# *RESPONSE OF CYANOBACTERIAL NITROGEN FIXATION TO ENVIRONMENTAL FACTORS*

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# *RESPONSE OF CYANOBACTERIAL NITROGEN FIXATION TO ENVIRONMENTAL FACTORS*

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*CERTIFICATE*

*This is to certify that the thesis entitled "Response of Cyanobacterial Nitrogen Fixation to Environmental Factors", submitted to the Faculty of the Post-Graduate School of the Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology embodies the results of bonafide research carried out by Shri P.A. Chandrasekkaran under my guidance and supervision and that no part of thesis has been submitted for any other degree or diploma. I further certify that such help or source of information as has been availed of in this connection is duly acknowledged.*

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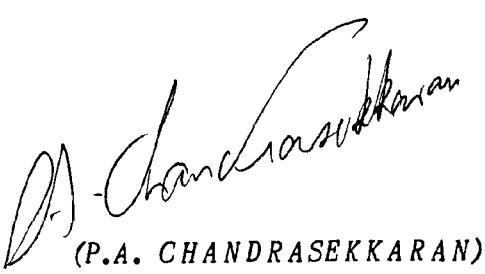

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# I. INTRODUCTION

Biological nitrogen fixation is restricted to certain prokaryotic diazotrophs like photosynthetic bacteria and cyanobacteria (blue-green algae), which accounts for 40-45% of the total nitrogen fixed annually in the biosphere. Cyanobacteria are $O_2$-evolving photosynthetic prokaryotes, many (but not all) species of which fix dinitrogen aerobically. The chief ecological and agricultural importance of cyanobacteria depends upon the ability of certain species to carry out both photosynthesis and nitrogen fixation, the photosynthetic process providing the energy required for nitrogen fixation. This trophic independence from carbon and nitrogen together with a great adaptability to variations of environmental factors makes cyanobacteria as a model system for biological investigations. Further, these algae are abundant in low land rice fields and the inherent fertility of these agricultural ecosystems has now been shown to be largely due to these algal activities. Although photosynthetic bacteria do fix nitrogen, their distribution in natural ecosystems is rather restricted by their exacting environmental requirements like anaerobic conditions and their ecological role is probably much less important than cyanobacteria. While biological nitrogen fixation in natural ecosystems is hardly limited by the number of nitrogen fixing microbes the actual nitrogen accretion through this process is rather small. This is largely because of a variety of environmental pressures which may prevent these organisms from performing what they otherwise do under pot culture or glass house conditions. Hence, if biological nitrogen fixation is to be harnessed advantageously in our crop

production systems, we have not only to identify such stresses, but also devise methods to overcome them.

The present investigation is aimed at examining the effect of the following factors on $N_2$ fixation by Anabaena variabilis ARM 394:

1. Temperature
2. Light
3. pH
4. Combined nitrogen ($NO_3$, $NH_4$, Urea)
5. Phosphorous
6. Fungicides (Dithane and Ceresan-M)
7. Herbicides (2,4-D, Dalapon, Diuron, Machete)
8. Insecticides (BHC and Phorate)

## II. REVIEW OF LITERATURE

Cyanobacteria are ubiquitous in distribution and are found in many different ecosystems. Frank (1889) and Prantl (1889) first indicated that these organism may fix $N_2$. The agronomic potential of cyanobacteria was pointed out by De (1939), who attributed the natural fertility of tropical paddy fields to the $N_2$-fixing blue-green algae. Since then many workers have intensively studied the basic and applied aspects of nitrogen fixation by these algae (see Stewart 1980; Gallon, 1980; Roger and Kulasooriya, 1980; Venkataraman, 1981a,b; Hallenbeck, 1987). Today our understanding has vastly increased about (i) the similarity of cyanobacteria to Gram-negative bacteria (Stanier and Cohen-Bazire, 1977); (ii) occurrence of $N_2$-fixing ability in almost all the major typological groups; heterocysts being the main site of dinitrogen-fixation (Haselkorn, 1978; Stewart, 1980); (iii) the ability of some non-heterocystous forms to fix nitrogen under anaerobic or microaerobic conditions (Bunt et al. 1970; Stewart, 1971; Carpenter & Price, 1976; Waterbury & Stanier, 1978; Rippka et al. 1979; Gallon, 1980); (iv) the involvement of glutamine synthetase (GS), often coupled with glutamate synthase (GOGAT) in ammonia assimilation, metabolism and regulation (Dharmawardene et al., 1973; Stewart et al., 1975; Thomas et al., 1975; Wolk et al., 1976; Meeks et al., 1977; Rowell et al., 1977); (v) presence of transcriptional control (Lau et al., 1977, 1980; Armstrong and Van Baalen, 1979; Gendel et al., 1979; Karagouni and Slater, 1979; Ownby et al., 1979; Haselkorn et al., 1983a,b); (vi) ability of certain forms for anoxygenic photosynthesis (Murai and Katoch, 1975; Smith and Hoare, 1977;

Bottomley and Van Baalen, 1978; Hallidal and Holman, 1979); (vii) oxidative pentose pathway as the major route of carbon dissimilation (Lex and Carr, 1974; Apte et al., 1978; Stewart, 1980); (viii) ability of the nitrogenase (Nase) to act as an ATP-dependent hydrogenase and generate hydrogen from water in presence of light (Kondratieva, 1976; Krasnovsky, 1976; Bothe et al., 1978; Eisbrenner, et al., 1978; Hallenbeck and Benemann, 1978; Hallenbeck et al., 1979a,b; Gallon, 1980); (ix) the presence of a membrane-bound uptake hydrogenase activity (Peschek, 1979 a,b,c, 1980); (x) presence of plasmids (see Lau et al., 1977, 1980; Reaston et al., 1980); (xi) regulatory effect of other factors like $O_2$ concentration, Mo concentration and light besides fixed N, on nif gene expression in the heterocystous cyanobacteria (Tozum and Gallon, 1979; Adams and Carr, 1981; Haselkorn et al., 1983a,b; Ramos et al., 1985); (xii) development of gene cloning and transformation systems for the unicellular non-N-fixing Anacystis nidulans (Van den Hondel et al., 1980; Kuhlemeier, 1981; Golden and Sherman, 1984); (xiii) construction of Hybrid Shuttle Vectors capable of transfer from E.coli to a number of N-fixing filamentous cyanobacteria (Wolk et al., 1984; Flores and Wolk, 1985); (xiv) isolation and sequencing of structural nif genes from Anabaena 7120 (Mevarech et al.,1980; Mazur and Chui, 1982; and its ability to restructure its own nitrogen fixing genes (Golden et al.,1985); and (xv) organization and sequencing of genes and molecular evidence for endosymbiotic origin (Cozens and Walker, 1987).

Nitrogen Fixation

From the view point of $N_2$ fixation, the most unique property of the cyanobacteria is their ability, in many cases, to carry out

the seemingly paradoxical simultaneous evolution of $O_2$ and reduction of dinitrogen. Many (but not all) species carry out these processes simultaneously by virtue of specialized, differentiated cells called heterocysts. The biochemistry and physiology of cyanobacterial nitrogen fixation and heterocysts, either exclusively or in a wider context, has been well documented (Stewart et al., 1978; Wolk, 1980; Adams and Carr 1981; Gallon, 1981; Lambert and Smith, 1981; Meeks, 1981; Bothe, 1982; Fay, 1976; Hallenbeck, 1987). With a few exception of the unicellular algae (Rippka et al., 1977; Singh, 1961) and the non-heterocystous blue-green algae Plectonema boryanum (Stewart and Lex, 1970), the heterocysts are present in most of the nitrogen fixing cyanobacteria.

Heterocystous cyanobacteria and unicellular Gloeocapsa fix nitrogen under aerobic as well as microaerobic conditions (Stewart, 1973), but non-heterocystous forms like P. boryanum 594, Phormidium sp., Oscillatoria sp., Lyngbya sp., Raphidiopsis sp. are known to fix nitrogen only under microaerobic conditions (Gallon, 1980).

There is now positive evidence for the nitrogen fixing ability of more than 125 strains or types of cyanobacteria (Table 1) (see Venkataraman, 1981a).

The regulation of heterocyst differentiation and the organization of the nif genes in Anabaena 7120 have received considerable attention (Meverech et al., 1980; Haselkorn et al., 1983a,b). The early studies showed that under aerobic conditions, the nitrogenase proteins are synthesized only in heterocysts (Fleming and Haselkorn, 1973)

*Table 1. Nitrogen fixing blue-green algal genera (After Gallon, 1980; Stewart, 1980; Venkataraman, 1981a) (Some of the strains occur symbiotically with other organisms).*

| | |
|---|---|
| *Unicellular* | *Synechococcus* |
| | *Gloeocapsa* |
| | *Aphanothece* |
| | *Dermocarpa* |
| | *Xenococcus* |
| | *Myxosarcina* |
| | *Chroococcoidiopsis* |
| | *Pleurocapsa group* |
| *Filamentous, non-heterocystous* | *Plectonema* |
| | *Lyngbya* |
| | *Trichodesmium* |
| | *Oscillatoria* |
| | *Pseudanabaena* |
| | *Microcoleus* |
| | *Schizothrix* |
| *Filamentous, heterocystous* | *Anabaena* |
| | *Nostoc* |
| | *Nodularia* |
| | *Cylindrospermum* |
| | *Scytonema* |
| | *Calothrix* |
| | *Anabaenopsis* |
| | *Mastigocladus* |
| | *Fischerella* |
| | *Tolypothrix* |
| | *Aulosira* |
| | *Stigonema* |
| | *Hapalosiphon* |
| | **Chlorogloea* |
| | *Camptylonema* |
| | *Gloeotrichia* |
| | *Nostochopsis* |
| | *Rivularia* |
| | *Scytonematopsis* |
| | *Westiella* |
| | *Westiellopsis* |
| | *Wollea* |

**Colonial heterocystous*

and the development of the heterocysts is by activation of sets of genes (Fleming and Haselkorn, 1974). The structural genes for nitrogenase and nitrogenase reductase as well as those for GS and the large subunit of RuBPCarboxylase have been cloned from Anabaena and physically mapped (Haselkorn, et al. 1983a,b). In the unicellular Gloeothece, the three genes nifH, nifK and nifD are adjacent and cotranscribed (Kallas et al., 1983) as in Klebsiella. In the filamentous Anabaena 7120, nifH and nifD are adjacent, whereas nifK is 11 kbp from nifD and transcribed independently (Haselkorn et al., 1983a). In Anabaena 7120, one nif RNA transcript has been shown to originate from a site that lacks good correspondence with a typical procaryotic strong promotor, suggesting the possibility of a need for positive activation. The nifH message is either unstable or repressed or both under aerobic conditions. This accounts for the need for heterocyst differentiation to fix nitrogen aerobically (Haselkorn et al., 1983a,b). In Anabaena 7120, heterocyst differentiation is accompanied by excision rearrangement. A site specific recombination between an 11 base-pair direct repeat sequence flanking the nifK and nifD genes removes 11 kilobase pairs of intervening DNA resulting in juxta position of the two genes and an alteration of the nifD protein-coding sequence. These rearrrangements are specific to the differentiation of heterocysts and are triggered by the environmental cue of nitrogen deprivation (Golden et al., 1985).

One of the approaches has led to the intriguing possibility of associating nitrogen fixing blue-green algae with crop plants.

Uptake of Anacystis nidulans into protoplasts of Parthenocissus tricuspidata (Davey and Power, 1975), Gloeothece into maize, tobacco and Neurospora protoplasts (Burgoon and Bottino, 1977) and Anabaena variabilis into tobacco (Meeks et al., 1978) has been demonstrated, but neither protoplast division nor nitrogen fixation could be demonstrated.

Nitrogenase

Although $N_2$-fixation by cell-free extracts was noted as early as 1960 (Schneider et al., 1960), biochemical studies on the Nase proteins from cyanobacteria have largely lagged behind many of the other diazotrophic organisms, probably due to the lower in vivo and in vitro activity. Early studies using cell free extracts were mainly to examine electron donation to Nase (see Codd et al., 1974). Cyanobacterial Nase is quite similar in its molecular characteristics to those of the Nase of other organisms (Hallenbeck et al., 1979a,b) and it catalyses the reduction of various substances like nitrogen, proton, cyanide and acetylene (Gallon, 1980). The requirement for ATP and reductant is nearly identical to that of other organisms, although unlike other Nase, excess dithionite has been reported to inhibit markedly A. cylindrica Nase (Haystead et al., 1972). Similar to other Nases, A. cylindrica Nase is composed of two oxygen-sensitive components that can fix dinitrogen only in combination (Hallenbeck et al., 1979a,b).

The Fe-protein binds MgATP and transfers electrons to the MoFe protein which binds and reduces the substrate (Burris et al., 1980). However, the most striking difference from other Nases that

has been found for the Nase from A. cylindrica in the pattern of cross reaction of its two protein components with the heterologous components from other N-fixing bacteria. The Mo-Fe protein of Anabaena cylindrica Nase forms active complexes with all the Fe-proteins examined. This is surprising in that antiserum to A. cylindrica Mo-Fe protein was found to only cross react with cyanobacterial Nase proteins from M. laminosus N. muscorum and A. variabilis, but not with these of A. vinelandii, R. rubrum or P. boryanum 401 (Hallenbeck, 1987). Surprisingly the Fe component of A. cylindrica Nase is very inactive with the Mo-Fe components from a variety of other organisms. These results suggest that a relatively small, specialised region of the Mo-Fe molecule is involved in Fe-protein binding and subsequent electron transfer.

Oxygen Protection

Nase is extremely $O_2$ labile and hence many cyanobacteria have evolved a variety of oxygen protection mechanisms (Weare and Benemann, 1973; Gallon, 1981). The most common oxygen protection mechanism is found in the members of the Nostacaceae, in which a specialized vegetative cell, heterocyst, allows the spatial separation of the basically incompatible processes of $O_2$ evolution and $N_2$ fixation (Almon and Bohme, 1980). Entry of external oxygen is thought to be restricted by the heterocyst envelope, which contains four unique glycolipids (Haury, and Wolk, 1978). Mutants that lack these glycolipids are deficient in aerobic Nase activity (Haury and Wolk, 1978). The ability of the heterocyst to exclude $O_2$ appears to vary with growth conditions (Murry et al., 1984) with growth in

the presence of moderate to high concentrations of $O_2$ apparently being required for the development of maximum protection, suggesting that $O_2$ is required for the induction of some key $O_2$ - excluding components. In this connection, it has been reported that adaptation of A. cylindrica to high $O_2$ tensions involves, at least in part, the induction of superoxide dismutase and catalase (Mackey and Smith, 1983) and Nase synthesized under strictly anaerobic conditions is markedly $O_2$ sensitive (Rippka and Stanier, 1978). At any rate, it is obvious that $O_2$ cannot be totally excluded from the heterocyst. Additional factors that are capable of protecting Nase from $O_2$ inactivation include enhanced rates of respiration in the heterocyst (Lex et al., 1972), the oxy-hydrogen reaction, (Houchins and Burris, 1981a,b) and $H_2$-stimulated $O_2$ uptake catalysed by the uptake hydrogenase localized in the heterocyst. Recently, it has been suggested that the ability of heterocystous cyanobacteria to rapidly recover Nase activity after a short-term exposure to high $O_2$ tension may be due to a type of conformational protection mechanism, as proposed for Azotobacter (Robson and Postgate, 1980) as well as de novo synthesis of Nase (Pienkos et al., 1983).

