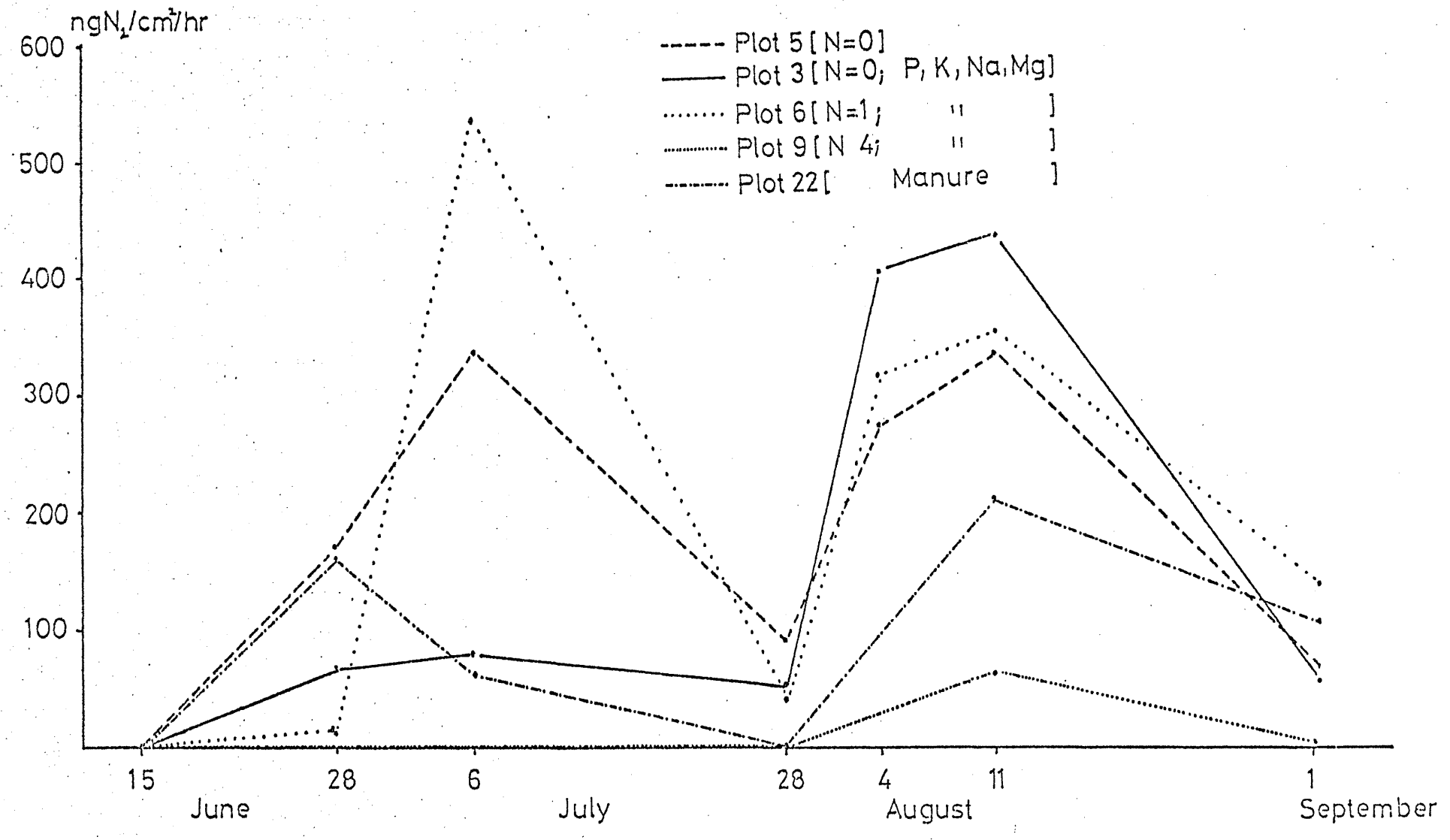


FIG 7.6 Seasonal nitrogen fixation on Broadbalk 1971 ; plots with no herbicide treatment.

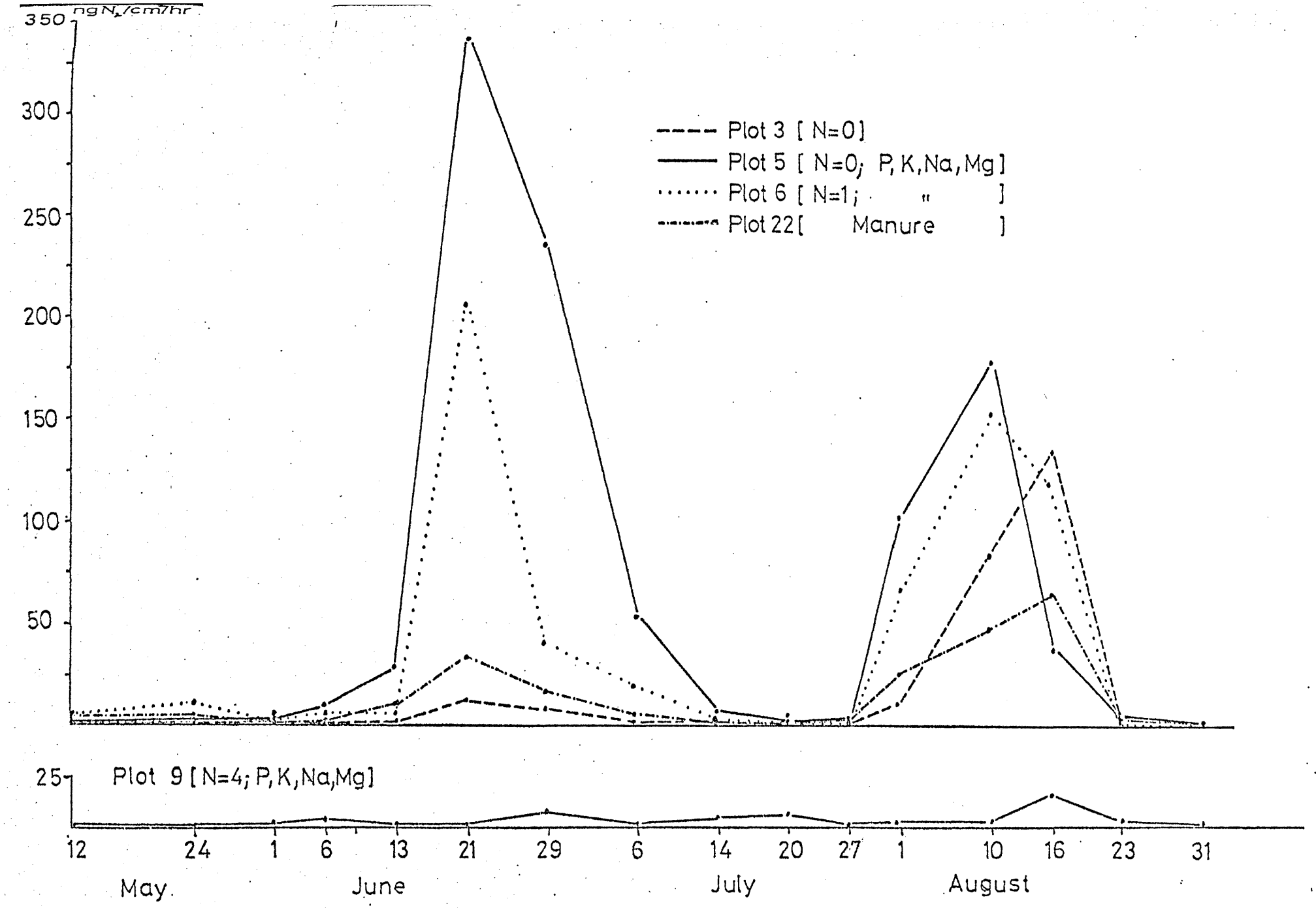


FIG 7.7 Seasonal nitrogen fixation on Broadbalk 1972 ; Herbicide treated plots.

previous maximum. The soil remained damp and the nitrogenase activity reached that of the previous high period after about 9 days and a maximum value after 14 days. The lag is, as might be expected, substantially less than the lag at the beginning of the season when the algal crust must develop from the relatively small inoculum remaining after the field has been ploughed and subjected to the rigors of winter weather.

(2) The effect of N,P,K,Na and Mg

It is difficult to separate the direct effect of fertilizer treatment on the algae from the indirect effects associated with a greater growth of the standing crop which affects both the soil moisture and light intensity. The soil surface beneath the closed wheat canopy on the higher nitrogen plots provides an equable algal habitat in that it remains damp for long periods after heavy rainfall because it is protected from the drying action of the sun and wind.

This effect is increased further by the weed cover which occurs on non herbicide treated plots (see Results 7.1.1.(3) The effect of herbicide treatment).

The light intensities recorded at ground level on various plots during bright sunlight are shown in table 7.3 overleaf. The percentage of the incident light which is absorbed has been calculated to give some indication of the relative density of the canopies.

The fixation values obtained for plot 3 (no fertilizer) were low at the beginning of the season, but increased in August as the sparse cover of vegetation closed over (see Figs. 7.5, 7.6 and 7.7). On the no herbicide areas, where the weeds assist in forming the canopy, the fixation on plot 3 increased to an August maximum which exceeded that of all other plots (see Fig. 7.6).

This suggests that algal growth and nitrogenase activity is not limited by the low nutritional status per se.

Plots 5(N=0,P,K,Na,Mg) and 6(N=1, P,K,Na,Mg) achieve a moderate degree of plant cover combined with a low nitrogen status and these plots give the highest overall fixation in herbicide treated areas.

Table 7.3

Light intensities at ground level on Broadbalk winter wheat plots. July 6th, 1971.

Plot No.	Herbicide	Range of light intensities (Lux)	% of light absorbed by canopy *	Comments (readings under normal crop unless otherwise stated)
9	-	570-3000	95-99	
9	+	2700-3600	94-96	
22	-	110-117	99	Under vetch
22	-	440-600	99-99	
22	+	1200-2300	96-98	
6	-	410	99	Under vetch
6	-	1800-4200	93-97	
6	+	4000-5000	92-94	
5	-	2000-4000	96-97	
5	-	1500	98	Under vetch
5	+	1800-22500	63-70	
5	+	2400-2700	95-96	Under Equisetum
3	-	1900-2000	67-69	
3	-	40,000	44	Between rows
3	+	20,000-27,000	55-67	
3	+	41,500	32	Between rows.

* Unshaded light intensity at ground level = 61000 Lux

The fixation rates generally decreased with increased nitrogen application from plot 6 to plot 9. Plots 7 and 8 were not monitored regularly, but estimations of fixation were made on four occasions. The results are shown in table 7.4 below. Although this effect might be partly due to excessive shading, nitrogen is implicated because, as Figs. 7.5 and 7.6 show, the contribution from plot 9 increases substantially towards the end of the season when much of the nitrogen has been taken up by the crop or washed down the soil profile.

Table 7.4 The effect of various levels of fertilizer nitrogen on nitrogen fixation.

Plot No.	Spring N application KgN/ha	Nitrogen fixation $ngN_2/cm^2/hr$			
		Sept.1st 1971	June 6th 1972	July 6th 1972	Aug.10th 72
6	48	139.9	4.05	17.5	150.5
7	96	116.5	3.85	6.60	76.3
8	144	8.0	0	2.25	2.25
9	192	12.0	0.28	1.20	1.40

The figures shown are the means of 6 replicate samples.

Williams and Rangeley (1972) note that the concentrations of nitrate in soil on plots 6-9 drops to less than 5 ppm towards the end of the season. Nair and Talibudeen (1973) used specific iron electrodes to investigate the movement of NO_3^- and K^+ in Broadbalk soils. The results obtained on plots 7 and 9 show that the nitrate concentration 5cm below the surface decreased from about 10^{-2} gm ions/l NO_3^- to about 10^{-4} gm ions/l NO_3^- (about 600 ppm to 6 ppm).

Although the affect of nitrate concentration on algal nitrogen fixation may operate, to some extent, by repressing nitrogenase synthesis and heterocyst formation, microscopic examination of samples taken from plot 9 showed that blue green algae were scarce, but that those present did have heterocysts. Visual observations during the early part of the

growing season indicated that the surface of plots 8 and 9 bore large areas of green algae and systematic examination of cover using a random quadrat method at the end of the season showed that plot 9 had a predominance of greens (see Table 7.5).

Although this effect may not become manifest over a single season, the repeated applications of high levels of nitrogen fertilizer over a long period, such as has occurred on Broadbalk, might be expected to bias the indigenous population towards species which are favoured by the higher soluble nitrogen concentration.

It would seem probable that the lack of fixation on plot 9 early in the season is attributable to a lack of blue-green algae as well as the possible deactivation of those present.

The fixation by plot 22 (farmyard manure) is greater than that of plot 9, but the general pattern of nitrogenase activity is similar with the herbicide treated sections reaching a maximum rather later than the other plots at the end of August.

The estimate of cover on this plot in September 1971 showed that 0.37% of the area was covered by green algae and 0.58% by blue-green algae (Table 7.5). The distribution of blue-green algae differed from that on other plots in being very noticeably clumped into small dense patches 1-2cm across. This effect is also manifested in the standard deviation of the weekly fixation estimates which are higher than those of other plots. It is possible that this distribution reflects the non uniform original application of manure which remains in quite large lumps producing local areas of high nutrient concentration. Similarly high standard deviations are produced by plot 9 where the application of nitro-chalk was reasonably even, and variations in soil topography and structure, which allow localized leaching of nutrients may also be important.

(3) The effect of herbicide treatment

Various herbicides have been used on some sections of Broadbalk since 1957. The herbicides have been chosen on a year to year basis to be effective against the emergent weed species.

The most prominent weeds encountered over the two year monitoring period were vetch and various grasses on the higher nitrogen plots and Equisetum on plot 3. Some idea of the density of weed cover can be gained from the light intensities at ground level shown in table 7.3.

The growth of weeds in the absence of herbicides is more manifest on well manured plots.

The spring herbicide treatments used in 1971, 1972 and 1973 are all mixtures of MCPA (4-chloro-2-methylphenoxyacetic acid), Mecaprop (2-(4-chloro-2-methylphenoxy)proprionic acid)and dicamba (3,6-dichloro-2-methoxybenzoic acid) marketed as Banlene and Baslene Plus.

All three operate by creating a hormonal inbalance in broad leaved plants. MCPA and Mecaprop are substituted phenoxyacetic and phenoxyproprionic acids which are degraded quite rapidly in the soil (half life about 1 week)whereas dicamba is a substituted benzoic acid which is degraded somewhat more slowly (half life about 1 month).

Much of the applied herbicide is lost to the plant canopy at the time of application and radioactive labelling experiments indicate that herbicide which does reach the ground is washed quite rapidly down the soil profile (G.G.Briggs, Personal Communication).

The use of herbicides reduced the annual fixation (see table 7.1) and a comparison of Fig.s 7.5 and 7.6 shows that in general the herbicide treated plots fix less well at the beginning of the season than the untreated plots, but better at the end of the season. This may be attributable to both the direct action of the herbicide on the algae and to indirect effects via the standing crop.

Lunkvist (1970) found that the nitrogenase activity of liquid cultures of N.punctiforme, N.muscorum and Cylindros·perman sp. was suppressed

by both DCMU and 2,4-D at concentrations which are normally applied in the field. At lower concentrations (10^{-4} - 10^{-5} M), which might occur in the field later in the season, Lunkvist reports that fixation is actually stimulated.

This direct effect may be exagerated by the weed cover which protects the soil surface on the untreated plots from desiccation, enhancing algal development early in the season.

The histograms shown in Fig. 7,8 compare nitrogen fixation by plots with and without herbicide in relation to soil moisture on several occasions between July and September 1971.

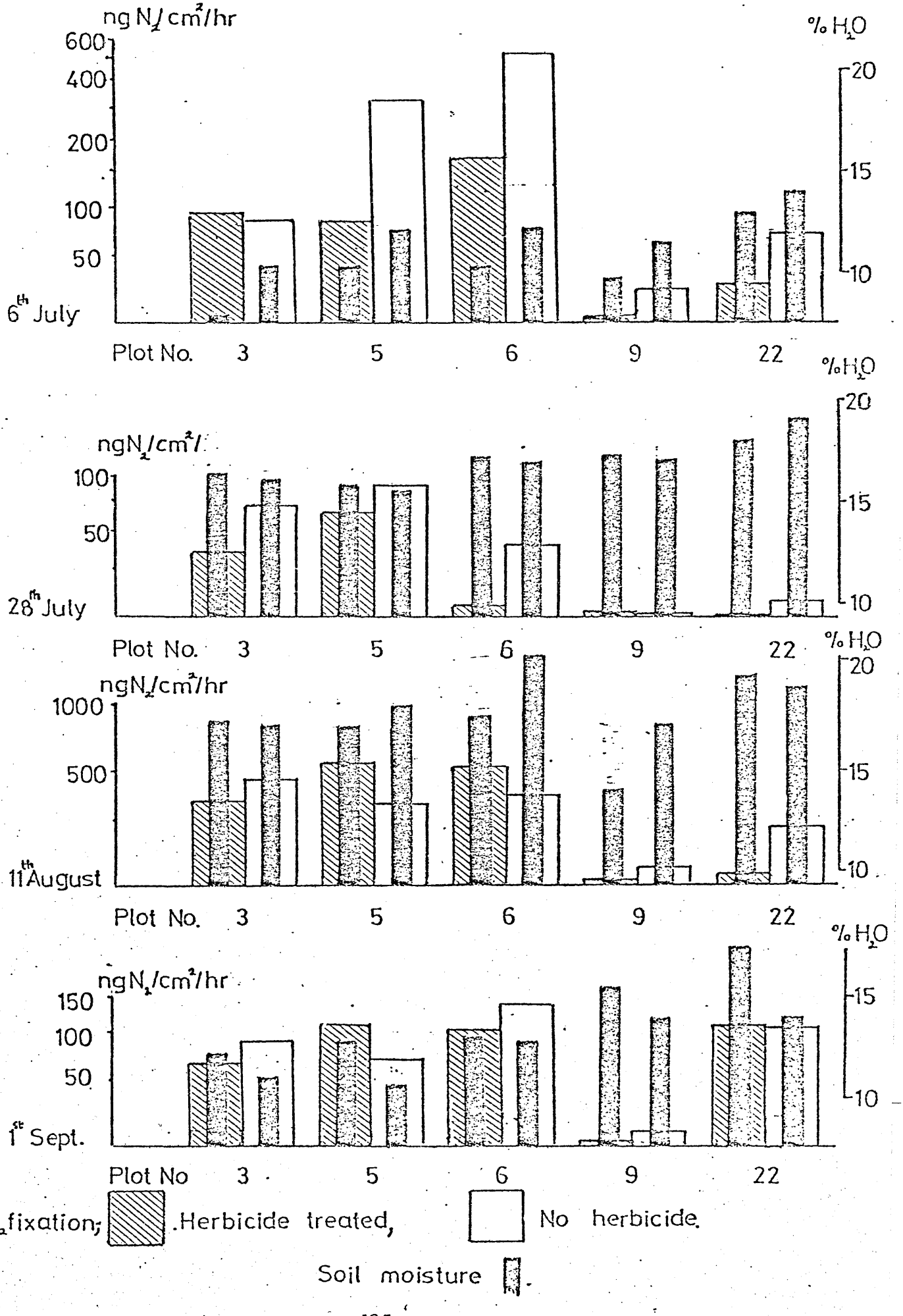
The effect of weed cover can be clearly seen on the 6th July three weeks after heavy rainfall. The no herbicide sections are wetter than the others and with the exception of plot 3, all show a higher fixation rate. On the 28th July, after two days rainfall, the effects of evaporation are not yet apparent and the soil moisture contents are generally similar, but by the 1st of September the no herbicide plots are drier than the others, particularly on the high nitrogen plots 9 and 22 where the canopy is so dense than many of the light showers in the previous fortnight probably did not reach the ground.

As Fig. 7.6 shows that the untreated areas of plots 5 and 6, which support a reasonable plant cover, give the greatest fixation in July whilst plot 3 does not contribute substantially until August when its cover is at maximum.

(4) Algal cover and species composition

The percentage algal cover on herbicide treated plots was estimated in September 1971 after the wheat had been harvested, using the random quadrat method described earlier. It was not possible to estimate the cover earlier in the season when fixation was at a maximum, because of the standing crop and the figures shown in table 7.5 overleaf probably fail to reflect the maximum seasonal cover.

FIG 7.8 The effect of herbicide treatment on soil moisture and N_2 fixation.



Each value is derived from 32 quadrat estimates each covering 100 square cms.

Table 7.5. Percentage of plot area with evident visual algal cover.

Plot No.	Green algae	Blue-green algae
22	0.37	0.58
9	9.36	1.56
6	0.29	17.48
5	0.09	21.37
3	0.03	6.51

The species composition of algae on Broadbalk was first studied by Bristol Roach (1939) who noted the presence of a number of species of blue-green algae of which Nostoc muscorum and Cylindrospermum licheniforme are now accepted as fixing elemental nitrogen. Attempts were made by Bristol Roach to enumerate the various species present using the serial dilution method, but as she observes, the technique is most unsuitable for filamentous blue-green algae. The cells, which form the often thick gelatinous lamina covering certain areas, do not separate during dilution and give a falsely low estimate of numbers. Estimates obtained in this manner are dependant on the degree of which such colonies are broken down and the estimated numbers of filamentous algae given by many authors are, in any case, largely dependant on their interpretation of what constitutes a single alga.

Numerical estimates were not attempted in this study but a number of species were isolated in uni-algal culture and identified by E.A.George, the curator of the Cambridge Collection of Algae and Protozoa, who was also kind enough to include the isolates in his collection.

The most prominent forms developing on nitrogen free agar were Cylindrospermum licheniforme, Nostoc ellipsosporum, N.muscorum and an Anabaena species, possibly A.cylindrica, designated in the Cambridge Culture Collection as 'Col.'