In non-heterocystous forms, a temporal separation of photosynthesis and nitrogen fixation has been suggested. There is also some evidence for compartmentalization in Plectonema (Meeks, et al., 1978) and Trichodesmium (Bryceson and Fay, 1981). In the unicellular Gloeothece, the oxygen-protection is achieved by several means such as temporal separation of photosynthetic $O_2$ evolution and nitrogen fixation; an oxygen-linked hydrogen uptake; presence

of tocopherols which may serve as an antioxidant (Newton et al., 1977) and certain internal membranes which may serve as intracellular protective compartment (Gallon, 1980). In addition, Gloeothece possesses an active superoxide-metabolizing system (Tozum and Gallon, 1979). Evidence for conformational protection in these non-heterocystous organisms is equivocal (Gallon and Hamadi, 1984).

Reductant and ATP

There is no evidence in any diazotroph for specialised mechanisms for supplying ATP to Nase. Hence, as might be expected, photophosphorylation, oxidative phosphorylation and substrate level phosphorylation probably have varying degrees of importance in meeting this requirement, depending on particular environmental conditions (Stewart, 1980; Gallon, 1980). In heterocystous species, the energy expenditure due to $N_2$ fixation appears to cause a much lower energy charge in heterocysts, relative to vegetative cells (Privalle and Burris, 1983) and the energy charge, which is responsive to changes in light intensity or $O_2$ tensions, appears to be a major factor controlling Nase activity, at least in isolated heterocysts (Ernst et al., 1983). If adequate reductant is available, cyclic photophosphorylation can, by itself, meet the ATP demand in the light (Benemann and Weare, 1974) and oxidative phosphorylation can do so in the dark, supporting about 50% of the light rate of Nase activity (Fay, 1976). In Gloeothece, prolonged rates of $N_2$ fixation supported by substrate level phosphorylation have been reported (Gallon, 1980).

Although many non-heterocystous cyanobacteria are capable of synthesizing Nase and supporting its activity under strictly anaerobic conditions (Rippka and Waterbury, 1977) virtually nothing is known regarding the reductant supply under these circumstances. Likewise, filamentous heterocystous species are capable of anaerobic $N_2$ fixation (Rippka and Stanier, 1978). Whether Nase is synthesized and active in the vegetative cells under these conditions is still a matter of controversy, but if so, the pathways of reductant flow to Nase under these conditions are unknown. Photosynthesis may directly supply reductant for light-driven $N_2$ fixation in Gloeothece (Gallon, 1980). It has been reported that Nase activity is dependent both in the light and in the dark on respiration in Gloeothece, which supplies both ATP as well as the reductant in the light (Mullineaux et al., 1980). In vitro studies have suggested that isocitrate and possibly malate might supply reductant via $NADP^+$, ferredoxin and the corresponding dehydrogenase (Gallon et al., 1973). Additionally, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase may play major roles, since the specific activity of these enzymes is four to ten fold greater in nitrogen fixing cultures (Gallon et al., 1974). In heterocystous aerobic cyanobacteria, nitrogen fixation is believed to be confined to the heterocyst, since it lacks the Mn containing component of the $O_2$ evolving system of photosystem II (Tel-Or and Stewart, 1977) is unable to reduce directly ferredoxin with water derived electrons. Since heterocyst lacks ribulose 1-6-bis phosphate carboxylase, it is largely incapable of $CO_2$ fixation. Hence, the reductant derived from C-metabolism must be provided by the C compound(s) imported from the vegetative cell.

The nature of this carbon compound is at present uncertain. Of a variety of sugars tested, only D-erythrose supported high rates of $N_2$ fixation by isolated heterocysts (Privalle and Burris, 1984). Heterocysts when incubated for short time with $CO_2$ accumulates significant labelled quantities of alanine, suggesting transport of alanine, glutamate and sugars (Juttner, 1983). If the imported alanine is oxidized in the heterocyst to glutamine, several reducing equivalents would be produced offering a possible explanation for the high activities of alanine dehydrogenase previously found in heterocysts (Rowell et al., 1977). Other reductant generating enzymatic pathways like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase whose activities in the heterocyst fraction are five to ten times that of vegetative cells have been reported (Apte et al., 1978). NADPH appears to donate electrons directly to Nase in the dark via a ferredoxin: NADPH oxido-reductase, whereas in the light DMBIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) inhibition of this reaction indicates an involvement of photosystem I. In the same way, NADH appears to support Nase activity in the light by donating electrons to photosystem I at the plastoquinone level (Schrautemeier et al., 1984, 1985).

In cyanobacteria, it has long been recognised that $H_2$ is capable of supporting Nase activity in reducant-limited cultures (Benemann and Weare, 1974) and accordingly, high rates of $H_2$ supported acetylene reduction by isolated heterocysts have been reported (Smith et al., 1985). The light-mediated flow of reductant from $H_2$ to Nase appears to follow the same pathway as NADH, namely, entering photosystem I at the plastoquinone level

(Ernst and Bohme, 1984). Cyanobacterial ferredoxin is widely believed to be the immediate electron donor to Nase (Houchins, 1984). Ferredoxins have been isolated and sequenced from a large number of cyanobacteria. Many cyanobacteria, including Nostoc strain Mac (Hutson et al., 1978), N. verrucosum (Shin et al., 1977), Aphanothece sacram and Spirulina maxima (Cammack et al., 1977) contain two distinct species of ferredoxins, an apparent result of ancestral gene duplication (Hase et al.,1978). These two ferredoxins are present in the same proportion in photoautotrophically and dark heterotrophically grown cells. Thus, their synthesis appears to be independent of growth conditions. The specific role of each ferredoxin in cellular metabolism has not yet been determined (Hutson et al., 1978). A ferredoxin fraction obtained from heterocysts has also been shown to differ markedly in its biological properties from that of the vegetative cells (Schrautemeier and Bohme, 1985). Preliminary evidences indicate that it is a (2Fe-2S) ferredoxin and immunologically distinct from the vegetative cell ferredoxin. This suggests that heterocysts contain a specific electron carrier for Nase, although this requires further proof from genetic and biochemical analysis.

Ammonia Assimilation

The major route of primary ammonia assimilation in most of these algae is through glutamine synthetase (GS) coupled with ferredoxin linked glutamate synthase (GOGAT) (Wolk et al., 1976; Rowell et al., 1977). GS and aminotransferases are present in heterocysts, while GOGAT and GS in vegetative cells. Nitrate has a variable and transitory inhibitory effect on nitrogen fixation by blue-green algae (Haselkorn, 1978) and presence of high concentrations of ammonia severely

decreases the nitrogenase activity (Tuli and Thomas, 1980) and glutamine synthetase (Quinto et al., 1977) and this has been shown to be mediated by a variety of mechanisms including repression (Woolfolk et al., 1966), degradation (Quinto et al., 1977) and disaggregation (Sims et al., 1974) of the enzyme or modulation by adenylylation cascade. GS is known to exist in adenylylated and unadenylylated states in bacteria (Wohlhueter et al., 1973), but evidence for this in blue-green algae is lacking. The marine Anabaena CA is perhaps the only form that expresses nitrogenase even in presence of excess of ammonia, whereas the enzyme activity is not expressed in presence of nitrate-nitrogen (Bottomley et al., 1979). This anomolous response could be probably because of a naturally occurring mutation in the regulatory mechanism. The nitrogen-derived ammonia and glutamine formation occur only in heterocysts (Thomas, 1978) and most of the glutamine is transported to vegetative cells where it is metabolized to glutamate and various other amino acids by glutamate synthase and amino-transferases located in vegetative cells. Part of the glutamine could be converted into glutamate in the heterocysts by the aminotransferases located there. The glutamate thus produced may function as a substrate for more glutamine formation. Glutamate could also be transported to heterocysts from vegetative cells either directly or as aspartate, which could be produced by transamination (see Meeks, 1981).

Besides ammonia, the overall mechanism of nitrogenase regulation seems to involve intracellular level of amino acids. In Plectonema, arginine and glutamine prevent induction of nitrogenase

(Nagatani and Haselkorn, 1978). In Anabaena, intracellular glutamine pools increase in seconds in response to ammonia, followed by a decrease in nitrogenase activity (Rowell et al., 1977). Recently, it has been shown that low levels of exogenous glutamate inhibit nitrogenase, the inhibition being, however, alleviated by aspartate (Thomas, 1978). The overall mechanism of nitrogenase regulation involves the conversion of ammonia to the level of amino acids. Subsequent steps in the regulatory circuit between amino acids and nif operon(s) remain largely unknown. It is, however, tempting to speculate that amino acids work as effectors of glutamine synthetase protein, which has been hypothesized to serve as a positive genetic activator for the expression of the nif genes (Shanmugham and Morandi, 1976). Antibiotics like rifampicin, streptomycin and chloramphenicol inhibit nitrogenase activity, suggesting that transcription of DNA as well as translation of mRNA (protein synthesis) might be involved (Gallon, 1980; Chandrasekkaran and Venkataraman, 1985). The removal of these antibiotics from the medium allows the nitrogenase activity to return to its original level in Anabaena variabilis (Chandrasekkaran and Venkataraman, 1985).

GS and/or a product, like ammonia is involved in the inhibition of nitrogenase synthesis and heterocyst production, although the relationship between nitrogenase and heterocyst is not clear. It is also possible that repression of nitrogenase in these systems may not be regulated by GS (Tuli and Thomas, 1980; Khan et al., 1985) and the newly identified glnF product may be involved (Gaillardin and Magasanik, 1978). The regulation of heterocysts has been attributed

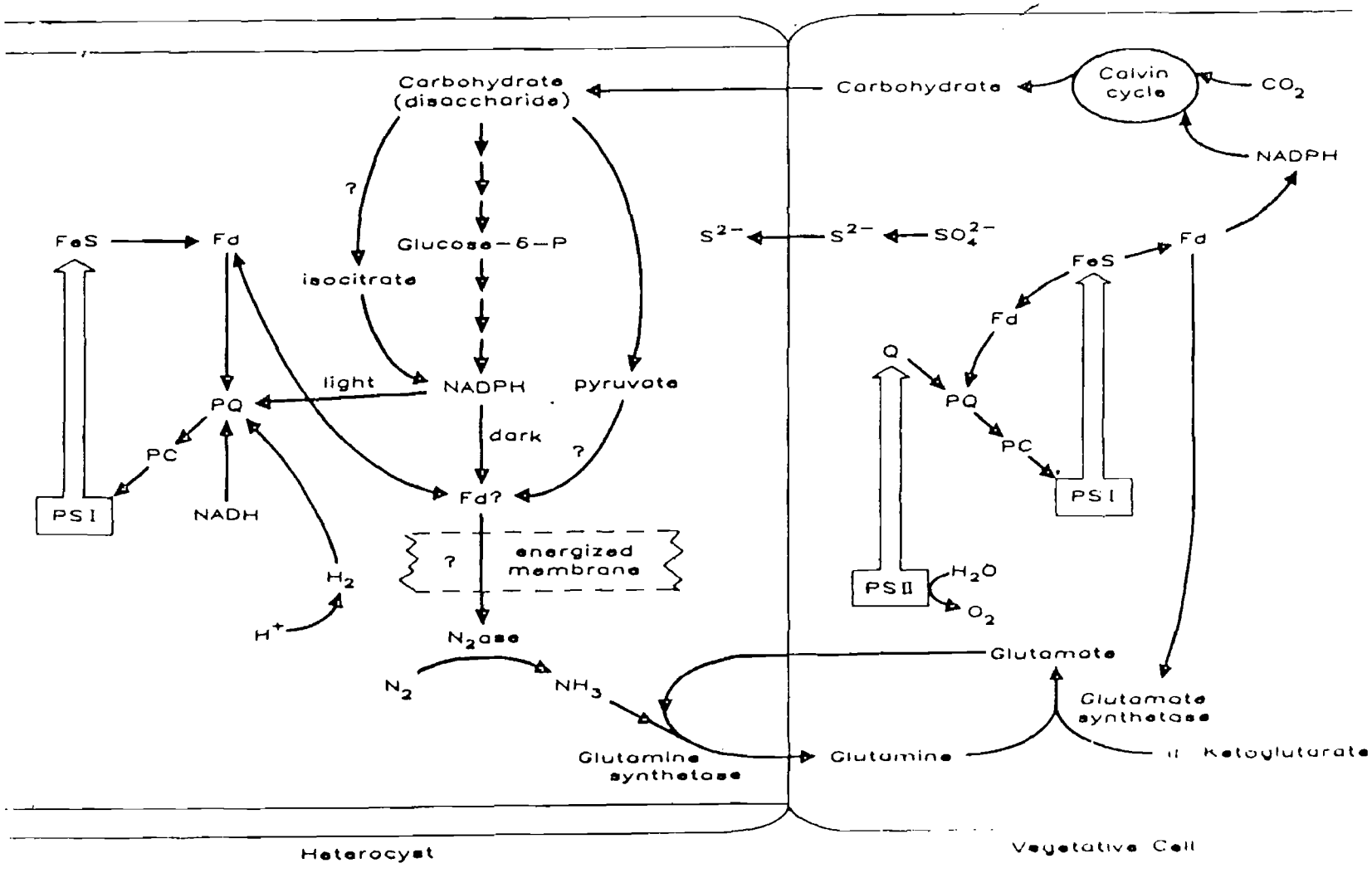
to a "field of inhibition", according to which the heterocysts inhibit the nearby vegetative cells from becoming heterocysts (Wolk, 1967; Wilcox et al., 1973). This inhibition is thought to be mediated by a diffusible substance(s) produced in heterocysts. The only nitrogenous compound that has hitherto been shown to be produced by and exported from heterocysts in glutamine (Thomas et al., 1977). In Anabaena L-31, extracellular polypeptides have been implicated in heterocyst regulation (David and Thomas, 1980). Cyclic AMP (Hood et al., 1979) or cyclic GMP, independent of GS may also be involved (Stewart, 1980).

The first stable product of nitrogen fixation is ammonia. The first major organic product of assimilation of ammonia is glutamine (Meeks, 1981) and glutamate is the second product. Alanine appears to be formed primarily by alanine dehydrogenase in Anabaena cylindrica, Cylindrospermum licheniforme, Plectonema boryanum and Anacysts nidulans, while in Anabaena variabilis, by transamination reaction, probably from glutamate, rather than by direct amination with ammonia (Meeks et al., 1978). The possible pathways of nitrogen fixation in heterocystous cyanobacteria are shown in Fig. 1.

*Hydrogen production*

The nitrogenase enzyme can also function as an ATP-dependent hydrogenase and generate hydrogen from water (Hallenbeck et al., 1979a; Stewart and Gallon, 1980). There appears to be two types of uptake hydrogenases, one linked to photoreducing activity and another to oxygen-dependent activity of the cells (Peschek, 1980).

*Fig. 1. Schematic representation of interrelationships between heterocysts and vegetative cells in nitrogen fixing cyanobacteria (Hallenbeck, 1987).*


Carbohydrate (disaccharide)
Carbohydrate
Calvin cycle
$CO_2$
NADPH
?
Glucose–6–P
isocitrate
FeS
Fd
$S^{2-}$
$S^{2-}$
$SO_4^{2-}$
Fd
FeS
Fd
Q
PQ
PC
PS I
light
NADPH
pyruvate
dark
?
Fd?
PQ
PC
PS I
NADH
$H_2$
$H^+$
?
energized membrane
PS II
$H_2O$
$O_2$
$N_2$ase
$N_2$
$NH_3$
Glutamate
Glutamate synthetase
Glutamine synthetase
Glutamine
α Ketoglutarate
Heterocyst
Vegetative Cell

A soluble inducible, reversible hydrogenase activity has also been demonstrated in vegetative cells and heterocysts (Hallenbeck and Benemann, 1979). Both the particulate hydrogenase and soluble hydrogenase have been considered to be expression of a single multifunctional enzyme. The uptake hydrogenase is beneficial, as it helps to recycle hydrogen lost through nitrogenase activity.