D. SUMMARY

The seasonal nitrogen fixation on Broadbalk, one of the Rothamsted Classic plots, was monitored over a two year period. The plot is subdivided into strips, each of which has received a separate fertilizer treatment which has remained substantially the same for the past 140 years. The treatments range from nothing at all through farm yard manure, to the spring application of 192 Kg N/ha as nitro-chalk with the addition of Na, P, K and Mg, with and without herbicide treatment. Jenkinson (1971) calculated that the crop removed an unexplained 35 Kg N/ha/annum from the soil of plots receiving no nitrogenous fertilizer, and Day et al (1973) suggest that this deficit is primarily met by the surface crust of blue-green algae.

Estimates of algal nitrogen fixation were inferred from acetylene reduction by small soil cores. Six replicate samples were taken from each plot at weekly intervals throughout the season.

The seasonal fixation rates in 1972 varied, according to the manurial treatment of the plots, from 1.44 Kg N_2/ha/season (herbicide treated plots receiving 192 Kg N/ha) to 28.22 Kg N_2/ha/season (plots receiving 42 Kg N/ha without herbicide). In 1973 the quantities of nitrogen fixed were substantially smaller. The weekly fixation values are closely correlated with rainfall in the previous fortnight and the lower fixation rates in 1973 are associated with a seasonal precipitation of 145 mm compared with 232 mm in 1972.

The lag between rainfall and developing nitrogenase activity, even after dry periods later in the season when a visible algal crust has already developed, suggests that many of the algal cells have died during the dry periods, and that a large part of the recovery is by regrowth.

Soil moisture appeared to be the most important single factor controlling nitrogen fixation, and it is difficult to separate the effects of fertilizer treatment from indirect effects associated with the standing crop which protects the soil surface from desiccation. Algal nitrogen fixation did not, however, appear to be limited by a low nutritional status since, although the fixation on unfertilized plots was low early in the

season when the plant cover was sparse, it increased towards the end of the season in 1972 to a value which exceeded that of all other plots.

The inhibitory effects of high soluble nitrogen concentrations are manifested on plots receiving 192 Kg/haN. The fixation on these plots was very low at the beginning of the season despite a good crop cover, but increased markedly in August as the nitrogen concentration fell.

The greatest overall fixation was produced by plots of moderate nutritional status, which had a good crop cover enhancing algal development, but insufficient soluble nitrogen to affect nitrogenase activity or species composition. The fixation rates generally decreased with increasing nitrogen application at levels about 48 Kg N/ha.

The seasonal fixation was greatest on plots which received no herbicide treatment. Measurements of soil moisture showed that these plots remained moist for longer after heavy rainfall because of the increased weed cover, and it seems probable that the greater fixation under these circumstances is partly associated with soil moisture and shading and partly with the direct action of the herbicide on algal development and nitrogen fixation.

SECTION 8. ALGAL INOCULATION EXPERIMENTS

A. INTRODUCTION.

B. EXPERIMENTAL DESIGN.

C. PREPARATION OF MATERIAL.

D. ESTIMATION OF NITROGEN FIXATION.

E. TREATMENT OF DATA.

F. RESULTS 1972.

1) Effect of Rainfall and Irrigation.

2) Effect of added nitrogen.

3) Effects of species of inoculum and method of application.

G. RESULTS 1973.

1) General.

2) The Effects of Nitrogen Application Over Two Successive Years.

3) The Effects of Algal Application Over Two Successive Years.

H. SUMMARY.

8. ALGAL INOCULATION EXPERIMENTS

A. INTRODUCTION

Experiments were carried out at Rothamsted Experimental Station in 1972 and 1973 to investigate the effect of algal inoculation on estimated nitrogen fixation and wheat yield. In the first year several species of algae were used with two methods of application on irrigated and un-irrigated plots with and without added nitrogen. In the second year only one method of application (liquid inoculum) was used with two algal species (N.ellipsosporum and A.cylindrica).

Analyses of the data obtained in 1972 showed no statistically significant interaction between the algal inoculation and the wheat yield, and the experiment was continued in 1973 using the same plots to see if the algal nitrogen became available to the crop in the second year.

Unfortunately the 1973 wheat crop became infected with Take-all (Gaeomannomyces graminis). This fungal infection of the roots, which is not uncommon when wheat is grown in same ground for two consecutive years, causes a radical reduction in crop, and the harvest was so poor that the statistical analyses of crop yield were unfortunately valueless.

The data pertaining to algal fixation in relation to the other field parameters are given in this section.

B. EXPERIMENTAL DESIGN

An area of approximately 0.1ha on Great field II was divided into 60 7' x 15' plots which were sown to Winter Wheat (Maris Ranger).

The plots were separated by paths 2ft. wide and the edge effects minimized by using the outer 9" margins of each plot as guard rows which were not included in the harvest. (see Fig. 8.1).

P and K were applied to all plots at the rates shown for Broadbalk and nitrogen as nitro-chalk was applied at 80 Kg/ha in accordance with the randomized experimental plan.

Fig. 8.1 Test plots for algal inoculation experiments. The difference between the plots is due to the added nitrogenous fertilizer.

Fig. 8.2 One of 420 incubation chambers in position in the test plot.

Each plot was subject to 4 variables; Species of algal inoculum, method of application, irrigation, and nitrogen status.

i) Species of algal inoculum

a) none

b) N.ellipsosporum (indigenous strain)

c) N.punctiforme

d) A.cylindrica

ii) Method of application

a) Liquid culture applied at a known volume per plot with a watering can.

b) Dry sand crust broadcast by hand.

iii) Irrigation

a) none

b) To compensate for water deficit.

c) Continuously moist surface.

In practice irrigation proved difficult because the available water supply is under heavy use during the summer, and it was not possible to compensate for the water deficit even when the hose was used continuously for 10 hours a day. Under these circumstances plots which should have been thoroughly irrigated were merely wetted as much and as often as possible.

iv) Applied nitrogen

a) none

b) 80 Kg/ha N as nitro-chalk

C. PREPARATION OF MATERIAL

About 80 l of each species of algae for liquid application was grown in the glass columns previously described (See section 2). The nitrogen content, dry weight, and volume of the cultures applied is shown in table 8.1 overleaf.

Table 8.1 Dry weight and N content of algal cultures used as liquid inocula

Species	Algal dry wt. mg/L	Kjeldahl N mgN/L	Volume applied per plot	Dry wt./ha *	N/ha *
N.ellipsosporum	100	4.20	9L	0.927 Kg	0.038 Kg
N.punctiforme	102	3.98	9L	0.946 Kg	0.037 Kg
A.cylindrica	46	5.07	9L	0.426 Kg	0.0447 Kg
Mixture	74	5.04	9L	0.686 Kg	0.046 Kg

* Plot area = 0.00097 ha

For the dry sand inocula algae were grown on 6' x 4' trays on quartz sand about 3" deep, moistened with the medium of Allen and Arnon (1956). The trays were placed in a greenhouse and heavily inoculated with the desired algal species.

The algal crust which developed was turned in every 7-10 days so that the algae were forced to continually re-colonize the surface. After 2 months the trays were allowed to dry slowly over about a week. The contents were then thoroughly mixed and transferred, in 1Kg samples, to polythene bags where they were stored for a week before being broadcast onto the plots.

The estimated quantities of applied nitrogen are shown in table 8.2 below.

Table 8.2 Algal nitrogen in sand inocula.

Species	Kjeldahl N/Kg dry sand	Wt. of sand inoculum per plot (Kg)	Total algal N/ha (Kg)
N.ellipsosporum	15.6 mg	3.5	0.056
N.punctiforme	9.03 mg	3.5	0.032
A.cylindrica	11.2 mg	3.5	0.040
Mixture	11.94 mg	4.0 Kg	0.04

Both the liquid and sand cultures were applied to the plots on the 9th May.

D. ESTIMATION OF NITROGEN FIXATION.

Five in situ incubation chambers were driven into each plot at the beginning of May when the Wheat was 3-4" high (Fig. 8.2). The nitrogenase activity was monitored at approximately weekly intervals thereafter until the field was ploughed in September, using the technique described in Section 2.

E. TREATMENT OF DATA

The ethylene production within the incubation chambers has been calculated using the method shown in Appendix 1 with the container volume and gas loss being inferred from the acetylene concentration.

The mean value from five chambers was obtained for each plot on each sampling occasion together with the standard deviation. In cases where replicate plots have been run together means and standard deviations have been re-calculated from the original chamber values.

The standard deviation values for individual points shown on the graphs were, in some cases, larger than 50% of the mean, and for this reason some of the points graphically compared are not significantly different. The difference between the various treatments does, however, become highly significant when the results are taken collectively over the season to give estimates of annual fixation.

The annual fixation values have been obtained by cumulating the estimated quantity of nitrogen fixed each week and the variance associated with this value. The relative error is decreased during this process because it is the variance rather than the standard deviation which is summed.

F. RESULTS 1972

1) Effect of rainfall and irrigation

Although the fixation rates on the different plots vary considerably according to the treatments they have received, all show similar trends in

response to rainfall, (for example Fig. 8.6). A graphical comparison of overall fixation and rainfall is shown in Fig. 8.3. The values plotted are the mean weekly fixation rates for all plots irrespective of treatment.

The pattern of seasonal fixation is similar to that obtained for Broadbalk in the same year with fixation increasing markedly during August after two weeks of relatively heavy rainfall.

It is not the rainfall per se which affects the algal development, but rather the soil moisture which is a summation of precipitation and evaporation which is related to humidity, sunshine, air temperature and wind.

The lack of fixation early in the season with an average rainfall of about 7mm a week may be attributable to the drying periods between showers which caused observable surface desiccation at some time during most weeks , but it is odd that, as Fig. 8.4 shows, the fixation pattern is similar even in the irrigated plots.

The estimated seasonal fixation on irrigated plots of 2.55 KgN_2/ha (s.d.= 1.01 Kg) is higher than the 1.58 Kg/ha (s.d.= 0.54 Kg) recorded on the un-irrigated plots, but the difference only becomes manifest towards the end of the season. The t-test shows that the differences are significant at the 1% level.

It is possible that the lack of fixation early in the season is attributable to desiccation even on irrigated plots since, as was mentioned earlier, the availability of water was such that it was impossible to maintain the irrigated plots in a continuously wet state.

If akinetes, which have survived the winter, start to germinate during only transitory periods of moisture, it is possible that a partial wetting is worse than none at all. As section 5 shows, desiccation can cause cellular lysis and the germinating cells may be killed by short dry periods because they are unable to ~~re-akinetize~~. reform akinetes

FIG 8.3 Effect of rainfall on nitrogenase activity; Inoculation exp.1972

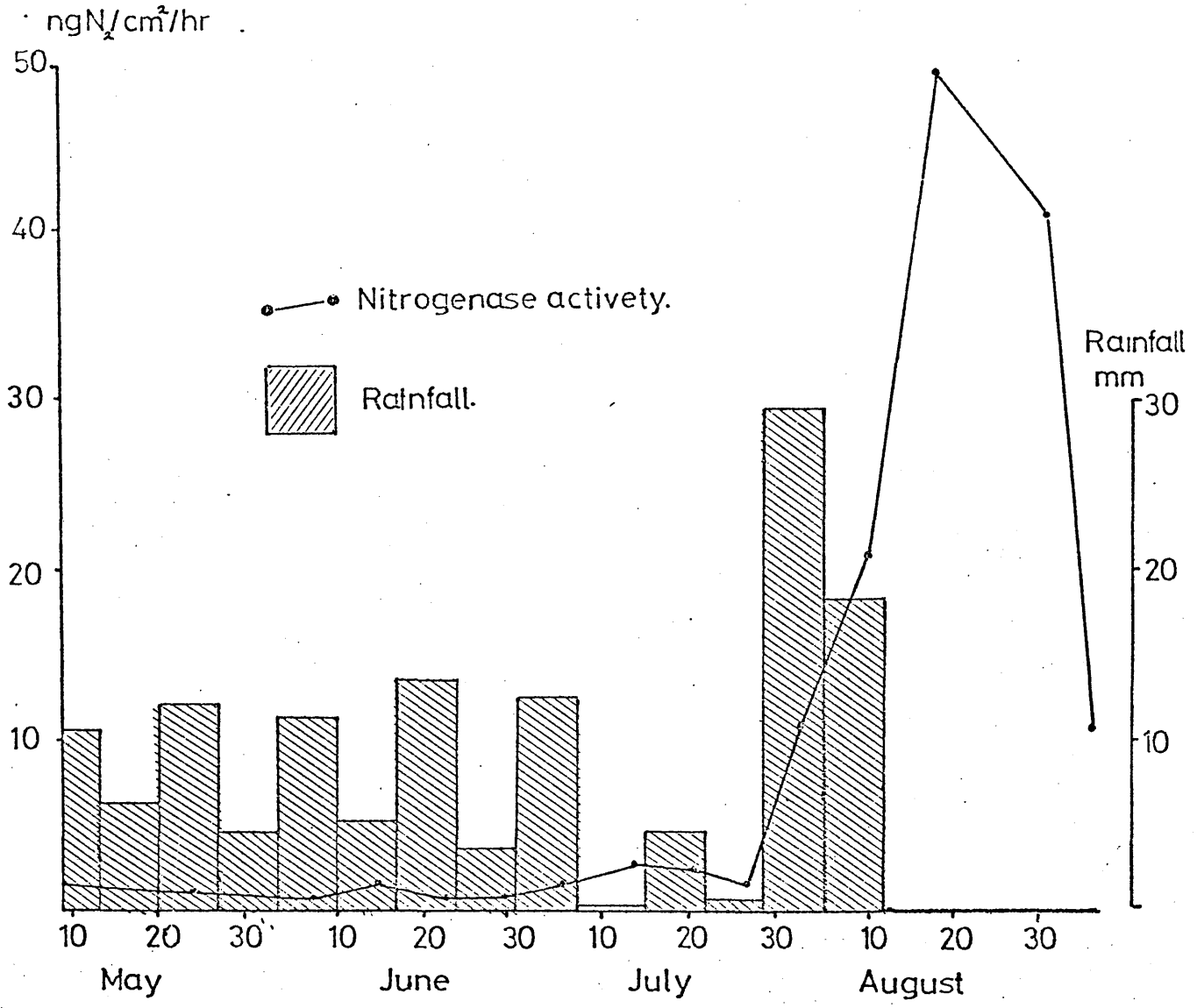
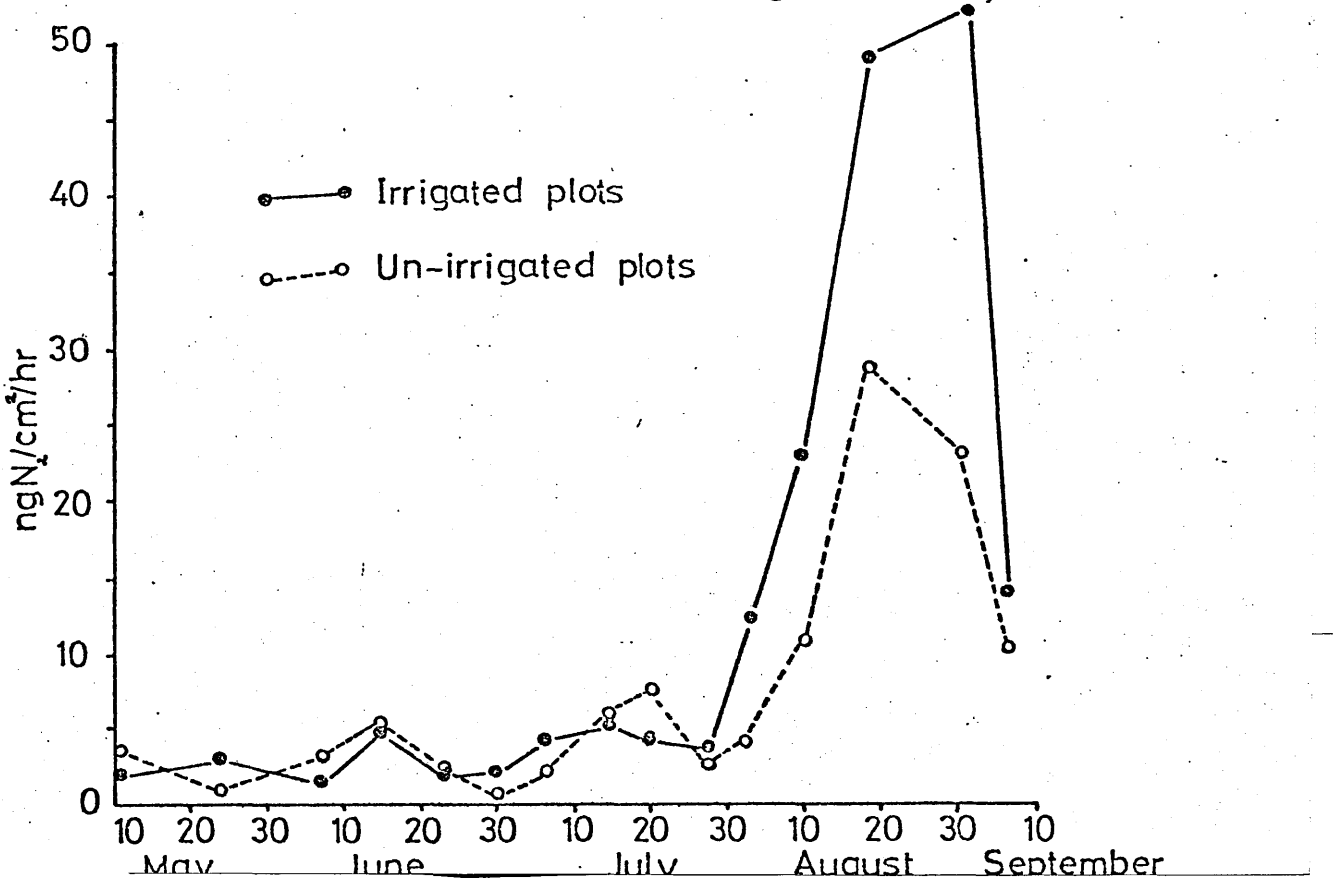


FIG 8.4 Effect of irrigation on seasonal nitrogenase activity. 1972.



2) Effect of added nitrogen

The addition of nitrogenous fertilizer in the spring at 80 kg N/ha produced an increase in nitrogen fixation on all plots. The mean seasonal fixation values for plots inoculated with the various species of algae with and without added nitrogen are shown in Table 8.3 below.

Table 8.3 Estimated seasonal nitrogen fixation with and without added nitrogen

Species of inoculum	80 Kg/ha applied N		No applied N		t value * between means
	KgN_2/ha	s.d	KgN_2/ha	s.d.	
Uninoculated controls	0.79	0.20	0.49	0.14	4.91
N.ellipsosporum	5.96	1.34	3.15	0.91	6.95
A.cylindrica	3.11	1.48	2.28	0.67	2.04
N.punctiforme	1.71	0.38	1.35	0.35	2.99

The values shown are derived from the summed weekly fixation values and variances on 16 occasions throughout the season.
* Tables indicate that 't' values are; 10% = 1.75, 2% = 2.60; 0.1% = 4.07

The t-test indicates that the increases are significant beyond the 0.1% probability level for the control plots and those inoculated with N.ellipsosporum. The differences between the nitrogen and no nitrogen plots inoculated with A. cylindrica and N.punctiforme, which are somewhat smaller, are significant at the 10% and 2% levels respectively.

The differences became more manifest towards the end of the season and, as Fig. 8.5 shows., in the case of N.ellipsosporum, spring nitrogen application appears to actually repress fixation in the first half of the season.

The total quantities of nitrogen fixed at the beginning and end of the season are shown in table 8.4 overleaf.

FIG 8.5 Effect of nitrogen fertilizer on seasonal nitrogen fixation by plots inoculated with Nostoc Ellipsosporum .

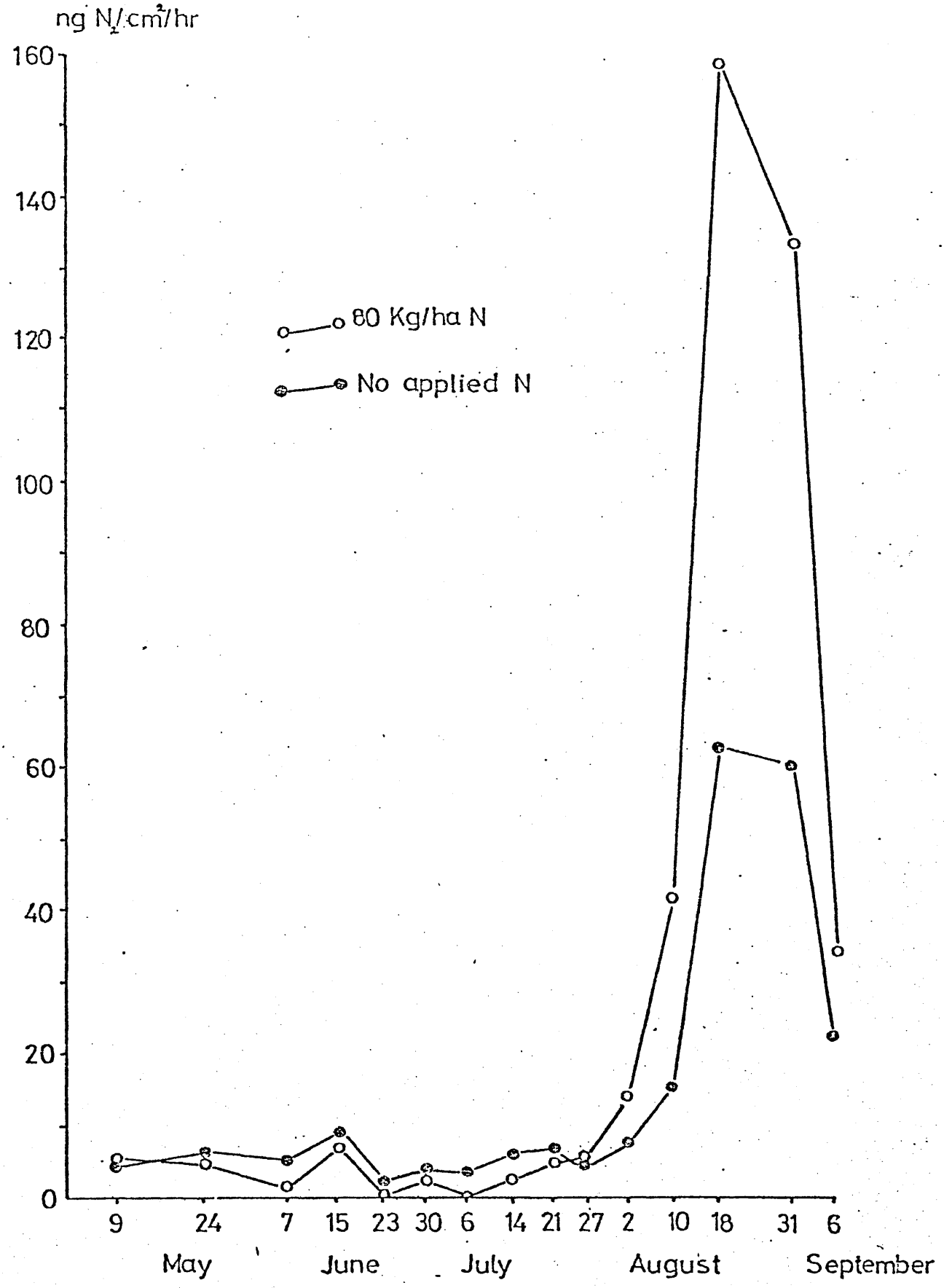


Table 8.4 Effect of nitrogenous fertilizer on plots inoculated with N.ellipsosporum.