Ecological Aspects

The ability to fix nitrogen confers on blue-green algae an agricultural importance and also a good survival value under conditions of limitations of fixed nitrogen.

The last two decades have witnessed remarkable advancements in our efforts to domesticate and harness the nitrogen fixing blue-green algae as a biological input in lowland rice ecosystems.

Although rice fields provide an ideal environment for cyanobacteria, it is not always true that all cultivated lowland fields support an abundant nitrogen fixing cyanobacteria. However, recurrent combinations of algal species might occur in comparable habitats. Large number of nitrogen fixing species belonging to different genera have been recorded from many parts of the world under water logged conditions (Henriksson et al., 1972; El-Nawawy and Hamdi, 1975).

In India, only 33% of 2213 rice field soil samples examined were found to harbour useful nitrogen fixing forms and their relative abundance showed wide variations with localization of specific forms in certain regions (Venkataraman, 1975). The occurrence, succession

and biomass production of some of these algae are influenced by crop growth and other management practices (Srinivasan, 1979; Roger and Kulasooriya, 1980; Venkataraman, 1981a). An interesting antagonism between cyanobacteria and sulphate reducing bacteria has been observed which suggests the possibility of preventing sulphate reduction by introducing blue-green algae in such situations (Roger and Jacq, 1972; Roger, 1973).

Crop growth and management practices often influence the seasonal periodicity and succession of indigenous cyanobacteria. The maximum algal biomass in rice fields has been observed after about two weeks (Kurasawa, 1956) or one month (Ichimura, 1954) before transplantation of paddy in Japan; just before tillering in Ukraine (Prikhodkova, 1968); between tillering and panicle initiation in Senegal (Roger and Reynaud, 1976) and after harvesting during wet season and after heading during dry season in Philippines (Watanabe et al., 1977, 1978a,b). Nitrogen fixing forms have been observed throughout the cultivation cycle in many areas, although generic and species composition differs and shows a succession.

Cyanobacteria, being photoautotrophic show definite responses to the quality and quantity of light (Venkataraman, 1979). In general they prefer low light intensities. Nevertheless forms like Cylindrospermum (Traore et al., 1978) and Aulosira (Singh, 1976) can grow well under high light intensities. The dense plant canopy of the dwarf high yielding rice varieties plays a definite role in regulating the availability of light during the cultivation cycle which, in turn, affect the growth and activity of cyanobacteria in the soil and water (Kulasooriya et al., 1980a,b).

These algae are seldom affected by any violent changes in temperature in lowland rice fields because the paddy water itself acts as a good temperature conditioner. Most of the cyanobacteria can withstand a high degree of dessication and regenerate on waterlogging. Wind (Roger and Reynaud, 1979), rain (Singh, 1976) and typhoons (Roger and Kulasooriya, 1980) may have an effect on the nitrogen fixing activity through reduction in the light and by erosion of algal biomass.

Various microorganisms like protozoa, fungi, bacteria and cyanophages play an important role in regulating algal growth in natural ecosystems. Many heterocystous forms are attacked by chytridiceous fungi (Canter, 1963). Since Safferman and Morris (1963) isolated the first lytic cyanophage, several phages pathogenic to cyanobacteria have been reported from Russia (Rubenchik et al., 1966), India (Venkataraman and Kaushik, 1974; Kaushik and Venkataraman, 1982b), Israel (Padan et al., 1967) and Sweden (Granhall and Hofsten, 1969). Many myxobacterialean members have a powerful cyanobacterial lysing activity in nature (Daft and Stewart, 1971). Cyanobacterial growth is also influenced by grazers like copepods, ostracods, snails and mosquito larvae (Grant and Alexander, 1981; Grant et al., 1983; Watanabe et al., 1981; Wilson et al., 1981). Chironomid larvae which graze extensively on Nostoc commune could be biologically controlled by an integrated rice-fish culture (Martinez et al., 1977).

Among the chemical factors which influence the growth and nitrogen fixation of cyanobacteria pH, nutrient status of the soil and

redoxpotential are important (Roger and Kulasooriya, 1980). The subterranean algal flora is also influenced by the type and mode of application of inorganic fertilizers like nitrogen (Roger et al., 1980, 1984), phosphorous and potassium. In general, nitrogen fertilizers show a selective action on the diazotrophs and non-diazotrophic cyanobacteria (Rinaudo, 1974). Usually nitrogenous fertilizers repress the nitrogenase activity of the diazotrophic strains. However, heterocyst differentiation and nitrogenase activity of Anabaena CA (Bottomley et al., 1979) is not repressed by ammonia. Further, only very little inhibition by nitrate and ammonia is noted in Scytonema schmidlei and Westielopsis prolifica (Khan et al., 1985).

Phosphatic fertilizer stimulates algal growth and nitrogen fixation (Araragi et al.,1978; Srinivasan, 1978; Yamaguchi, 1975). Lime application also stimulates their growth (Amma et al., 1966). Organic manures like green manure, compost and crop residue have both a stimulatory (Matsuguchi and Yoo, 1979) or depressive effect (Lehri and Mehrotra 1970).

Cyanobacterial growth results in an improvement in the soil aggregation (Roychoudhury et al., 1979, 1983), nitrogen content (Chopra and Dube, 1971) and to a lesser extent in the organic matter content (Sankaram, 1971). Possibly due to photosynthetic oxygenation, a significant reduction in the reduced substances has also been reported (Aiyer et al., 1972). Cyanobacteria have been implicated in soil reclamation of salt-affected soils (Kaushik et al., 1981; Kaushik and Krishnamurti, 1981; Kaushik and Venkataraman, 1982a,b).

The response of cyanobacteria to various agricultural chemicals like fungicides, herbicides and insecticides shows wide variations (Venkataraman and Rajyalakshmi, 1971a,b; Dasilva et al., 1975; Das and Singh, 1977; Kaushik and Venkataraman 1983), although their biological activity is little affected by these chemicals at the recommended field doses. In vitro studies indicated that some of these chemicals are stimulatory (Ahmad and Venkataraman, 1973; Srinivasan and Emayavaramban, 1977; Srinivasan and Ponnuswamy, 1978; Gangawane and Saler, 1979) or inhibitory (Venkataraman and Rajyalakshmi, 1971b; Gangawane and Saler, 1979) or neutral. Chemicals like stam F-34 and DCMU inhibit both growth and heterocyst differentiation (Vaishampayan et al., 1978), while few like Machet have been shown to have a mutagenic action (Singh and Vaishampayan, 1978; Singh et al., 1979).

Venkataraman and Rajyalakshmi (1971a) using fungicides Ceresan and dithane-M observed tolerance of high concentrations of these fungicides by T.tenuis, Aulosira fertilissima and Anabaena sp. The growth of Westiellopsis sp. was affected more than others in the presence of fungicides; on the other hand, Aulosira sp. and Calothrix sp. were resistant to these fungicides and their overall growth was not affected (Venkataraman, 1972). Ceresan from 0.1 to 100 ppm inhibited the growth of 28 strains of blue-green algae (Venkataraman and Rajyalakshmi, 1971a).;

The in vivo studies show that in general, herbicides with the ability to inhibit photosynthesis are considerably more toxic to algae in the soil than those inhibiting the other functions.

*Cylindrospermum licheniforme, isolated from soil was not affected by 2,4-D upto 400 ppm. Singh (1974) also noted a high tolerance of Cylindrospermum sp to 2,4-D at 100 ppm (Torres and O'Flaherty, 1976) and Anabaena upto 500 ppm (Venkataraman and Rajyalakshmi, 1971b). Anabaenopsis reciborskii could tolerate 2,4-D upto 800 µg/ml in liquid culture and higher concentrations were found to be lethal. The concentrations of 10 µg/ml showed slightly better growth than the control and growth was almost unaffected upto 100 µg/ml concentration (Das and Singh, 1977). Oscillatoria was found to be more sensitive to 2,4-D and fairly tolerant to MCPA (Cullimore and McCann, 1977). The herbicides 2,4-D and MCPA affected nitrogen fixation in Nostoc muscorum, N-punctiforme and Cylindrosepermum sp. at concentrations recommended for field application, but stimulate nitrogen fixation at $10^{-4}$ to $10^{-5}$ M concentrations (Inger, 1970). Das and Singh (1977) studied the effect of 2,4-D on nitrogen fixation in Anabaenopsis raciborskii where the alga gradually reduced its nitrogen fixing ability at higher concentrations (100-1000 µg/ml) but the concentration of 10 µg/ml slightly enhanced the nitrogen fixation. Singh et al., (1979) showed that the herbicides alachor and butachor showed mutagenic properties when tested in Nostoc muscorum systems.*

*Economic Importance of Blue-Green Algae*

*The role of $N_2$-fixing blue-green algae in the maintenance of the fertility of rice fields has been well substantiated and documented (Watanabe et al., 1977; Roger and Kulasooriya, 1980;*

Venkataraman, 1981a). The global experiments in which the rice plants have been shown to respond to algalisation provide presumptive evidence of their nitrogen-fixing ability (Pantastico and Gonzales, 1976). In India, the beneficial effect of blue-green algae on yields of many rice varieties has been demonstrated in a number of localities. In many areas, 10 to 20% increase in the grain yield has been observed as a result of algal application in the absence of any added chemical N fertiliser. In a large number of trials, complementation of recommended high levels of N by blue-green algae has been observed to result in about 10% higher grain yields, giving farmers an additional income of Rs. 490 (Venkataraman, 1981a and b). A residual effect of algae has also been observed in subsequent season in many localities (Srinivasan, 1979). In a few locations, algalisation has been reported to have no significant effect (Cole, 1977), the cause for which may be physico-chemical or biological (Roger and Kulasooriya, 1980).

Beneficial effects of algal inoculation have also been reported on a number of other crops such as tomato (Kaushik and Venkataraman, 1979), radish (Rodgers et al., 1979), cotton, sugarcane and maize (Singh, 1961), Capsicum annum and Lactuca sativa (Dadhich et al., 1969).

## III. MATERIAL AND METHODS

### A. ORGANISM

Axenic culture of Anabaena variabilis ARM 394 from the National Facility For Bluegreen Algal Collections, Indian Agricultural Research Institute, New Delhi was used in these investigations.

Anabaena variabilis Kutz ex Born et Flah

Thallus gelatinous, dark-green, trichome without any sheath, flexuous, 4-6 μ broad, more often 4.2-5 μ broad, slightly contricted at the cross-walls, and cells conical, obtuse, cells barrel-shaped sometimes with gas-vaculoles, 2.5-6 μ long, heterocysts spherical or oval, 6 μ broad, upto 8 μ long, spores formed centrifugally, in series, sometimes all the cells in a filament forming spores, 7-9 (-11) μ broad, 8-14 μ long, epi spore wall smooth, colourless.

### B. MEDIUM

The medium used in all experiments was the nitrogen-free BG 11 medium (Stanier & Cohen-Bazire, 1977) (mg/l)

| | |
|---|---|
| $MgSO_4.7H_2O$ | 75 |
| $CaCl_2.2H_2O$ | 36 |
| $K_2HPO_4$ | 40 |
| $Na_2EDTA$ | 1 |
| Citric acid | 6 |
| Iron (111) citrate | 6 |
| $Na_2CO_3$ | 20 |
| $H_3BO_3$ | 2.86 |
| $MnCl_2.4H_2O$ | 1.81 |

| | |
|---|---|
| $Na_2MoO_4.2H_2O$ | 0.39 |
| $ZnSO_4.7H_2O$ | 0.22 |
| $CuSO_4.5H_2O$ | 0.08 |
| $Co(NO_3)_2.6H_2O$ | 0.05 |

*The pH of the culture medium was adjusted to 7.1.*

## *C. STERILIZATION*

*The glasswares were subjected to chromic acid rinsing, followed by and washing in running tap water overnight. Before use, all the glasswares were rinsed in glass distilled water and sterilized at 160°C for 6 hrs in a hot air oven. Culture media were sterilized at 15 lb pressure for 20 min in an autoclave.*

## *D. MAINTENANCE OF THE CULTURE*

*The alga was maintained in BG 11 liquid and solid (1.5% agar) media at 2500 lux and 30 ± 1°C.*

## *E. GROWTH MEASUREMENT*

*All experiments including growth were done in triplicate unless otherwise stated. Growth was estimated in terms of total chlorophyll content. Algal cells were harvested by centrifugation at 3000 rpm. The pellet was washed twice with distilled water. The chlorophyll pigment was extracted with 10 ml of 96% methanol at 60°C for 30 min. Care was taken to compensate the evaporation loss. The extract was allowed to cool and the volume made up. After centrifugation, the absorption of the pigment was measured at 650 and 665 nm in a Systronics Spectrophotometer 106 (MK 11). Chlorophyll*

was quantified according to the following formula (McKiney, 1941).

$$mg\ Chl/ml = 255 \times 10^{-2} \times E_{650} + 0.4 \times 10^{-2} \times E_{665}$$

where

$E_{650}$ = absorption coefficient at 650 nm

$E_{665}$ = absorption coefficient at 665 nm.

## F. ACETYLENE REDUCTION ASSAY

Nitrogenase activity was measured in terms of acetylene reduction (Kaushik and Venkataraman, 1983) and expressed as $\mu$ mol $C_2H_4$. mg $Chl^{-1}$ $hr^{-1}$. Acetylene equal to 10% of the total volume was injected and vials were incubated for 90 min at 30°C under 2500 lux. The reaction was terminated by injecting 0.1 ml TCA (50%) and the gas phase was assayed for ethylene, using a Nucon Model GLC 5500 with flame ionization detector and a 2 meter long Porapak R stainless steel column. The column temperature was maintained at 100°C and nitrogen gas at a flow rate of about 35 ml per min served as a carrier gas.

## G. CHEMICALS USED

| | | | |
|---|---|---|---|
| $KNO_3$ | - | BDH (AR) | |
| $NH_4Cl$ | - | BDH (AR) | |
| $NH_4NO_3$ | - | BDH (AR) | |
| Urea | - | BDH (AR) | |
| $K_2HPO_4$ | - | BDH (AR) | |
| Dithane | - | Zinc ethylene dithiocarbamate | Rallis India Ltd. |
| Ceresan | - | N-ethylmercuric p-toluene sulphonilide | " " " |
| 2,4-D | - | 2,4-Dichlorophenoxy acetic acid | " " " |

| | | |
|---|---|---|
| *Dalapon* | - *2,3-dichloropropionic acid* | *Rallis India Ltd.* |
| *Diuron* | - *3,3,4-dichlorophenyl 1,1-dimethyl Urea* | " " " |
| *Machet* | - *Butachlor* | " " " |
| *BHC* | - *Y-1,2,3,4,5,6-hexachlorocyclohexane* | " " " |
| *Phorate* | - *O,O-diethyl S-ethyl thiomethyl diphosphate* | " " |
| *D-Erythrose* | - *Loba-chemil Indoaustranal Co. Bombay* | |
| *Glucose* | - " " " " " | |
| *Fructose* | - " " " " " | |
| *Glucose-6-Phosphate* | - " " " " " | |
| *Sucrose* | - *Glaxo laboratories (India) Ltd.* | |
| *Lysozyme* | - *(Sigma Chem. Co., USA)* | |
| *Tris-Buffer* | - *(Sigma Chem. Co., USA)* | |
| *EDTA* | - *BDH (AR)* | |
| *NaCl* | - *BDH (AR)* | |
| *NaOH* | - *BDH (AR)* | |
| $MgCl_2$ | - *BDH (AR)* | |
| *Sorbitol* | - *Glaxo Laboratories (India) Ltd.* | |
| *Bovine Serum Albumin* | - *(Sigma Chem. Co., USA)* | |

## *H. EFFECT OF TEMPERATURE ON NITROGENASE ACTIVITY*

*Filaments from mid-log cultures were incubated at* $5^{o}C$, $15^{o}C$, $25^{o}C$, $35^{o}C$ *separately in an illuminated B.O.D. incubator at 2500 lux. Acetylene reduction activity was measured separately at each temperature upto 52 hrs. Illumination of 2500 lux was kept constant throughout the experiment.*

## I. EFFECT OF LIGHT ON NITROGENASE ACTIVITY

$N_2$-growing mid-log cultures were incubated at 500, 1000, 2000, 3000, 4000 and 10,000 lux and in total darkness separately in an illuminated B.O.D. incubator at constant temperature of 30 $\pm$ 1°C. Acetylene reduction activity was measured at each light intensity upto 52 hrs.