Spring nitrogen application	Total seasonal fixation		Fixation 9th May - 21st July		Fixation 27th July - 6th Sep	
	KgN_2/ha	S.d.	KgN_2/ha	S.d.	KgN_2/ha	S.d.
80KgN/ha	5.96	1.34	0.38	0.17	5.58	2.19
None	3.95	0.91	0.75	0.20	2.40	0.89
't' values between means	4.97		5.66		5.39	

Tables indicate that means are different at the 0.1% probability level if experimental 't' values $>$ 4.07 .
The values shown are derived from the summed weekly fixation values and variances on 16 occasions throughout the season.

The t-test shows that the fixation on the nitrogen treated plots is lower than that on the untreated plots at the 0.1% probability level between the 9th May and 21st July, and higher than the untreated plots at the 0.1% level thereafter.

This phenomenon is similar to that noted on the higher nitrogen plots of Broadbalk (see section 7). Early in the season the high soluble nitrogen concentration may repress nitrogenase synthesis and heterocyst formation, although the greater wheat growth enhances algal establishment by protecting the soil surface from desiccation. The nitrogen concentration decreases towards the end of the season because of leaching and removals by the crop (Williams and Rangely 1972) with a consequent increase in fixation.

3) Effects of species of inoculum and method of application

The various algal species were applied as either a liquid inoculum or as dry sand grown cultures prepared as described earlier.

The effectiveness of the two methods of inoculation with the three algal species are compared in Fig 8.6 and Table 8.5. overleaf.

FIG 8.6 Effect of species of inoculum and method of application on seasonal nitrogen fixation

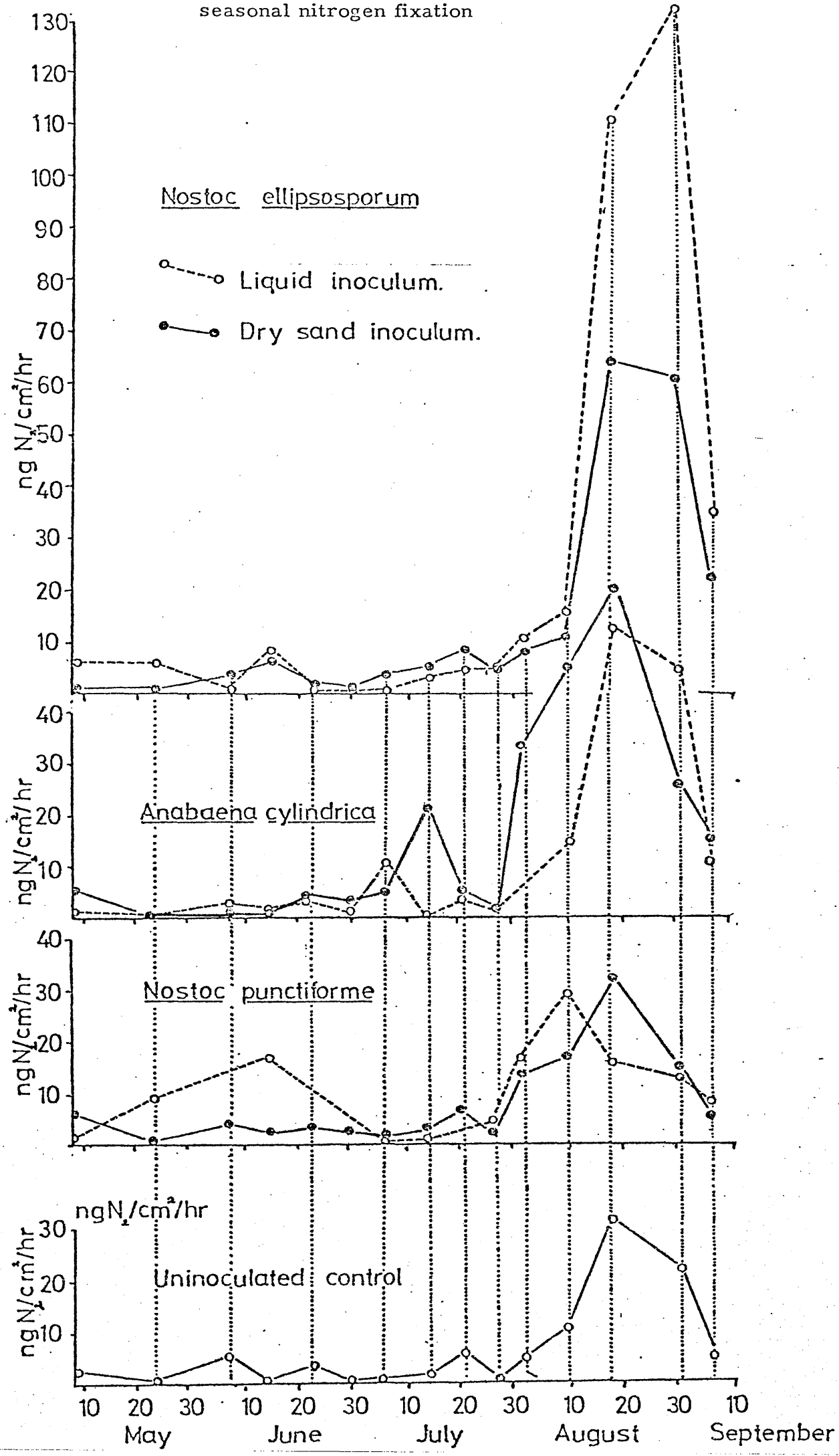


Table 8.5 Effect of species of algal inoculum and method of application

Algal species	Liquid inoculum		Sand inoculum		't' values between * means
	KgN_2/ha	s.d.	KgN_2/ha	s.d.	
N.ellipsosporum	4.75	1.78	2.93	0.84	3.646
A.cylindrica	2.20	0.66	3.13	0.81	3.580
N.punctiforme	1.50	0.33	2.08	0.55	2.628
Uninoculated control; 1.34 KgN_2/ha; s.d. = 0.38					

* The means are significantly different at the 1% level if 't' experimental $>$ 4.073.
The figures are derived from the mean and variance values for 16 weekly readings.

With the exception of liquid cultures of N.punctiforme all the inoculated plots gave an increase in fixation over the control which is significant at the 1% probability level. The 't' values obtained by comparing the mean of the two methods of application are shown in the right hand column of Table 8.5. The differences are all significant at the 1% level. The most effective treatment was a liquid inoculation with N.ellipsosporum which gave an increase over the basal level of 250%.

It is of interest that this species was more effective when applied as a liquid while both A.cylindrica and N.punctiforme gave better results when they were applied as dry sand cultures. Since, as section 5 shows, the vegetative cells are killed by severe desiccation, the effectiveness of the sand inocula is probably largely dependent on relative numbers of akinetes present. This will presumably depend on the algal species, the state of growth of the cultures and the degree and rate of desiccation.

Regularly moistened laboratory cultures of A.cylindrica and N.ellipsosporum have been observed to undergo extensive akinetization on several occasions when other, apparently similar, cultures continue to show healthy vegetative growth. The factors which induce such changes were not investigated, but they would seem to be of

considerable interest since heavily akinetized cultures of this type could be dried to form a highly viable, readily stored algal inoculum.

It was found that the algae could be applied to the plots with equal ease as either liquid or sand and both types of culture are easily prepared in the laboratory,although sand cultures may have a slight advantage in that the apparatus required for their production is extremely simple.

Akinetized sand cultures may also offer an advantage if the material is to be applied during dry weather. Under these conditions the akinetes can remain dormant until suitable conditions occur, whilst the sudden desiccation which has been noted when relatively small volumes of liquid culture are applied to field plots may cause extensive cellular lysis.

G. RESULTS 1973

1) General

The 1972 experiment was continued into the second year using the same plots, again sown to winter wheat. Some of the plots were, however, subdivided so that the effect of nitrogen application and algal inoculation over two consecutive years could be investigated.

As was mentioned in the introduction, the crop became severely infected with take-all and the monitoring was abandoned after six weeks when the effects of the Gaeumannomyces infection became manifest. At this time the wheat, even on the nitrogen fertilized plots, was extremely sparse with large open areas and extensive patches of various weeds.

The results obtained over this period generally compliment those described for 1972, although the recorded fixation rates were rather higher at the beginning of the second year, presumably because of the greater rainfall (The rainfall between June and July 1972 was 56.2 mm compared with 142.9 mm over the same period in 1973).

2) The effects of nitrogen application over two successive years

The 1972 results indicated that although applied nitrogen stimulated fixation towards the end of the season it produced a significant inhibition earlier on. This effect is borne out in the second year. The cumulative nitrogen fixation at the beginning of the 1973 season with various combinations of applied nitrogen treatment are shown in Table 8.6 below. The values given are not directly comparable with those from the previous year since they represent only a six week period.

Table 8.6 The effect of nitrogen applications in successive years.

Nitrogen treatment	Uninoculated		A.cylindrica		N.ellipsosporum	
	KgN_2/ha	s.d.	KgN_2/ha	s.d.	KgN_2/ha	s.d.
No applied N	1.12	0.71	1.43	0.73	1.72	1.15
N applied in 1st year	1.60	0.68	1.90	1.17	1.40	0.50
N applied in 2nd year	1.18	0.69	1.35	0.75	1.15	0.74
N applied in both years	1.11	0.76	1.12	0.44	1.08	0.60

The results shown are derived from the summed weekly fixation values and variances on 6 weekly occasions from June 21st to July 24th, 1973.

Plots with the lowest nitrogen concentration, which had received either no nitrogen at all, or nitrogen only in the first year, gave the greatest fixation values. Plots which had received nitrogen in the second year fixed somewhat less effectively while the most deleterious treatment, at least at the beginning of the season, was a nitrogen application in both years.

3) The effects of algal application over two successive years

The effects of inoculation in 1972 became manifest only later in the season and it is difficult to evaluate the effect of the various combinations of inoculum used in 1973 from the data at the beginning of the season. The only fact which clearly emerges from the cumulative fixation values

is that the inoculated plots generally fixed better than the uninoculated ones. The data are shown in Table 8.7 below.

Table 8.7 The effect of algal inoculation in two consecutive years.

(A) A.cylindrica

Algal treatment	80Kg/ha applied N		No applied nitrogen	
	KgN_2/ha	s.d.	KgN_2/ha	s.d.
Inoculated in both years	1.12	0.44	1.43	0.73
Inoculated in 1st year	0.92	0.46	1.53	0.80
Inoculatedin 2nd year	0.95	0.54	1.00	0.45
Uninoculated	1.11	0.76	1.12	0.71

(B) N.ellipsosporum

Inoculated in both years	1.08	0.60	1.72	1.15
Inoculated in 1st year	2.02	1.00	1.73	0.80
Inoculated in 2nd year	2.28	1.51	1.29	0.85
Uninoculated	1.09	0.76	1.12	0.71

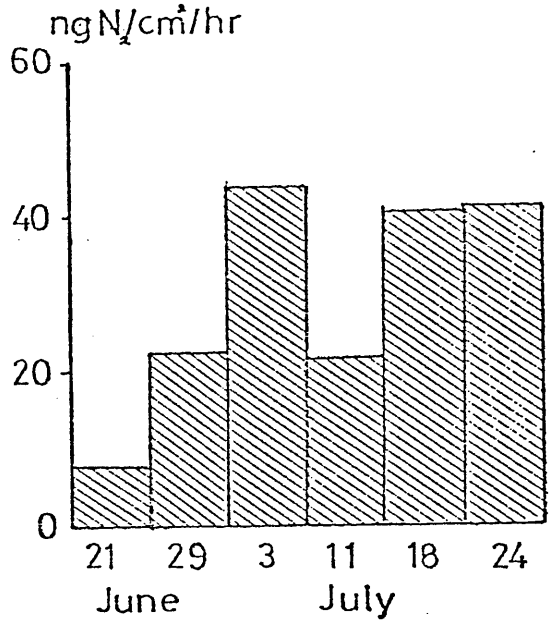
The results shown are derived from the summed weekly fixation values and variances on 6 weekly occasions from June 21st to July 24th 1973.

The weekly fixation values appear to show that this difference may be attributable to the lag in fixation on the uninoculated plots. The fixation by unfertilised plots inoculated in 1972, inoculated in 1973 and inoculated in neither year are compared in Fig. 8.7.

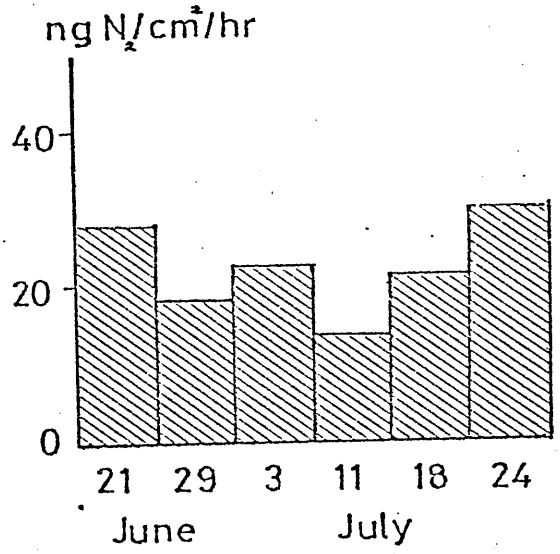
The plots inoculated in 1973 (8.7B) achieve a relatively high level of fixation immediately, whereas fixation on the uninoculated plots increases

FIG 8.7 EFFECT OF ALGAL INOCULATION ON THE DEVELOPMENT NITROGENASE ACTIVETY

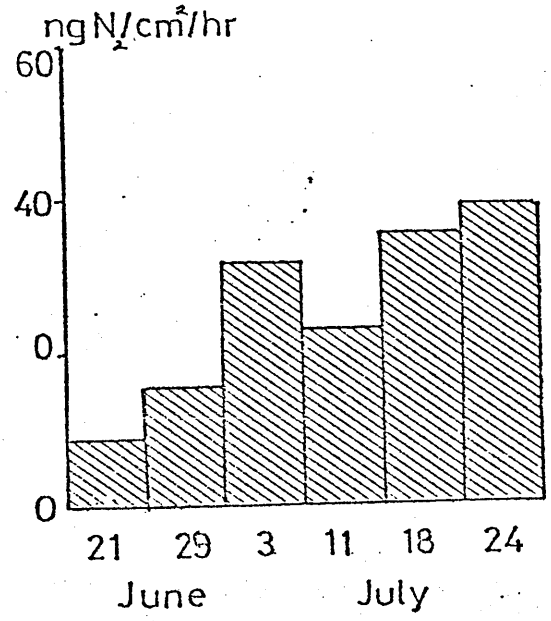
(A) Plots inoculated in neither year.



(B) Plots inoculated in 1973



(C) Plots inoculated in 1972.



mor slowly (8.7A).

A similar progressive increase in activity also occurs on plots which were inoculated only in the first year (Fig. 8.7C).

The development of nitrogenase activity on nitrogen treated plots is slow irrespective of inoculation probably because of the initial inhibitory effects of the high soluble nitrogen concentration.

H. SUMMARY

The effect of algal inoculation with liquid or dried sand cultures of three species of algae was investigated on 60 field plots sown to winter wheat, with and without added nitrogen and irrigation.

The algae had no significant effect on the wheat yield in a single year.

The weekly fixation values, like those of Broadbalk, are closely correlated with rainfall.

The quantity of water available for irrigation was such that the plots could not be kept continuously moist, and although a greater rate of fixation was given by irrigated plots later in the season, the initial effects were negligible. It is possible that transitory wetting is worse than none at all since the vegetative cells which develop from akinetes may be killed by desiccation (see also section 5).

The spring application of 80Kg N/ha as nitro chalk significantly reduced the nitrogen fixation on N.ellipsosporum inoculated plots early in the season, but stimulated fixation later on when much of the nitrogen had been used by the crop and leached out by rainfall. The stimulation appears to operate primarily via the wheat crop which is sufficiently dense, on nitrogen treated plots, to protect the soil surface from desiccation. A similar effect was observed in the second year when the fixation rates in the first six weeks of the season were lowest on plots

which had received applied nitrogen for two successive years.

N.ellipsosporum applied as a liquid inoculum gave the greatest increase in nitrogen fixation followed by A.cylindrica and N.punctiforme which, in contrast to N.ellipsosporum, were most effective when applied as a dried sand crust. The differences between inoculated and uninoculated plots became manifest in the first year only after wet weather at the end of the season. Data obtained in the second year suggest that differences may also occur early in the season if the weather is wet. Under these circumstances inoculated plots achieve a high level of fixation much more rapidly than those plots where the algal flora must develop from a small inoculum.

SECTION 9. GENERAL DISCUSSION

9. GENERAL DISCUSSION

Donze, Raat and Van Gorkom (1974) have produced data in a very recent paper which substantiates the conclusion, described in Section 3 of this Thesis, that the ATP requirement for nitrogenase activity under normal conditions in the light is met by both photophosphorylation and oxidative phosphorylation. Their evidence is based on the effect of oxygen and inhibitors on acetylene reduction by carbon starved cultures of Anabaena cylindrica in the light and in the dark.

They observe that the rate of acetylene reduction by normal cultures under anaerobic conditions in the light is similar to that given under aerobic conditions in the dark. (The work described in Section 3 suggests that this would, in fact, be dependant on the light intensity) and the rate under anaerobic conditions in the dark represented only about 5% of that in the presence of oxygen. The injection of oxygen into anaerobic cultures in the dark produced a rapid increase in nitrogenase activity associated with the production of ATP by oxidative phosphorylation.

In carbon starved cells the anaerobic fixation in the light was similar to that before starvation, but the oxygen dependant dark fixation was halved. The authors conclude that since respiration in these cells does not appear to be reduced, the lower rate of nitrogenase activity is due to lack of reductant. This is supported by the DCMU sensitivity of cells under these conditions and the fact that this sensitivity can be relieved by the addition of a DPIP (2,6-dichlorophenol indophenol) / ascorbate electron generating system.

The oxygen-dependant continuation of acetylene reduction, observed in the field overnight, may be partially dependant of daylength since the photosynthetically produced endogenous substrates, which accumulate during the day, are depleted at night. The contribution from overnight fixation may thus become limited as the days shorten towards the end of the summer. Conversely the high levels of nitrogenase activity, recorded by Henriksson (1971) during the short Swedish nights, are presumably

associated with a high level of endogenous substrate.

It must be emphasised that the results pertaining to overnight fixation are only tentative since they are based on estimates of acetylene reduction.

The estimates of total fixation in the field presented in sections 7 and 8 are given as seasonal rather than yearly values because the plots monitored were ploughed in September in accordance with normal agricultural practice. Forays into the field during periods of relatively warm weather in the winter failed to produce any samples which showed positive nitrogenase activity, and it would seem that very little nitrogen fixation persists in the winter in the temperate agricultural habitats sampled, although the soil moisture, which appeared to limit algal development in the summer, was high. The development of algal nitrogen fixation has been recorded by Fogg and Stewart (1968) during the antarctic summer in the South Orkney Islands at a temperature which ranged from 4 to 10^{o}C. It is possible that the lack of nitrogen fixation during periods of similar temperature in this country is attributable to either the lack of a suitable algal species, or the shorter daylength. If the former factor is indeed operative, it would lead to the interesting possibility of exploiting arctic and antarctic algal species for winter fixation in temperate soils.

The severe cellular lysis attendant on the desiccation of soil crusts in the laboratory (Section 5), combined with the low recovery of desiccated samples on the field (Section 6), and the long delay between rainfall and the recovery of a magnitude of nitrogen fixation equal to that achieved in the previous fixation maximum, all support the conclusion that the algal development on the temperate soils investigated is severely limited by desiccation.

The effect of soil moisture on algal distribution is shown by both the observations on the interaction between crop cover and algal development and the general observation that, within the range of habitats investigated, the algae were normally limited to areas of soil which were sheltered

from the drying action of the wind, compacted (improving capillary rise) and often locally depressed.

Although the effect of algal nitrogen did not become noticeable in the first year of the inoculation experiments, they are reflected by the crop yields on Broadbalk, where the estimated yearly fixation rates approach the nitrogen deficit implied by the crop yields (Jenkinson 1971).

As Section 7 shows, the cumulated nitrogen fixation is closely related to rainfall. The mean rainfall since 1840 between May and September is 234 mm which is very close to the 232 mm of rainfall recorded over the same period in 1971 when a fixation of 22.8 Kg N_2/ha was recorded on herbicide treated plots receiving only P,K,Na and Mg. Day et al calculated that the rhizosphere fixation on these plots accounted for about 2-3 Kg N_2/ha giving a total of about 25 Kg N_2/ha compared with Jenkinson's estimated deficit of 30 Kg N_2/ha.