## J. EFFECT OF pH ON NITROGENASE ACTIVITY

$N_2$-growing filaments from mid-log culture were collected by centrifugation (400 xg, 5 min) and the cell pellets were resuspended in $N_2$-free BG 11 medium with a graded pH of 4.0, 6.0, 7.0, 8.0, and 10.0. Acetylene reduction activity was measured upto 30 hrs.

## K. Effect of $NO_3^-$, $NH_4^+$ AND UREA ON NITROGENASE ACTIVITY

To $N_2$-growing mid-log cultures, 1mM, 2mM, 3mM $NH_4Cl$, $KNO_3$, $NH_4NO_3$ and Urea were added and acetylene reduction activity was measured upto 48 hrs.

## L. EFFECT OF PHOSPHATE ON NITROGENASE ACTIVITY

1 mM, 5mM, 10mM $K_2HPO_4$ was added to mid-log cultures and time course acetylene reduction activity was measured upto 48 hrs.

## M. EFFECT OF PESTICIDES ON NITROGENASE ACTIVITY

Two fungicides (Dithane and Ceresan M), four Herbicides (2,4-D, Dalapon, Diuron and Machet) and two insecticides (BHC and

Phorate) were examined for their effect on nitrogenase activity. Concentrations equivalent to field dose, 10% and 50% of field dose and twice the field dose of all pesticides were added to mid-log cultures growing in $N_2$-free BG 11 medium and acetylene reduction activity was measured upto 55 hrs.

## N. EFFECT OF DIFFERENT SUGARS ON NITROGENASE ACTIVITY

To $N_2$-growing mid-log cultures, 10 mM of various sugars like Erythrose, Sucrose, Glucose, and Fructose were added and acetylene reduction activity was immediately measured. The same treatment was repeated with filaments whih have been darktreated for one hour by wrapping the test tubes with aluminium foil and the acetylene reduction activity was measured immediately.

## O. ISOLATION OF HETEROCYSTS

Algae used for heterocyst isolation were from mid-log culture with a chlorophyll concentration of 0.09 mg Chl.$ml^{-1}$ and nitrogenase activity of 9.84 µmol $C_2H_4$ mg $Chl^{-1}hr^{-1}$. After harvesting by centrifugation (400 x g, 5 min), the filaments were washed once in medium A (pH 7.6) containing (mM) : Tes-NaOH, 10; $MgCl_2$, 10; Sodium phosphate, 5. Thereafter, preparation was performed in Septum-stoppered centrifugation tubes under anaerobic conditions. All buffers were sparged with hydrogen. After a 3 minute centrifugation at 750 x g, the filaments were resuspended in incubation medium B (pH 7.1) containing (mM) : Sorbitol, 300, Tes-NaOH, 10; $MgCl_2$, 10, Sodium phosphate, 5, bovine serum albumin (0.5%) and lysozyme (1.5 mg/ml).

The Algal suspension was incubated at 30°C for 1 hr under $H_2$ and stirred continuously. The lysozyme treated cells were sedimented by short centrifugation (250 x g, 5 minutes) and resuspended in 20 ml of medium A. After homogenizing in a tissue homogenizer (Merchantile Engineers) for 5 minutes, heterocyst were sedimented by centrifugation at 250 g for 5 min. The heterocyst preparation was washed two to three times in 2.0 ml medium B (without lysozyme) until it appeared on microscopic inspection essentially free from contaminating vegetative cells. Dithionite was omitted from all buffers since no consistent beneficial effect had been observed. To the isolated heterocysts kept in anaerobic conditions, various sugars like Erythrose, Glucose-6-phosphate, Sucrose, and Glucose were added at a concentration of 10 mM and nitrogenase activity was measured in isolated heterocyst (no. of heterocyst/ml) after flushing the vial with $H_2$ for 15 minutes.

## IV. RESULTS

(a) Inoculum concentration dependent growth of Anabaena variabilis

Table 2 and Fig. 2 shows the effect of different concentrations of initial inoculum on the growth curve of Anabaena variabilis as measured in terms of chlorophyll per ml of medium. Sample No. 1 with the initial concentrations of 0.001 mg clorophyll per ml showed the maximum linear increase in growth rate upto 14 days. Sample no. 2 with the initial concentration of 0.002 mg chlorophyll per ml showed linear increase only upto 7 days and then stationary phase was reached. With the increase in initial inoculum in Sample No. 3 and Sample No. 4 at 0.006 and 0.010 mg per ml respectively, growth increased upto 4 days only and thereafter reached stationary phase. Maximum growth rate of 1200 per cent was achieved in case of Sample No. 1. Hence in all further experiments the initial concentration of 0.001 mg Chlorophyll per ml was used for growth of Anabaena variabilis. All other experiments were performed with the filaments collected on the 9th day representing the mid-log culture.

(b) Temperature and the nitrogenase activity

Table 3 and Fig. 3 show the effect of different temperatures on the acetylene reduction. When the filaments from mid-log cultures were incubated at $5^{o}C$ in an illuminated BOD incubator there was an inhibition in the nitrogenase activity to the extent of 97% at 2 hrs and the activity never recovered. When incubated at $15^{o}C$ there was 55% reduction in the activity at 2 hrs and the activity declined further with the increased period of incubation and was only 15% at 52 hrs.

At $25^{o}C$ the activity was 100% at 2 hrs and the same high levels of activity was present throughout the experimental period and was at par with control. At $35^{o}C$, like $15^{o}C$ there was an initial reduction of 57% at 2 hrs which further declined to 17% activity at 52 hrs. Temperature of $25^{o}C$ and $30^{o}C$ is the optimal temperature for maintaining maximum nitrogenase activity.

Table 2. *Inoculum concentration dependent growth of* Anabaena variabilis *(Figures in parenthesis show the relative increase in growth).*

| Incubation (Days) | mg Chl. $ml^{-1}$ | | | |
|---|---|---|---|---|
| | Sample Nos. | | | |
| | 1 | 2 | 3 | 4 |
| 0 | 0.001 (100) | 0.002 (100) | 0.006 (100) | 0.010 (100) |
| 1 | 0.001 (100) | 0.002 (100) | 0.006 (100) | 0.010 (100) |
| 4 | 0.005 (500) | 0.006 (300) | 0.010 (167) | 0.016 (160) |
| 7 | 0.008 (800) | 0.011 (550) | 0.013 (216) | 0.019 (190) |
| 9 | 0.09 (900) | 0.011 (550) | 0.014 (233) | 0.019 (190) |
| 10 | 0.010 (1000) | 0.011 (550) | 0.014 (233) | 0.020 (200) |
| 11 | 0.011 (1100) | 0.011 (550) | 0.014 (233) | 0.020 (200) |
| 14 | 0.012 (1200) | 0.010 (500) | 0.015 (250) | 0.020 (200) |

*Fig. 2. Effect of inoculum concentration on growth.*

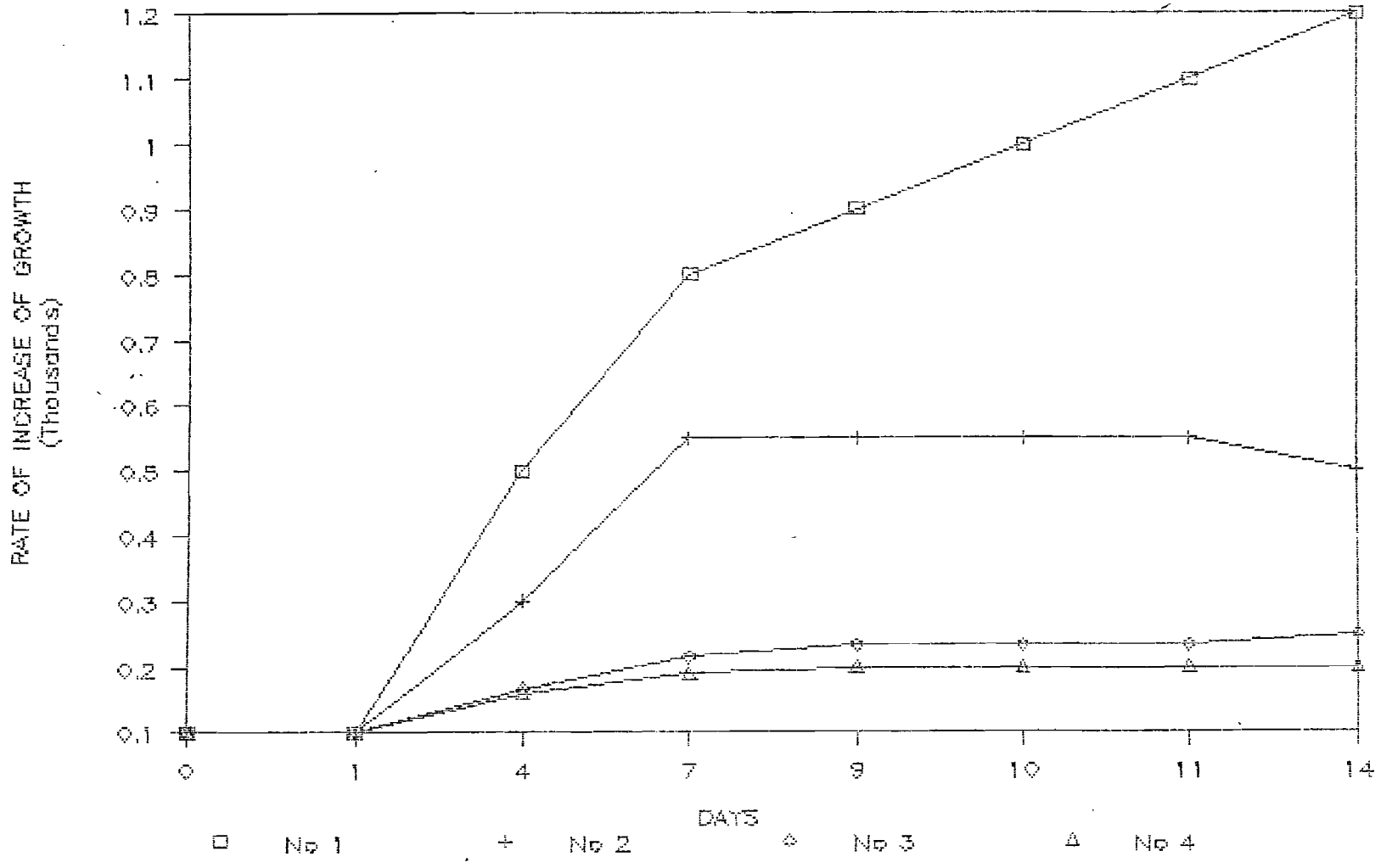
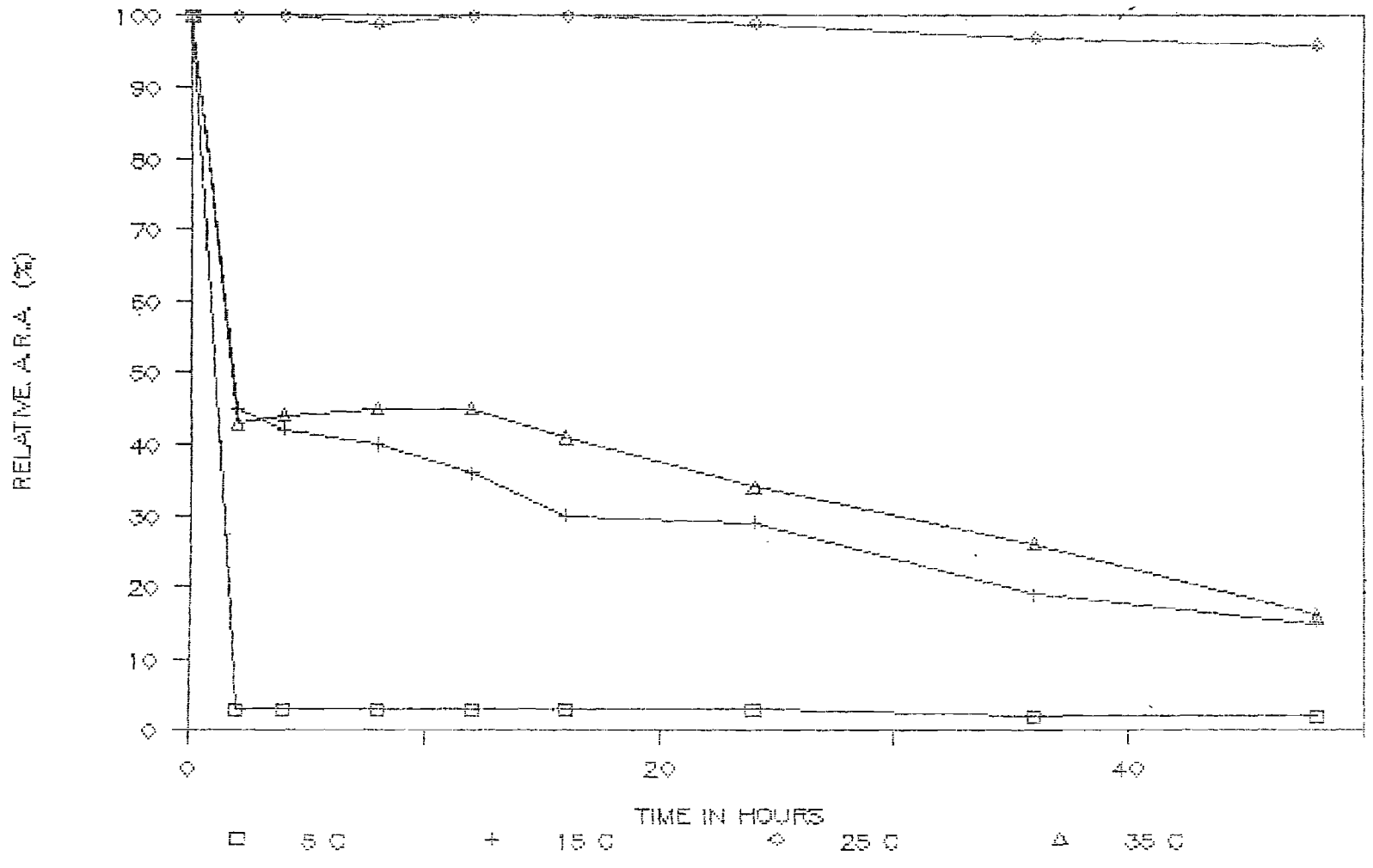

RATE OF INCREASE OF GROWTH
(Thousands)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
4
7
9
10
11
14
DAYS
No 1
No 2
No 3
No 4

Table 3. Temperature and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity) (Control 10.1 μ mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ at 30°C).

| Temperature °C | μ mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Hours | | | | | | | | |
| | 2 | 4 | 8 | 12 | 16 | 24 | 36 | 48 | 52 |
| 5 | 0.30 (3) | 0.31 (3) | 0.32 (3) | 0.30 (3) | 0.33 (3) | 0.28 (3) | 0.2 (2) | 0.2 (2) | 0.1 (1) |
| 15 | 4.6 (45) | 4.32 (42) | 4.07 (40) | 3.64 (36) | 3.10 (30) | 3.0 (29) | 1.98 (19) | 1.6 (15) | 1.6 (15) |
| 25 | 10.14 (100) | 10.12 (100) | 10.0 (99) | 10.0 (100) | 10.1 (100) | 10.0 (99) | 9.8 (97) | 9.7 (96) | 9.6 (95) |
| 35 | 4.40 (43) | 4.50 (44) | 4.60 (45) | 4.54 (45) | 4.23 (41) | 3.5 (34) | 2.63 (26) | 1.69 (16) | 1.74 (17) |

*Fig. 3. Effect of temperature (05, 15, 25 and 35°) on nitrogenase activity (acetylene reduction) of Anabaena variabilis.*


RELATIVE A.R.A. (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
TIME IN HOURS
□ 5 C
+ 15 C
◇ 25 C
△ 35 C

(c) Light and the nitrogenase activity

Fig. 4 and Table 4 show the effect of different light intensities on the acetylene reduction activity. When the blue-green alga was incubated in total darkness the activity fell by 95% in two hours and only 1% activity was present after 52 hrs. When incubated at 500 Lux the activity was 61% within 2 hrs and thereafter remained almost constant. Similarly a light intensity of 1000 Lux resulted in 34% reduction in activity at 2 hrs and the activity remained same throughout the 52 hr period with 53% activity recorded at the end of 52 hrs. At 2000 Lux light intensity, the activity was 89% at 2 hrs and was more or less constant throughout the experimental period with 82% activity at the end of 52 hrs. When incubated under 3000 Lux there was a slight stimulation in the nitrogenase activity upto 121% at 12 hrs and the activity remained at higher level upto 52 hrs although with minor variations.