The yields from plots receiving no added nitrogen are low and the use of algae as the sole nitrogen source in the production of temperate agricultural crops would not seem practicable. The crop returns from plots receiving 48 Kg N/ha applied nitrogen are, however, of considerable interest in this respect.

Such plots, which offer a compromise between a very sparse wheat cover and a consequently rapid desiccation of the soil surface, and high concentrations of soluble nitrogen which inhibit nitrogenase activity, fixed 25 Kg N_2/ha under average rainfall conditions. The total amount of nitrogen available to the crop approximates, on a yearly basis, to about 73 Kg N/ha of which more than half is provided by algal nitrogen fixation. The yield produced by these plots is about 4.7 tonnes of grain/ha compared with 5.3 tonnes/ha produced by plots receiving 192 Kg N/ha. The algal contribution in the latter case was 1.4 Kg which is less than 1% of the total.

Whether or not this method of agriculture is practicable will depend to a large extent on the degree of algal cover which can be achieved. Although highly significant increases in nitrogen fixation were produced by

algal inoculations the rates obtained represented only about half of those given by the natural algal population on Broadbalk in the same year. A number of factors might be responsible for this.

The size of inoculum applied was probably very small compared with the numbers of akinetes which have accumulated on Broadbalk over the last 140 years, and the effect of the inoculation might produce a cumulative increase in algal population in subsequent years. Another factor which may be of importance, is the topographical position of the plots in relation to soil moisture. The areas of Broadbalk studied lie at the bottom of a shallow slope, whereas the field trial plots were on flat, relatively high, ground. The affect of adding Mo, which has been shown to give an increase in algal nitrogen fixation under paddy conditions (De and Mandal 1956), might have been interesting in respect to algal establishment.

The most effective species of inoculum, Nostoc ellipsosporum, was an indigenous strain isolated in axenic culture from the field. In retrospect this finding might have been predicted since there is presumably considerable competition between the algal species for suitable niches within the micro-environment on the soil surface, and it would seem likely that species flourishing naturally under these conditions are well adapted to them. The suitability of a niche in this context would seem to be primarily controlled by soil moisture.

As the algal inoculation experiments show, irrigation would undoubtedly increase the contribution of biologically fixed nitrogen to temperate agricultural soils, but the water required is itself a limited commodity which is as necessary as the fixed nitrogen for which it may be exchanged.

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APPENDIX 1

CALCULATIONS

CALCULATIONS

Gas phase samples from acetylene/ethylene reducation assays were analysed by gas chromatography and the data thus produced was treated as shown below.

Let initial and final ethylene peak heights = E_1 and E_2

Let initial and final internal standard peak heights = A_1 and A_2.

$$\text{Corrected ethylene peak height} = E_2 \cdot \frac{A_1}{A_2} - E_1 = E_c$$

(If a blank sample is used as a control to avoid taking two successive gas phase samples from each bottle, it is subtracted from the expression shown above instead of the initial ethylene value).

Let the peak height given by ethylene standard containing Y ppm ethylene be E_s (A BOC special gas mix containing 100 ppm ethylene in argon was normally used). Then;

$$\text{Ethylene concentration in samples} = E_c \cdot \frac{Y}{E_s} \quad \text{ppm}$$

1 ppm = 1 µl/l, therefore;

$$\text{Ethylene produced by sample} = E_c \cdot \frac{Y \cdot V}{E_s} \mu l$$, where V = container volume in litres.

1 µmole of ethylene occupies 22.4 µl at STP, therefore;

the sample has produced $\frac{E_c \times Y \times V}{E_s \times 22.4}$ µmoles of ethylene or

$\frac{E_c \times Y \times V}{E_s \times 22.4} \times 10^3$ nmoles ethylene.

For the field samples the values were transformed to N_2 fixed on the assumption that $1 N_2 \equiv 3 C_2H_4$. Thus;

$$1 \text{ mole of } C_2H_4 \equiv \frac{28}{3} \text{ g } N_2 \text{ , or } 1 \text{ n mole } C_2H_4 \equiv 9.33 \text{ ng } N_2$$

The volume of data produced by the field experiments (about 5000 samples each year) was such that manual calculation was extremely time consuming, and the computer program shown below was designed to carry out the more tedious arithmetic.

The program calculates the ethylene production by each sample and the mean and standard deviation of each block of 'N' samples.

```
10  PRINT "INTERNAL STANDARD PROGRAM USING INFERED BOTTLE VOLUME"
20  PRINT "WHAT IS THE P.H. OF THE ETHYLENE STANDARD ?"
30  INPUT C1
40  PRINT "WHAT IS P.H. OF INTERNAL STANDARD ? "
50  INPUT C4
60  PRINT "WHAT IS INJECTION VOLUME ? "
70  INPUT I
80  PRINT
90  PRINT "AT WHAT ATTENUATION IS THE SAMPLE ACET. READING ? "
100  INPUT A
110  PRINT "WHAT IS THE REF.";
120  INPUT C$
130  PRINT
140  PRINT C$
150  PRINT "WHAT IS THE BLOCK SIZE";
160  INPUT N
170  IF N=0 THEN 410
180  LET S1=0
190  LET S2=0
200  FOR J=1 TO N
210  INPUT X1,F1,Y1
220  INPUT X2,F2,Y2
230  LET D=Y1*X2*F2/Y2-X1*F1
240  IF D>0 THEN 260
250  LET D=0
260  LET V=1010*C4*I/(Y1*A)
270  LET Y=100*D*V/C1
280  PRINT "THE          VOLUME IS.."
290  PRINT V
300  PRINT " MICRO MLS ETHYLENE PRODUCED ...."
310  PRINT Y
320  LET S1=S1+Y
330  LET S2=S2+Y*Y
340  NEXT J
350  LET M=S1/N
360  LET V=S2/N-M*M
370  LET W=SQR(V)
380  PRINT "BLOCK MEAN AND SD",M,W
400  GOTO 110
420  END
```

The constants for each block of data need be input only once (the program loops back from line 400 to line 110) and the variables designated X_1, F_1, Y_1 and X_2, F_2, Y_2 are the initial and final values for ethylene

peak height, chromatogram attenuation for ethylene, and the acetylene (internal standard) peak height. The acetylene attenuation setting is normally similar for all the members of a block, and it is therefore conveniently input only once with the constants at the beginning of the program.

The initial volume of the incubation chamber is inferred in line 260 from the volume of the injected acetylene, the initial acetylene peak height, the standard ethylene reading (compensating for variations in chromatogram response from occasion to occasion) and a constant derived experimentally using a container of known volume. Further losses during incubation are compensated for by the internal standard. The apparent volume of the container (that is the volume of the incubation chamber and the soil interstices into which the gas can diffuse) is printed out (line 290) so that values obtained from chambers with an excessive leakage can be disallowed. This was generally done if the apparent container volume exceeded 20 times the volume of injected acetylene, i.e. the concentration of the acetylene had dropped to less than 5%).

APPENDIX 2

SIMPLE APPARATUS FOR DETERMINING O_2 CONCENTRATION IN GAS SAMPLES

SIMPLE APPARATUS FOR DETERMINING 0_2 CONCENTRATION IN GAS SAMPLES

Construction

A diagram of the apparatus is shown overleaf. It was constructed from a 100 ml separating funnel and two 1 ml calibrated pipettes joined with thin ($1/8$ inch O.D.) butyl rubber tubing served to reduce the internal gas volume so minimizing volume changes due to temperature. The ends of the funnel and the pipettes were drawn out in a gas flame to accommodate the tube.

Pyrogallol packing

The alkaline pyrogallol must not be allowed to come into contact with atmospheric oxygen. The procedure used is described below.

A saturated solution of pyrogallol was prepared in a 500 ml medical flat capped with a subaseal. The solution was made alkaline by injecting 5N NaOH through the cap with a 20 ml hypodermic syringe furnished with a large needle.

The celite carrier was also placed in a medical flat (about $\frac{1}{2}$ full) and the alkaline pyrogallol was injected through the subaseal cap.

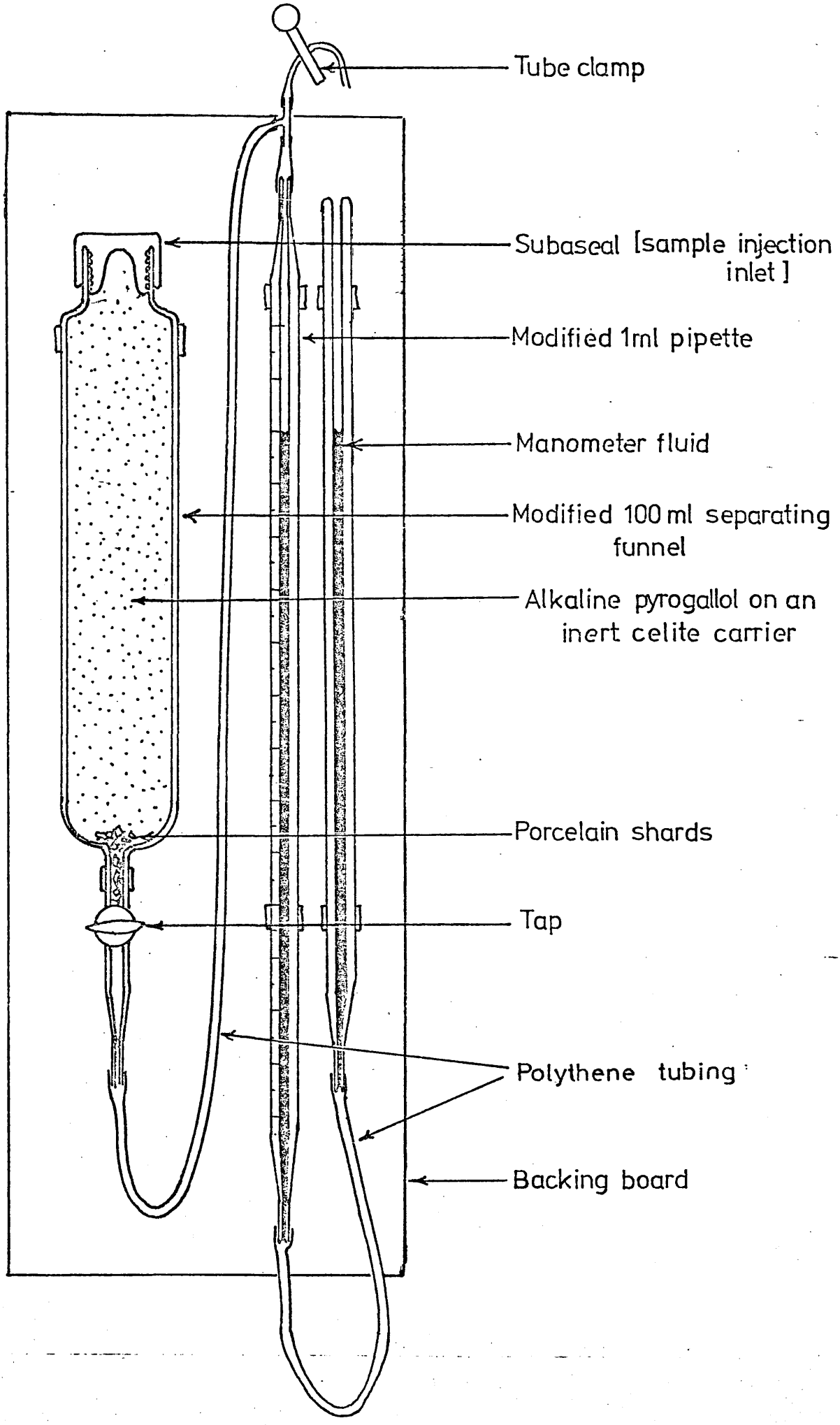
The atmosphere in the bottle can be replaced with A_r or N_2 but this is not absolutely necessary since the 0_2 is quickly adsorbed and the 20% decrease in internal pressure facilitates further injection of the solution.