A similar trend was obtained when the alga was incubated at 4000 Lux. However, when the alga was incubated at 10,000 Lux, there was a sharp decrease in the acetylene reduction to the extent of 87% at 2 hrs and the activity further declined to a level of 1% at the end of 52 hrs. The nitrogenase activity increased linearly as the light intensity increased upto 4000 Lux. But very high light intensities of 10,000 Lux inhibits nitrogenase activity drastically (Table 4).

(d) pH and nitrogenase activity

Table 5 and Fig. 5 show the effect of pH4, pH6, pH7, pH8 and pH10 on the nitrogenase activity as measured in terms of

Table 4. Light and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity) (Control 10.0 μ mol $C_2H_4$ mg $Chl^{-1}$ $hr^{-1}$ at 2500 Lux).

| Light Intensity (Lux) | μmol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Hours | | | | | | | | |
| | 2 | 4 | 8 | 12 | 16 | 24 | 36 | 48 | 52 |
| 0 | 0.53 (5) | 0.51 (5) | 0.32 (3) | 0.3 (3) | 0.30 (3) | 0.29 (2) | 0.14 (1) | 0.10 (1) | 0.1 (1) |
| 500* | 3.90 (39) | 3.8 (38) | 3.92 (39) | 3.81 (38) | 3.66 (36) | 3.64 (36) | 3.6 (36) | 3.5 (35) | 3.48 (34) |
| 1000 | 6.63 (66) | 6.54 (65) | 6.51 (65) | 6.48 (64) | 5.84 (58) | 5.81 (58) | 5.41 (54) | 5.38 (53) | 5.31 (53) |
| 2000 | 8.90 (89) | 8.71 (87) | 8.69 (86) | 8.54 (85) | 8.50 (85) | 8.50 (85) | 8.31 (83) | 8.29 (82) | 8.20 (82) |
| 3000 | 9.7 (97) | 10.9 (109) | 11.8 (118) | 12.0 (120) | 12.1 (121) | 11.8 (118) | 11.8 (117) | 11.1 (111) | 10.8 (108) |
| 4000 | 10.3 (103) | 11.4 (114) | 12.0 (120) | 12.1 (121) | 12.0 (120) | 11.5 (115) | 11.1 (111) | 10.0 (100) | 9.5 (95) |
| 10000 | 1.7 (17) | 1.64 (16) | 1.0 (10) | 0.52 (5) | 0.3 (3) | 0.2 (2) | 0.1 (1) | 0.1 (1) | 0.1 (1) |

*Data not included in figure

*Fig. 4. Effect of light (0, 1000, 2000, 3000, 4000 and 10000 Lux) on nitrogenase activity (acetylene reduction) of Anabaena variabilis.*

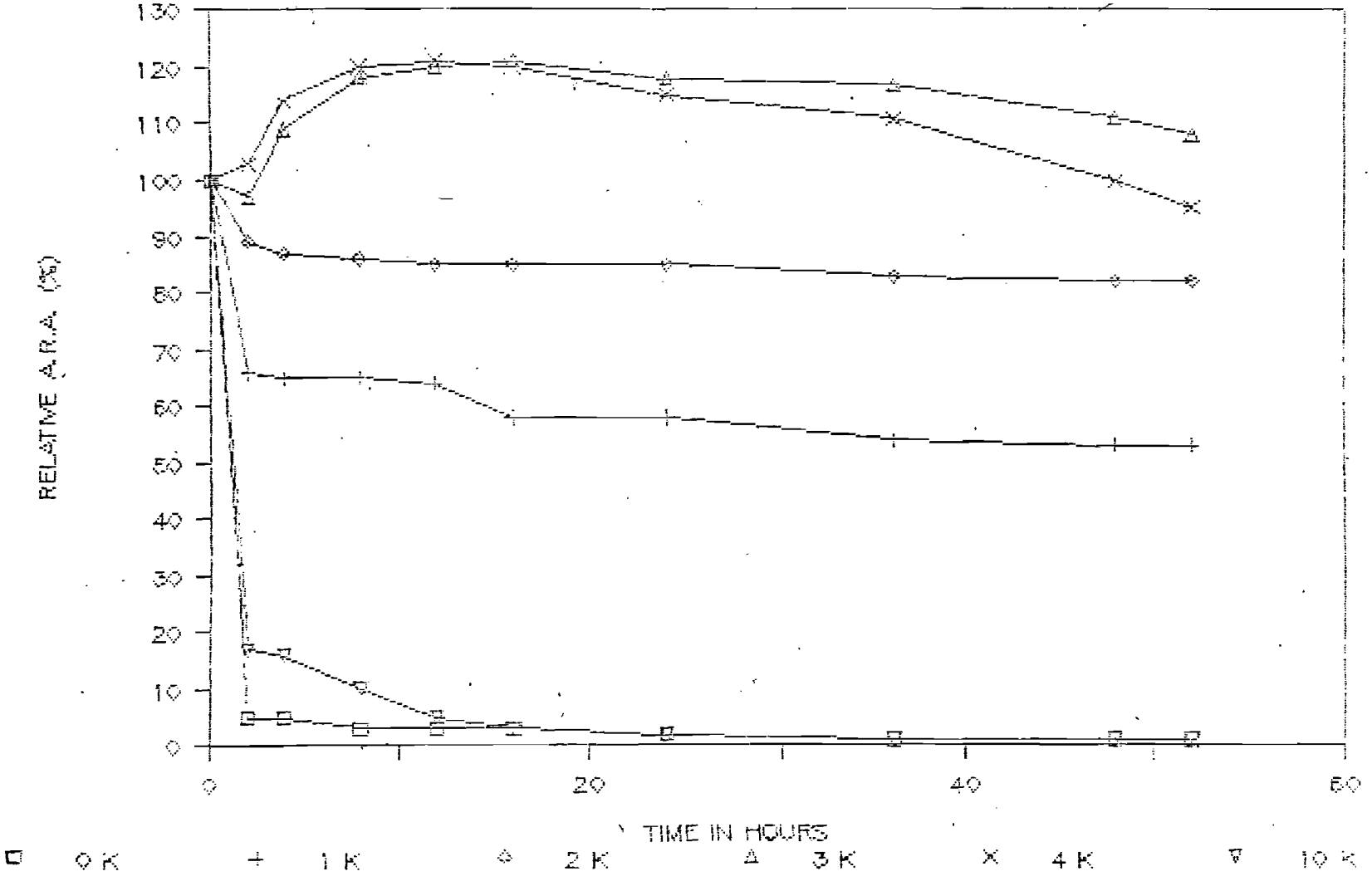
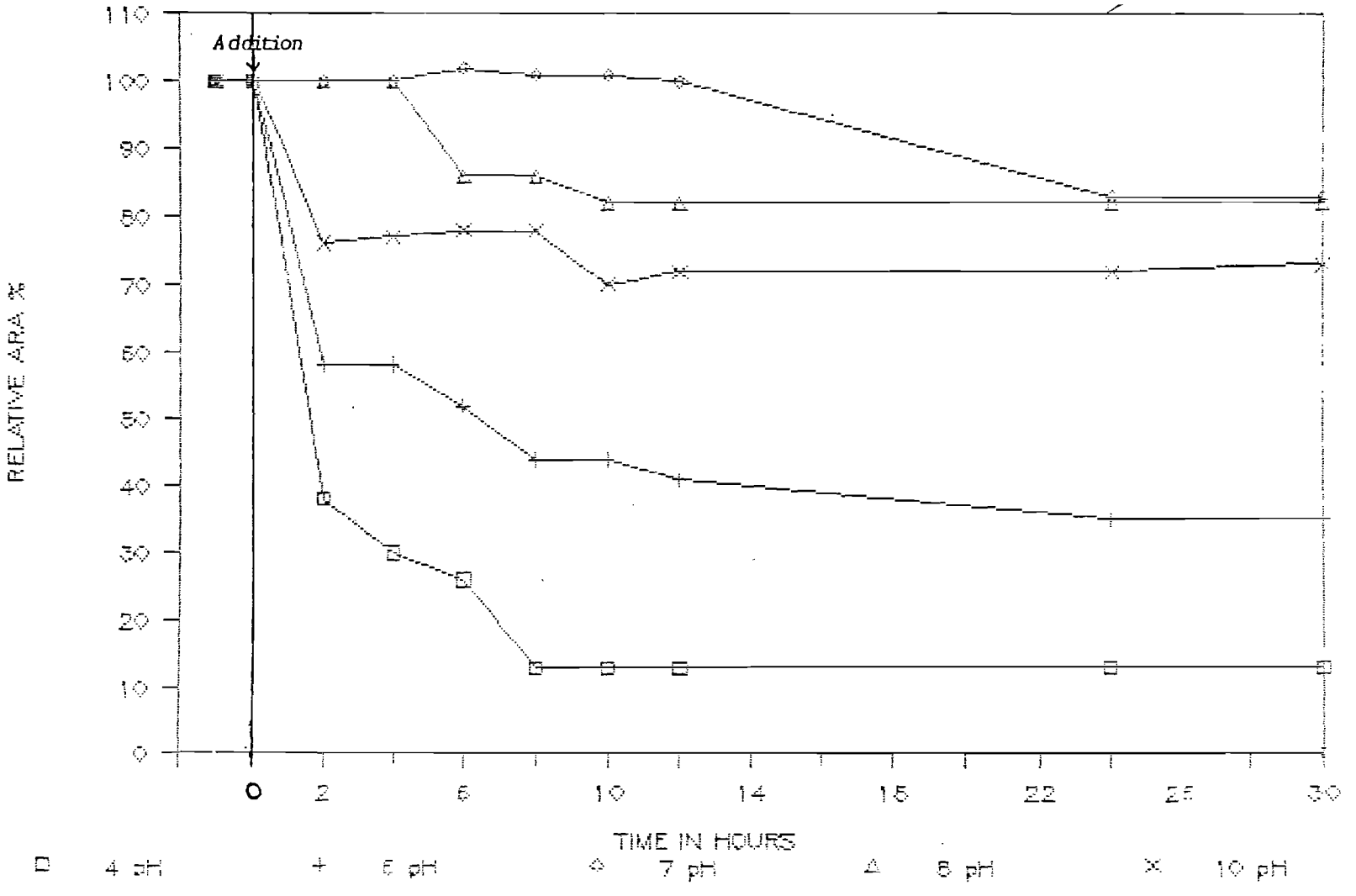

RELATIVE A.R.A. (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
TIME IN HOURS
0 K
1 K
2 K
3 K
4 K
10 K

Table 5. *pH and the nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity) (Control 9.18 μ mol $C_2H_4$ mg $Chl^{-1}$ $hr^{-1}$)*

| Treatment pH | μ mol $C_2H_4$ mg $Chl^{-1}$ $hr^{-1}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Hours of incubation | | | | | | | |
| | 2 | 4 | 6 | 8 | 10 | 12 | 24 | 30 |
| 4 | 3.52 (38) | 2.81 (30) | 2.6 (26) | 1.21 (13) | 1.21 (13) | 1.20 (13) | 1.18 (13) | 1.18 (13) |
| 6 | 5.33 (58) | 5.33 (58) | 4.81 (52) | 4.06 (44) | 4.06 (44) | 3.73 (41) | 3.21 (35) | 3.21 (35) |
| 7 | 9.18 (100) | 9.18 (100) | 9.33 (102) | 9.29 (101) | 9.29 (101) | 9.18 (100) | 7.61 (83) | 7.61 (83) |
| 8 | 9.18 (100) | 9.18 (100) | 7.91 (86) | 7.91 (86) | 7.54 (82) | 7.54 (82) | 7.54 (82) | 7.53 (82) |
| 10 | 6.98 (76) | 7.09 (77) | 7.16 (78) | 7.16 (78) | 6.45 (70) | 6.57 (72) | 6.57 (72) | 6.68 (73) |

*Fig. 5. Effect of pH (4, 6, 7, 8 and 10) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*


Addition
RELATIVE ARA %
110
100
90
80
70
60
50
40
30
20
10
0
0
2
6
10
14
18
22
26
30
TIME IN HOURS
4 pH
5 pH
7 pH
8 pH
10 pH

acetylene reduction. When the Anabaena culture was transferred to the nitrogen-free medium, whose pH was adjusted to pH4 there was a sharp linear decline in nitrogenase activity and only 13% activity could be detected after 8 hours. Within two hours the activity fell to 38% of the original activity. In case of pH6 also there was an immediate linear decrease and 44% activity could be detected at 8th hour. Thereafter reduction in ARA was gradual and at the end of 30th hour, 35% activity was present. When the culture was transferred to pH7 medium, there was no decline in the nitrogenase activity upto 12th hour. However, after 12th hour there was slight decrease in the activity. pH8 also showed a similar pattern where there was no reduction in the activity upto 14th hour. From 6th hour to 30th hour there was slight reduction in the activity although 82% activity remained even after 30 hours. At pH10 there was immediate fall in the nitrogenase activity upto 76% in the 2nd hour and thereafter showed marginal fluctuations. Thus although pH4, pH6, pH8 and pH10 inhibited the nitrogenase activity of this cyanobacteria to varying degrees, the effect of pH4 and pH6 were more drastic than pH10. pH7 supported the maximum nitrogenase activity.

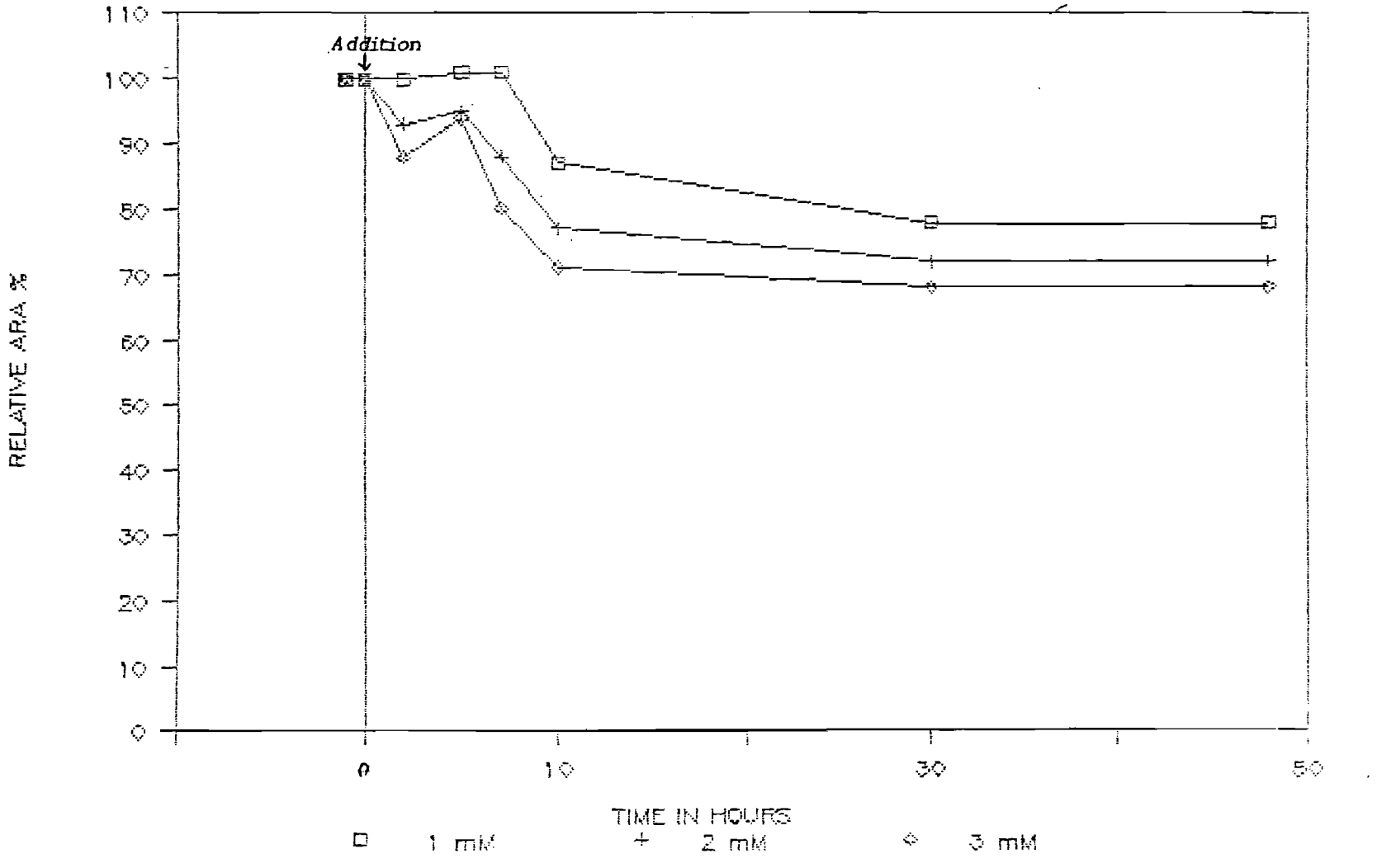
(e) Nitrate-N and nitrogenase activity

Table 6 shows the response of nitrogenase activity to the addition of 1mM, 2mM, 3mM $KNO_3$. When 1mM $KNO_3$ was added to the culture growing in the nitrogen-free medium there was no inhibitory effect upto 7 hours, after which there was a gradual reduction and varied from 13-22% (Table 6, Fig. 6). 2mM $KNO_3$

Table 6. $NO_3$-N ($KNO_3$) and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis.

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1}hr^{-1}$ | | | Relative activity (%) | | |
|---|---|---|---|---|---|---|
| | $KNO_3$ (mM) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| No addition (control) | 9.0 | 9.0 | 9.0 | 100 | 100 | 100 |
| After addition (Hrs) | | | | | | |
| 2 | 9.0 | 8.4 | 8.0 | 100 | 93 | 88 |
| 5 | 9.1 | 8.6 | 8.5 | 101 | 95 | 94 |
| 7 | 9.1 | 8.0 | 7.2 | 101 | 88 | 80 |
| 10 | 7.9 | 7.0 | 6.4 | 87 | 77 | 71 |
| 30 | 7.1 | 6.5 | 6.2 | 78 | 72 | 68 |
| 48 | 7.1 | 6.5 | 6.2 | 78 | 72 | 68 |

*Fig. 6. Effect of $KNO_3$-N (1, 2, 3 mM) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*


Addition
110
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
1 mM
2 mM
3 mM

had inhibitory effect to the extent of 7% after 2 hrs and even upto 48 hrs, 72% of activity was present. Similarly, 3mM $KNO_3$ also inhibited the nitrogenase activity.

(f) $NH_4$-N and nitrogenase activity

Tables 7 and 8 and Figs. 7 and 8 show the effect of 1mM, 2mM, 3mM $NH_4NO_3$ and $NH_4CL$ on nitrogenase activity as measured in terms of acetylene reduction. When 1mM, 2mM, 3mM, $NH_4NO_3$ was added, there was a sharp linear decline in nitrogenase activity upto 5th hr and only 20% activity could be detected at 5th hr. With 1mM $NH_4NO_3$, there was an immediate reduction of 14% after 2 hrs and 62% after 5 hrs and thereafter the reduction was gradual upto 48 hrs. With the increase in $NH_4NO_3$-N to 2mM, there was an initial reduction upto 30% after 2 hrs and 79% after 5 hrs. Drastic inhibition was noted with 3mM $NH_4NO_3$ which caused 64% reduction in activity within 2 hrs and 79% after 5 hrs (Table 7, Fig. 7).

Addition of 1mM, 2mM, 3mM $NH_4Cl$ also caused almost similar linear decline in nitrogenase activity (Table 8). At 1 mM $NH_4Cl$ a reduction of 33% and 78% in AR activity was observed after 5 and 48 hrs respectively. The 2 mM and 3 mM $NH_4Cl$ inhibited the ARA upto 44 and 53% respectively after 5 hrs and 78% and 84% after 48 hrs respectively. At 7 hrs although both $NH_4NO_3$ and $NH_4Cl$ inhibited the nitrogenase activity of the alga, the effect of $NH_4NO_3$ was more drastic ((Figs. 7 and 8).

Table 7. Ammonium-N ($NH_4NO_3$) and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis.

| Incubation | $\mu$mol $C_2H_4$ mg Chl$^{-1}$hr$^{-1}$ | | | Relative activity (%) | | |
|---|---|---|---|---|---|---|
| | $NH_4NO_3$ (mM) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| No addition (control) | 9.2 | 9.2 | 9.2 | 100 | 100 | 100 |
| After addition (Hrs) | | | | | | |
| 2 | 8 | 6.5 | 5 | 86 | 70 | 54 |
| 5 | 3.5 | 2 | 2 | 38 | 21 | 21 |
| 7 | 2 | 1-6 | 1.6 | 21 | 17 | 17 |
| 10 | 1.5 | 1.4 | 1.4 | 16 | 15 | 15 |
| 30 | 1.0 | 1.0 | 1.0 | 11 | 11 | 11 |
| 48 | 1.0 | 1.0 | 1.0 | 11 | 11 | 11 |

*Fig. 7. Effect of $NH_4NO_3$ (1, 2, 3 m M) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*

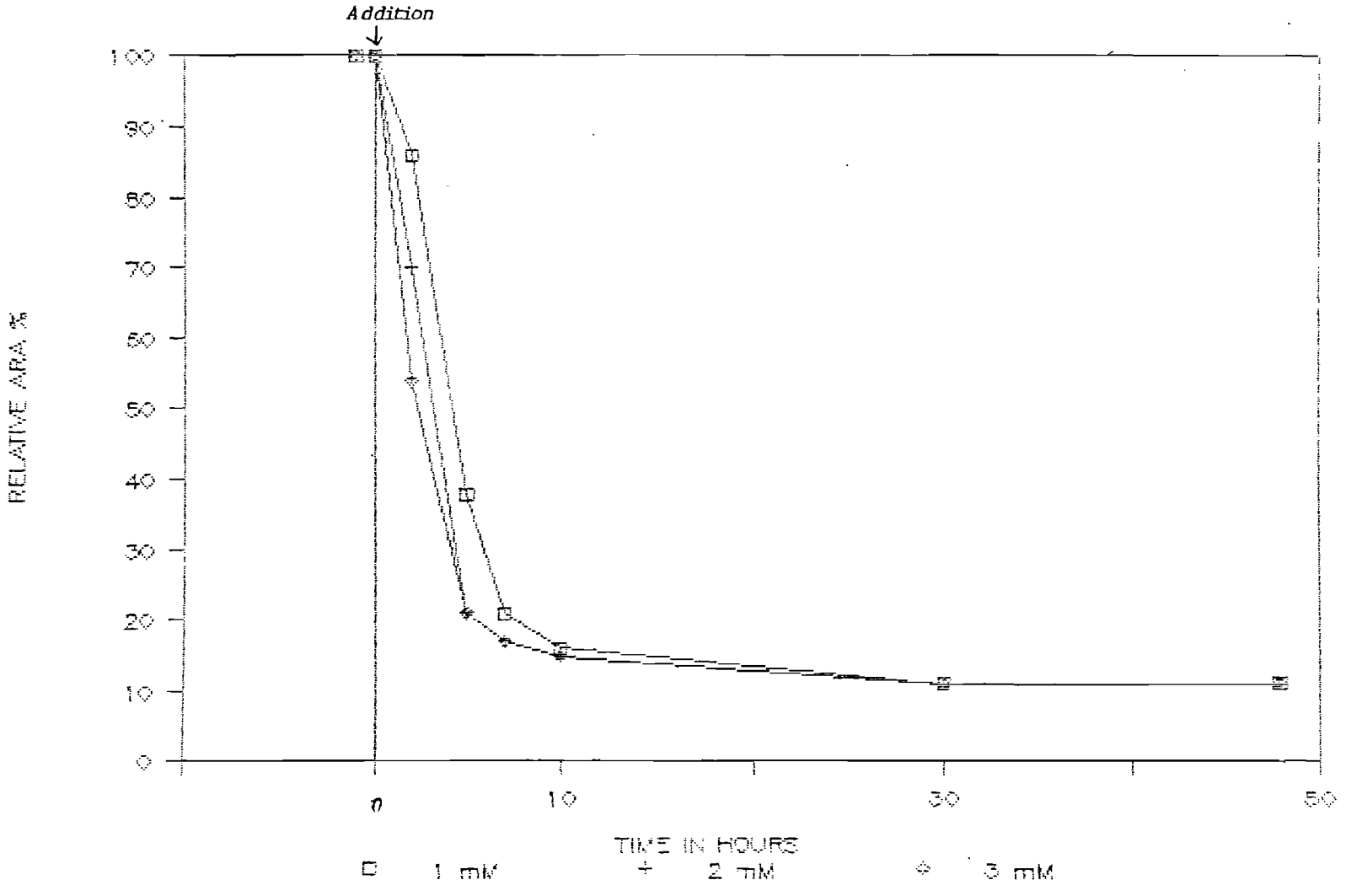
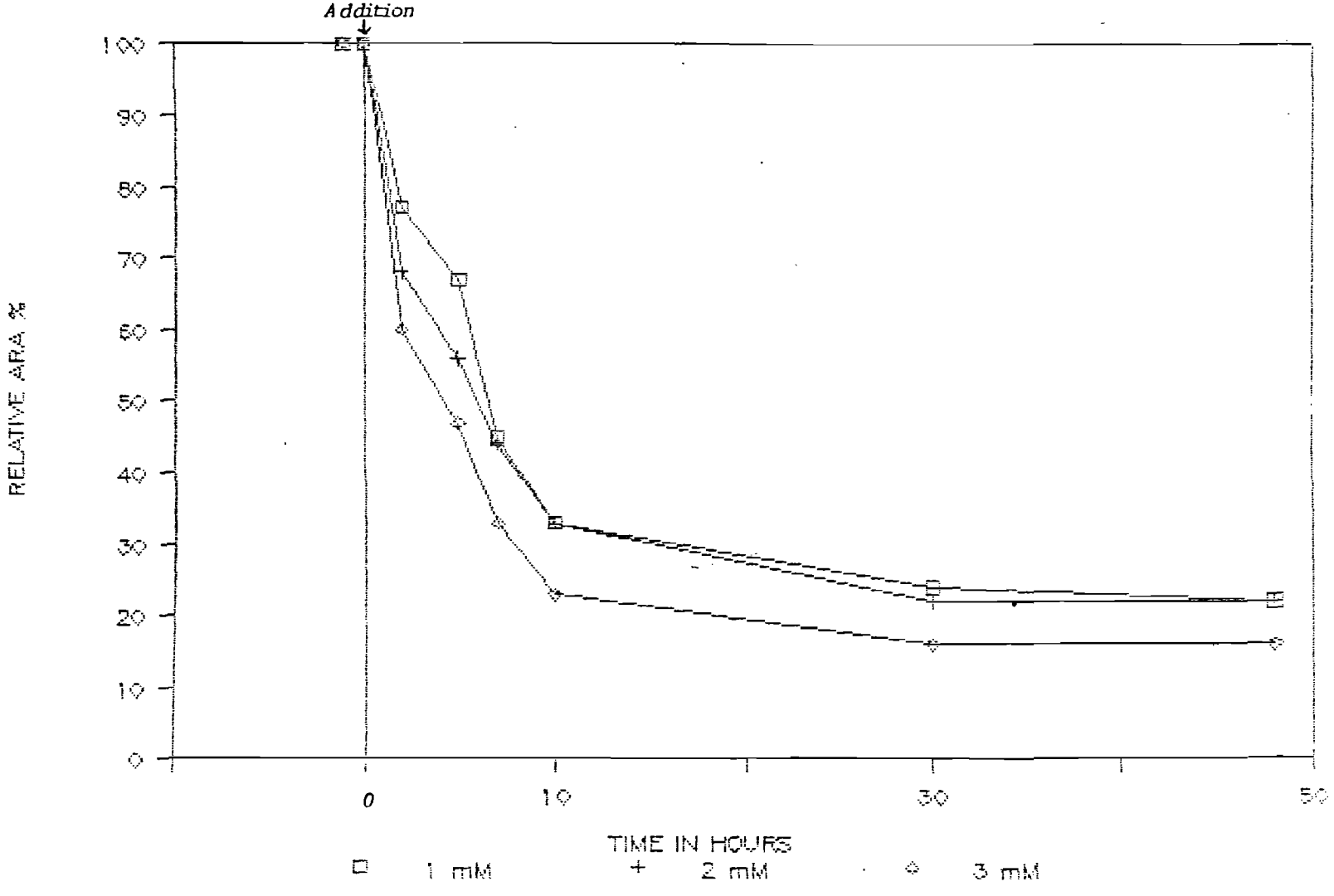

Addition
RELATIVE ARA, %
100
90
80
70
60
50
40
30
20
10
0
0
10
30
50
TIME IN HOURS
1 mM
2 mM
3 mM

Table 8. $NH_4$-N ($NH_4Cl$) and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis.

| Incubation | μmol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | Relative activity (%) | | |
|---|---|---|---|---|---|---|
| | $NH_4Cl$ (mM) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| No addition (control) | 9.0 | 9.0 | 9.0 | 100 | 100 | 100 |
| After addition (Hrs) | | | | | | |
| 2 | 7.0 | 6.2 | 5.4 | 77 | 68 | 60 |
| 5 | 6.1 | 5.0 | 4.2 | 67 | 56 | 47 |
| 7 | 4.1 | 4.0 | 3.0 | 45 | 44 | 33 |
| 10 | 3.0 | 3.0 | 2.1 | 33 | 33 | 23 |
| 30 | 2.2 | 2.0 | 1.5 | 24 | 22 | 16 |
| 48 | 2.0 | 2.0 | 1.5 | 22 | 22 | 16 |

*Fig. 8. Effect of $NH_4Cl$-N (1, 2, 3 m M) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*


Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
1 mM
2 mM
3 mM

(g) Urea and nitrogenase activity

Table 9 and Fig. 9 show the response of nitrogenase activity to addition of 1 mM, 2 mM, 3 mM Urea. Exposure to 1 mM urea caused an immediate reduction of 30% after 2 hrs and only 23% AR activity was present after 48 hrs. 2 mM urea inhibited slightly more to the extent of 37% after 2 hrs and only 12% AR activity was expressed after 48 hrs. Addition of 3 mM urea had 47% inhibition after 2 hrs and only 6% activity remained after 48 hrs. Urea inhibited nitrogenase activity to a considerable extent with the inhibition increasing on increase in concentration and the trend was almost similar to $NH_4Cl$-N.