The packing can be transferred to the separating funnel aneerobically by working with the hands inside a polythene bag under a stream of N_2.

Method of Use

Before the apparatus can be used, it must be flushed through with an inert gas to remove the 0_2 in the tubes which otherwise diffuses slowly back into the Pyrogallol giving a slow volume decrease.

With a tube clamp open the level of manometer fluid in the left



Simple apparatus for determining O_2 concentration in gas samples

hand pipette is adjusted to zero by raising or lowering the arm on the right hand side.

The clamp is closed off and a 1 ml gas sample is injected into the separating funnel through the subaseal. The 0_2 is adsorbed and the residual gas displaces the manometer fluid. The pipettes are again adjusted so that the level is the same in both and the volume of gas is read off. If the left hand pipette is mounted upside down with the "1 ml" mark at the top, the proportion of 0_2 in a 1 ml sample can be read off directly.

Precautions

1. The two pipettes used in constructing the apparatus must be, as nearly as possible, the same so that the capillary rise is the same in both.
2. Some types of butyl rubber tubing are permeable to certain gases (e.g. A_r and H_2) which diffuse out slowly giving a steadily decreasing volume reading. Suitable tubing such as polythene must be used for this reason.
3. The apparatus must be held in a vertical position.
4. The temperature must remain constant during the period between zero setting the manometer and reading the volume. If the laboratory temperature is subject to rapid fluctuations of more than 2 or $3^{o}C$, the apparatus may be placed in a water bath.

APPENDIX 3

CULTURE MEDIA

CULTURE MEDIA

Allen and Amon (1955)

0.001 M $MgSO_4$

0.0005 M $CaCl_2$

0.004 M NaCl

0.002 M K_2HPO_4

+ Micronutrient solution

Phosphate autoclaved separately and 0.02M K_2NO_3 added if desired.

Micronutrient solution

Fe (as EDTA Complex) 4 ppm.

Mn (as $MnSO_4 . 4H_2O$) 0.5 ppm.

Mo (as MoO_3) 0.1 ppm.

Zn (as $ZnSO_4 . 4H_2O$) 0.05 ppm.

Cu (as $CuSO_4 . 5H_2O$) 0.02 ppm.

B (as H_3BO_3) 0.5ppm.

V (as NH_4VO_3) 0.01 ppm.

Co (as $Co(NO_3)_2 . 6H_2O$) 0.01 ppm.

Ni(as $NiSO_4 . 6H_2O$) 0.01 ppm.

Cr(as $Cr_2(SO_4)_3K_2SO_4 . 24H_2O$) 0.01 ppm.

W(as $Na_2WO_4 . 2H_2O$) 0.01 ppm

Ti(as $TiO(C_2O_4)$ x.Y.H_2O) 0.01 ppm.

Henriksson (1951)

K_2HPO_4	0.5g
$MgSO_4 . 7H_2O$	0.15g
$CaCl_2 . 2H_2O$	0.05g
NaCl	0.05g
Fe Citrate	0.01g
Citric acid	0.01g
$NaMoO_4 . 2H_2O$	0.25g

Made up to 1 litre with distilled water.

Chu (1942)

K_2HPO_4	0.01 - 0.005 M
$MgSO_4 . 7H_2O$	0.025 M
Na_2CO_3	0.02 M
$NaSiO_3$	0.025 M
$FeCl_3$	0.008 M
$Ca(NO_3)_2$	0.04 M

Bunt (1942)

KH_2PO_4	0.25g
$MgSO_4 . 7H_2O$	0.125g
NaCl	0.125g
$FeSO_4 . 7H_2O$	2.5mg
$MnSO_4 . 4H_2O$	2.5mg
$NaMoO_4 . 2H_2O$	0.25mg
$CaCO_3$	5.5g

Made up to 1 litre with glass distilled water.

APPENDIX 4

ESTIMATED WEEKLY FIXATION VALUES ON BROADBALK

Estimated Nitrogen Fixation, Broadbalk 1972

May 12

Plot No	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	0.02	0.03	3
9	0	0	3
6	186.3	109.0	3
5	0	0	3
3	0	0	3

May 24

22	0	0	3
9	0.26	0.37	3
6	8.42	5.42	3
5	0.34	0.25	3
3	1.12	1.08	3

June 1

Plot No	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	0.14	0.20	3
9	1.43	1.70	3
6	0.41	0.51	3
5	0	0	3
3	0.41	0.33	3
7	0.38	0.38	2
8	0	0	1

June 6

22	4.24	3.30	4
9	2.85	4.76	4
6	1.70	1.89	4
5	8.53	32.62	4
3	0	0	4

June 13

22	8.77	1.81	2
9	0.12	0.12	2
6	3.74	3.74	2
5	26.73	26.73	2
3	0	0	2

June 21

Plot No	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	37.30	5.87	4
9	0.29	0.50	4
6	234.2	112.8	4
5	396.0	223.7	4
3	13.64	1.78	4

June 29

22	15.22	10.06	4
9	3.90	4.54	4
6	39.05	45.05	4
5	234.1	227.8	4
3	8.44	5.61	4

July 6

22	1.61	1.41	4
9	1.23	1.21	4
6	17.70	21.81	4
5	52.32	29.97	4
3	1.74	1.65	4
7	6.59	6.63	4
8	2.87	2.15	4

July 14

22	0	0	2
6	1.41	0.59	2
5	0	0	2
3	1.37	1.37	2

July 20

Plot No	Mean fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	1.06	2.5	4
9	4.21	3.34	4
6	0.16	0.24	4
5	0.76	0.26	4
3	0.75	0.79	4

July 27

22	0.06	0.09	4
9	0.29	0.09	4
6	0.51	0.46	4
5	0.45	0.47	4
3	0.15	0.16	4

August 1

22	24.24	6.16	4
9	0	0	4
6	65.07	2.03	4
5	103.0	40.05	4
3	8.80	1.36	4

August 10

22	45.85	48.94	4
9	1.39	1.23	4
6	155.0	112.8	4
5	176.5	138.6	4
3	80.63	5.03	4

August 16

Plot No	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	63.86	61.69	4
9	8.70	2.01	4
6	120.7	114.8	4
5	37.61	36.48	4
3	134.0	133.3	4

August 23

22	1.11	0.02	2
6	0.83	0.03	2
5	0.72	0.43	4
3	1.59	0.99	4

August 31

22	0.09	0.16	4
9	0.26	0.17	4
6	0.35	0.48	4
5	0.31	0.54	4
3	0.09	0.16	4

Estimated Nitrogen Fixation, Broadbalk 1971

June 28 Samples selected for the visual presence of algae.

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	3.915	0.62	3
22	-	162.45	42.35	2
9	+	0.85	1.2	3
9	-	-	-	
6	+	10.525	2.25	3
6	-	4.6	0.7	2
5	+	9.6	9.35	2
5	-	168.5	41.1	3
3	+	73.5	48.4	3
3	-	57.45	50.3	3

Samples selected for the visual absence of algae

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	22.215	20.375	2
22	-	0.16	0.225	3
9	+	1.95	1.5	3
9	-	-	-	2
6	+	2.45	6.1	3
6	-	3.35	2.6	3
5	+	9.15	7.05	3
5	-	49.6	27.4	3
3	+	-	-	-
3	-	13.85	17.95	3

July 6 Samples selected for the visual presence of algae

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	21.5	25.5	6
22	-	65.5	37.4	6
9	+	1.03	1.05	6
9	-	9.20	5.40	6
6	+	172.0	26.1	6
6	-	538.0	212.0	6
5	+	88.2	45.8	6
5	-	336.0	174.0	6
3	+	96.0	39.2	6
3	-	80.0	30.2	6

July 6 Samples selected for the visual absence of algae

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	2.21	1.90	6
22	-	13.8	6.80	6
9	+	2.73	1.04	6
9	-	7.40	5.80	6
6	+	16.6	19.6	6
6	-	48.0	38.0	6
5	+	9.78	4.85	6
5	-	65.8	89.8	6
3	+	36.5	22.6	6
3	-	62.6	23.6	6

July 28 Samples selected for the visual presence of algae.

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	0.415	0.8	6
22	-	8.8	7.35	6
9	+	1.9	4.87	6
9	-	1.975	2.85	6
6	+	11.0	19.35	6
6	-	40.55	58.5	6
5	+	65.8	44.0	6
5	-	91.75	48.0	6
3	+	35.35	26.5	6
3	-	52.0	28.5	6

Samples selected for the visual absence of algae.

Plot No	Herbicide	Mean Fixation $ngN_2/cm^3/hr$	Standard deviation	No of replicates
22	+	0.14	0.08	6
22	-	3.8	1.90	6
9	+	1.3	0.80	6
9	-	-	-	6
6	+	4.255	4.55	6
6	-	7.2	0.50	5
5	+	7.15	3.60	6
5	-	16.85	14.5	6
3	+	10.2	5.50	5
3	-	24.3	14.8	6

August 4 Samples selected for the visual presence of algae.

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
9	-	-	-	-
6	-	317.7	109.4	7
5	-	273.5	148.0	6
3	-	401.5	238.5	7

August 11 Samples selected for the visual presence of algae.

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	38.4	64	8
22	-	214.0	128.5	8
9	+	17.4	48.2	8
9	-	61.8	115.5	8
6	+	535.0	66.0	8
6	-	357.5	140.5	8
5	+	525.5	127.0	8
5	-	338.5	135.0	8
3	+	333.5	79.5	8
3	-	439.5	141.0	8

August 27 Samples selected for the visual presence of algae

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	520.0	289.5	6
9	+	52.0	92.5	5
6	+	399.5	91.0	6
5	+	346.0	292,0	6
3	+	126.0	50.2	6

September 1 Samples selected for the visual presence of algae

Plot No	Herbicide	Mean Fixation $ngN_2/cm^2/hr$	Standard deviation	No of replicates
22	+	109.4	56.5	6
22	-	108.5	48.9	6
9	+	16.3	35.0	6
9	-	1.20	0.70	6
8	+	111.8	68.9	6
8	-	0.80	0.67	6
7	+	132.9	61.6	6
7	-	116.7	82.5	6
6	+	84.4	29.5	6
6	-	139.5	38.4	6
5	+	79.5	36.2	6
5	-	69.4	44.6	6
3	+	50.1	9.45	6
3	-	58.6	37.4	4