(h) Phosphate and nitrogenase activity

Fig. 10 shows the relative nitrogenase activity as a function of time in presence of 1 mm, 5 mM, 10 mM Phosphate. Addition of 1 mM phosphate had a slight reduction in acetylene reduction to the extent of 5% at 2 hrs, but later it stimulated the nitrogenase activity greatly. The increase in nitrogenase activity till the end of 48 hrs was upto 80%. 5 mM Phosphate although did not inhibit nitrogenase activity drastically. The inhibition varied from 9% at 2nd hr to 6% at 48th hr. Similarly, 10 mM Phosphate also inhibited the nitrogenase activity and it varied from 14 to 23% during the entire period of incubation (Fig. 10). Phosphate in all the three concentrations used did not inhibit the nitrogenase activity of the alga drastically, instead at 1 mM concentration it greatly enhanced the nitrogenase activity (Table 10).

*Table 9. Urea-N and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis.*

| Incubation | $\mu$mol $C_2H_4$ mg. Chl$^{-1}$ hr$^{-1}$ | | | Relative activity (%) | | |
|---|---|---|---|---|---|---|
| | Urea (mM) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| No addition (control) | 9.3 | 9.3 | 9.3 | 100 | 100 | 100 |
| After addition (Hrs) | | | | | | |
| 2 | 6.6 | 5.9 | 5 | 70 | 63 | 53 |
| 5 | 5.8 | 5.6 | 4.5 | 62 | 60 | 48 |
| 7 | 2.0 | 1.4 | 1.0 | 26 | 15 | 11 |
| 10 | 2.5 | 1.2 | 0.6 | 26 | 13 | 6 |
| 30 | 2.2 | 1.1 | 0.6 | 23 | 12 | 6 |
| 48 | 2.2 | 1.1 | 0.6 | 23 | 12 | 6 |

*Fig. 9. Effect of Urea-N (1, 2, 3 m M) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*

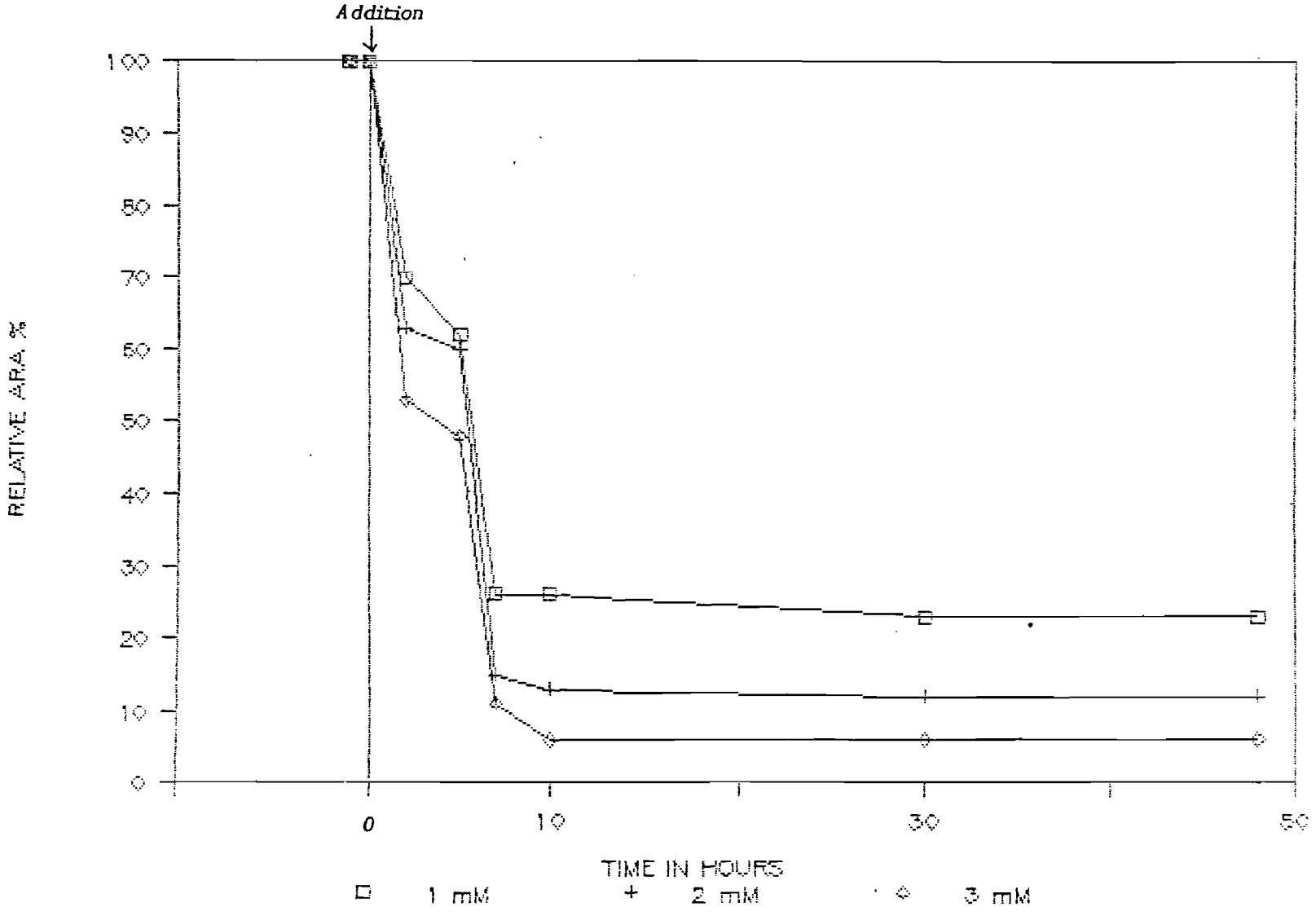
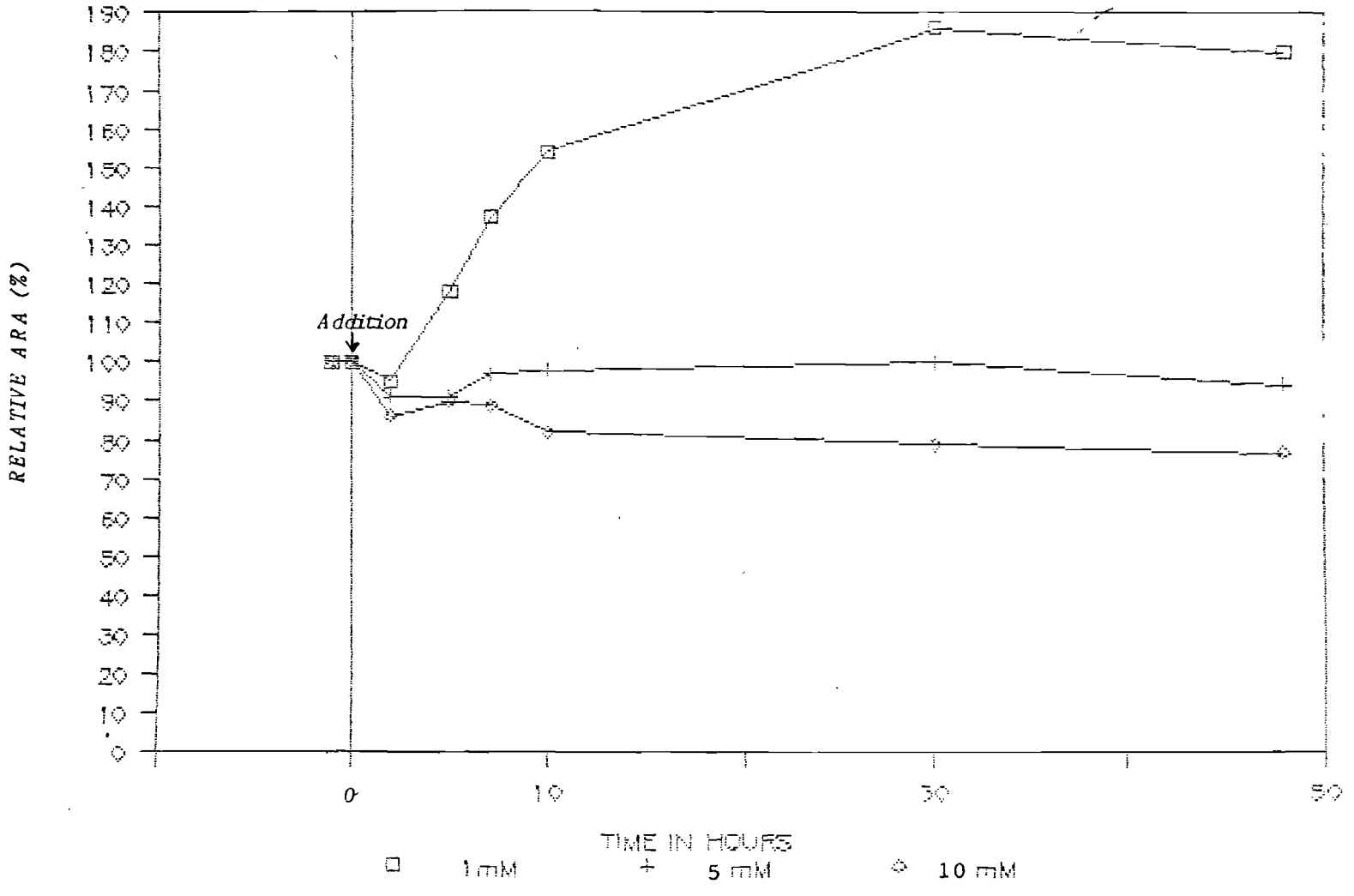

Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
1 mM
2 mM
3 mM

*Table 10. Phosphate and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis.*

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1}hr^{-1}$ | | | Relative activity (%) | | |
|---|---|---|---|---|---|---|
| | Phosphate (mM) | | | | | |
| | 1 | 5 | 10 | 1 | 5 | 10 |
| No addition (control) | 9.4 | 9.4 | 9.4 | 100 | 100 | 100 |
| After addition (Hrs) | | | | | | |
| 2 | 9.01 | 8.6 | 8.1 | 95 | 91 | 86 |
| 5 | 11.1 | 8.6 | 8.5 | 118 | 91 | 90 |
| 7 | 12.9 | 9.2 | 8.4 | 137 | 97 | 89 |
| 10 | 14.5 | 9.3 | 7.8 | 154 | 98 | 82 |
| 30 | 17.5 | 9.4 | 7.5 | 186 | 100 | 79 |
| 48 | 17.0 | 8.9 | 7.3 | 180 | 94 | 77 |

*Fig.10. Effect of phosphate (1, 5, 10 m M) on nitrogenase activity (acetylene reduction) of Anabaena variabilis*


RELATIVE ARA (%)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Addition
0
10
30
50
TIME IN HOURS
1 mM
5 mM
10 mM

(i) Fungicide Ceresan and nitrogenase activity

Fig. 11 and Table 11 show the effect of 0.5, 2.5, 5 and 10 ppm ceresan on the acetylene reduction activity. Addition of 5 ppm (field dose) of ceresan brought about a rapid decrease (46%) in the activity within 2 hrs, but gradually the activity was restored to 72% at 55 hours. 10 ppm ceresan equivalent to twice the field dose had a drastic reduction in activity (65%) within 2 hrs but from 5 hrs onwards the activity began to restore and by 55 hrs the activity was at par with 5 ppm. Addition of 0.5 ppm equivalent to 10% of field dose and 2.5 ppm equivalent to 50% of field dose also showed similar initial reduction and thereafter a gradual increase in activity until 88% and 84% activity was restored respectively after 55 hrs. Addition of ceresan in all the 4 concentrations had a drastic reduction in the activity within 2 hrs in varying degrees. The reduction in activity was maintained till 5th hour and later the activity increased to a maximum of 88% for 0.5 ppm and 71% for 10 ppm after 55 hrs.

(j) Fungicide Dithane M45 and nitrogenase activity

Table 12 and Fig. 12 shows the effect of the fungicide Dithane M45 on the acetylene reduction activity. In presence of 1 ppm (field dose) and 2 ppm (twice the field dose) there was a linear decline in activity until 58% activity was detected in both the concentrations after 55 hrs. Addition of 0.01 ppm Dithane M45 equivalent to 10% of the field dose inhibited only 30% of the activity at 55 hrs. Similarly 0.5 ppm equivalent to 50% of the field dose

Table 11. Fungicide ceresan and the nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).

| Incubation | $\mu$mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | Ceresan (ppm) | | | |
| | 0.5 | 2.5 | 5* | 10 |
| No addition | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| After addition (Hrs) | | | | |
| 2 | 5.2 (61) | 4.6 (54) | 4.5 (53) | 3.0 (35) |
| 5 | 5.41 (64) | 4.87 (57) | 4.65 (55) | 3.07 (36) |
| 10 | 6.6 (78) | 5.3 (63) | 5.3 (63) | 3.7 (44) |
| 20 | 7.8 (92) | 5.8 (69) | 5.7 (67) | 4.2 (50) |
| 30 | 7.92 (94) | 6.4 (76) | 6.01 (71) | 5.3 (63) |
| 40 | 7.54 (89) | 6.8 (80) | 6.6 (78) | 6.45 (76) |
| 50 | 7.51 (89) | 7.02 (83) | 6.5 (77) | 6.4 (76) |
| 55 | 7.4 (88) | 7.1 (84) | 6.13 (72) | 6.0 (71) |

*Field dose

*Fig.11. Effect of the fungicide ceresan (0.5, 2.5, 5.0 and 10 ppm) on nitrogenase activity (acetylene reduction) of Anabaena variabilis.*

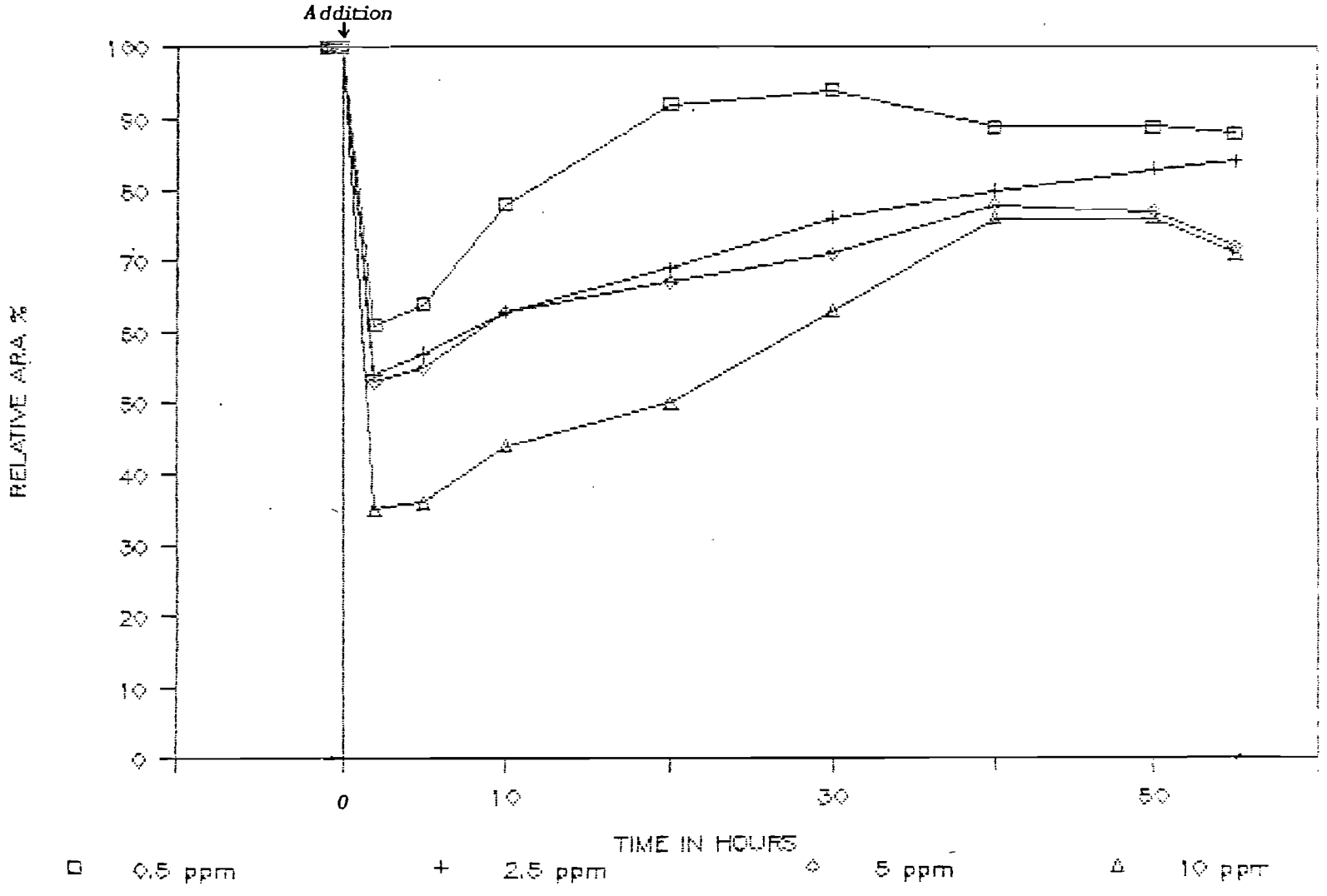
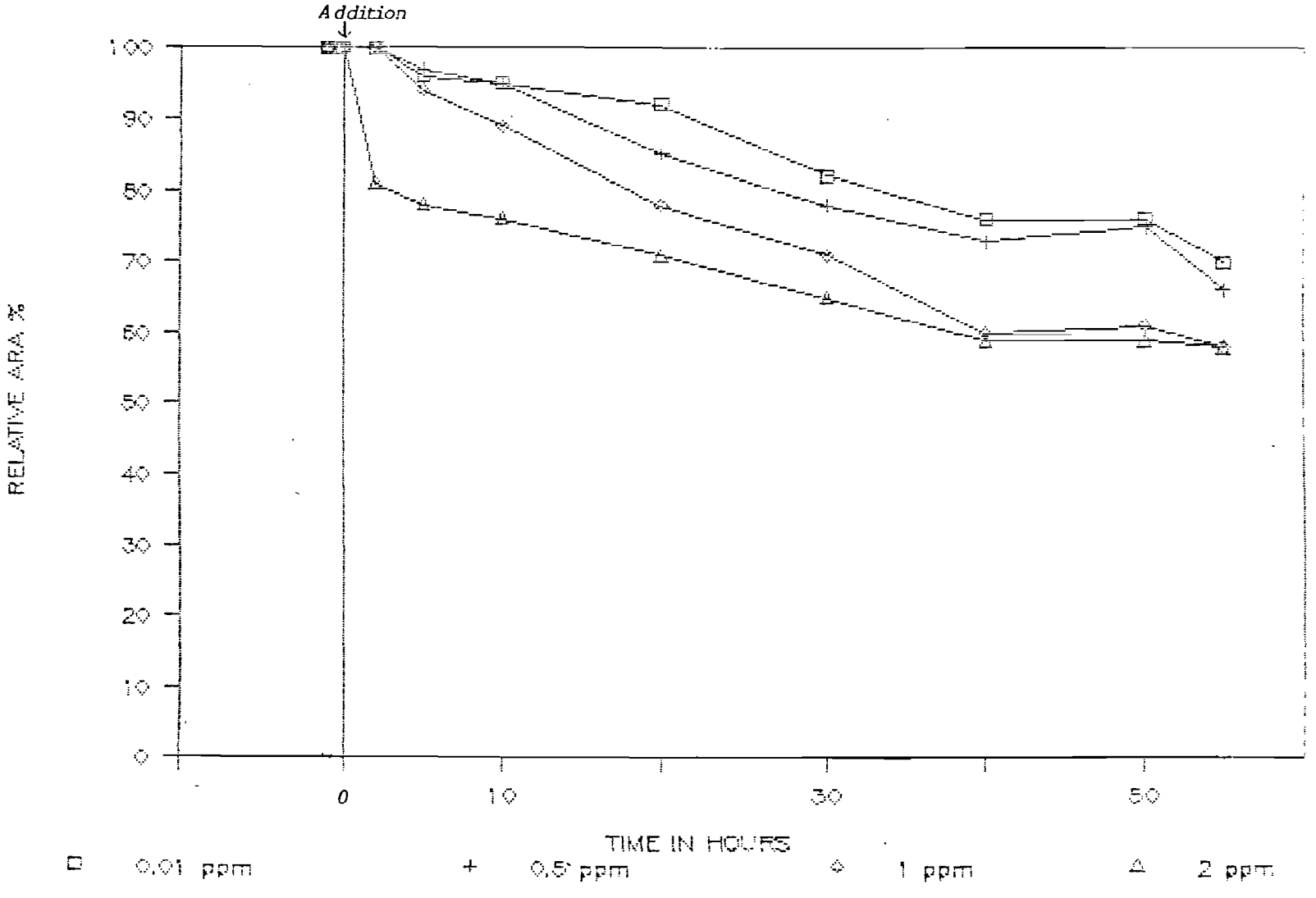

Addition
RELATIVE A.P.A. %
100
90
80
70
60
50
40
30
20
10
0
0
10
30
50
TIME IN HOURS
0.5 ppm
2.5 ppm
5 ppm
10 ppm

Table 12. Fungicide dithane and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis ( Figures in parenthesis show the relative activity).

| Incubation | $\mu$ mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | Dithane M-45 (ppm) | | | |
| | 0.01 | 0.5 | 1* | 2 |
| No addition | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| After addition (Hrs) | | | | |
| 2 | 8.4 (100) | 8.4 (100) | 8.4 (100) | 6.8 (81) |
| 5 | 8.1 (96) | 8.7 (97) | 7. (94) | 6.61 (78) |
| 10 | 8.0 (95) | 8.0 (95) | 7.5 (89) | 6.4 (76) |
| 20 | 7.8 (92) | 7.2 (85) | 6.56 (78) | 6.01 (71) |
| 30 | 6.9 (82) | 6.6 (78) | 6.04 (71) | 5.5 (65) |
| 40 | 6.4 (76) | 6.2 (73) | 5.08 (60) | 5.0 (59) |
| 50 | 6.4 (76) | 6.3 (75) | 5.2 (61) | 5 (59) |
| 55 | 5.9 (70) | 5.6 (66) | 4.9 (58) | 4.9 (58) |

*Field dose

*Fig.12. Effect of the fungicide Dithane M45 (0.01, 0.5, 1 and 2 ppm) on the nitrogenase activity (acetylene reduction) of Anabaena variabilis.*


Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
□ 0.01 ppm
+ 0.5 ppm
◇ 1 ppm
△ 2 ppm

had a reduction to an extent of 36% at 55 hrs. In general, inhibition was less severe with Dithane as compared to ceresan.

(k) Herbicide 2,4-D and the nitrogenase activity

Addition of 2,4-D at the recommended field dose of 0.5 ppm resulted in slight reduction to the extent of 25% at 2 hrs, but the activity gradually increased from 88-92% with the increased period of exposure (Fig. 13 and Table 13). 1 ppm 2,4-D equivalent to twice the field dose resulted in a drastic reduction in the acetylene reduction activity to the extent of 75% but the alga never recovered from the inhibition and only at best 27% activity was present at the end of 55 hrs. At 0.05 ppm (10% of field dose) and 0.25 ppm (50% of the field dose) a marginal reduction in activity was observed (16% and 17% respectively at 2 hrs) and with the period of incubation the activity increased in both cases to 92% and 90% respectively at 55 hrs. Only twice the field dose (1 ppm) inhibited the nitrogenase activity of the alga permanently (Fig. 13).

(l) Herbicide Machete and the nitrogenase activity

Table 14 and Fig. 14 show the effect of addition of 0.05, 0.25, 0.5 and 1 ppm Machete on acetylene reduction. At the recommended field dose of 0.5 ppm there was a sharp decline in the activity to the extent of 41% at 2 hrs but later there was linear increase in acetylene activity upto 97% at the end of 55 hrs. Twice the field dose of Machete (1 ppm) resulted in a further reduction in the nitrogenase activity and the inhibition was to the extent of 67% at 2 hrs, however, this inhibition was reversed with the increase

*Table 13. Herbicide 2,4-D and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).*

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | 2,4-D (ppm) | | | |
| | 0.05 | 0.25 | 0.5* | 1 |
| *No addition* | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| *After addition (Hrs)* | | | | |
| 2 | 7.1 (84) | 7.0 (83) | 6.3 (75) | 2.1 (25) |
| 5 | 7.02 (85) | 7.011 (83) | 6.44 (76) | 2.26 (26) |
| 10 | 7.6 (90) | 7.5 (89) | 7.2 (85) | 2.4 (28) |
| 20 | 7.91 (94) | 7.9 (94) | 7.8 (92) | 2.6 (30) |
| 30 | 7.91 (94) | 7.6 (90) | 7.54 (89) | 2.67 (31) |
| 40 | 7.74 (92) | 7.6 (90) | 7.51 (89) | 2.5 (29) |
| 50 | 7.89 (93) | 7.5 (89) | 7.4 (88) | 2.3 (27) |
| 55 | 7.81 (92) | 7.64 (90) | 7.41 (88) | 2.3 (27) |

*Field dose

*Fig.13. Effect of the herbicide 2,4-D (0.05, 0.25, 0.5 and 1 ppm) on the acetylene reduction activity of Anabaena variabilis.*

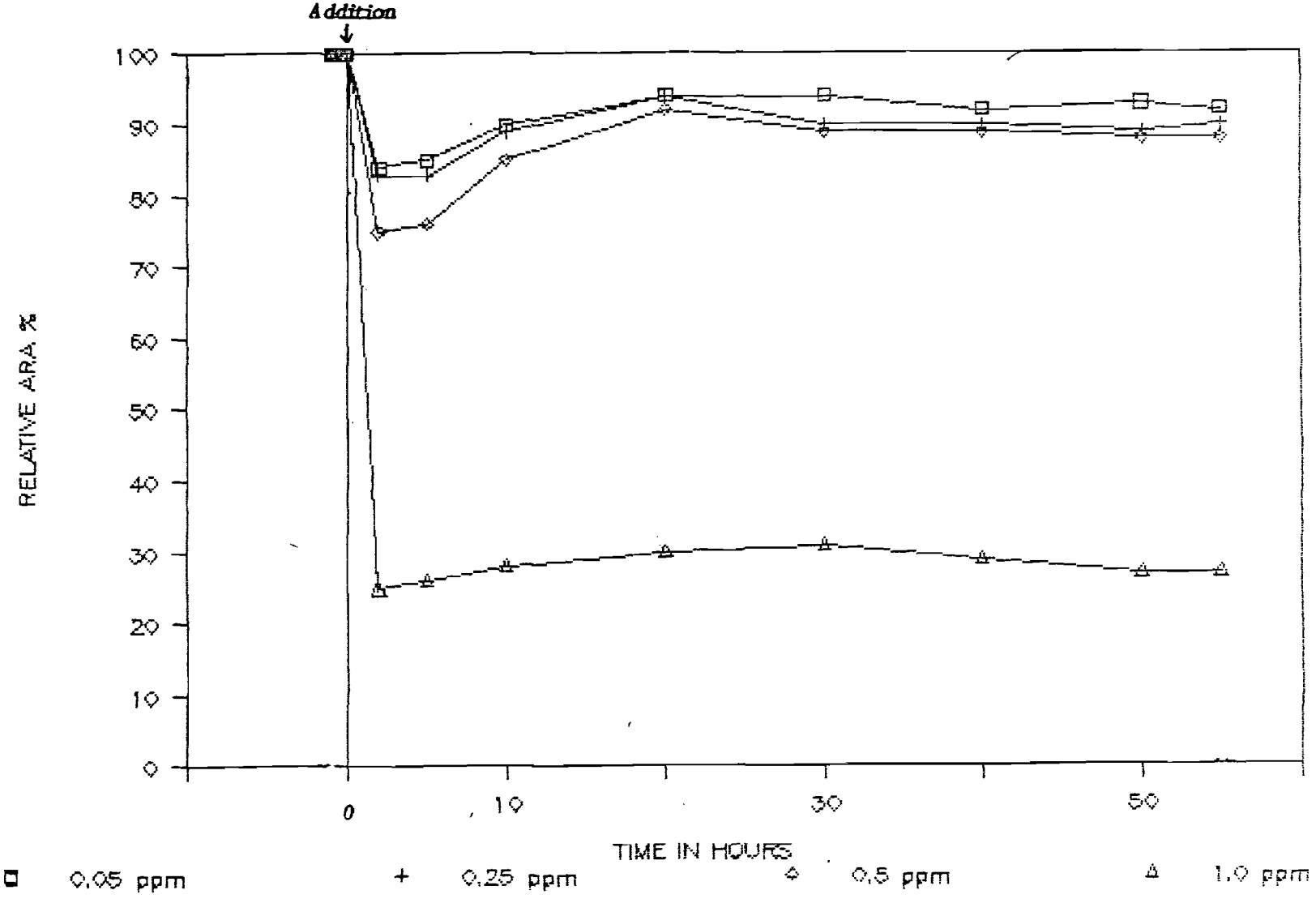
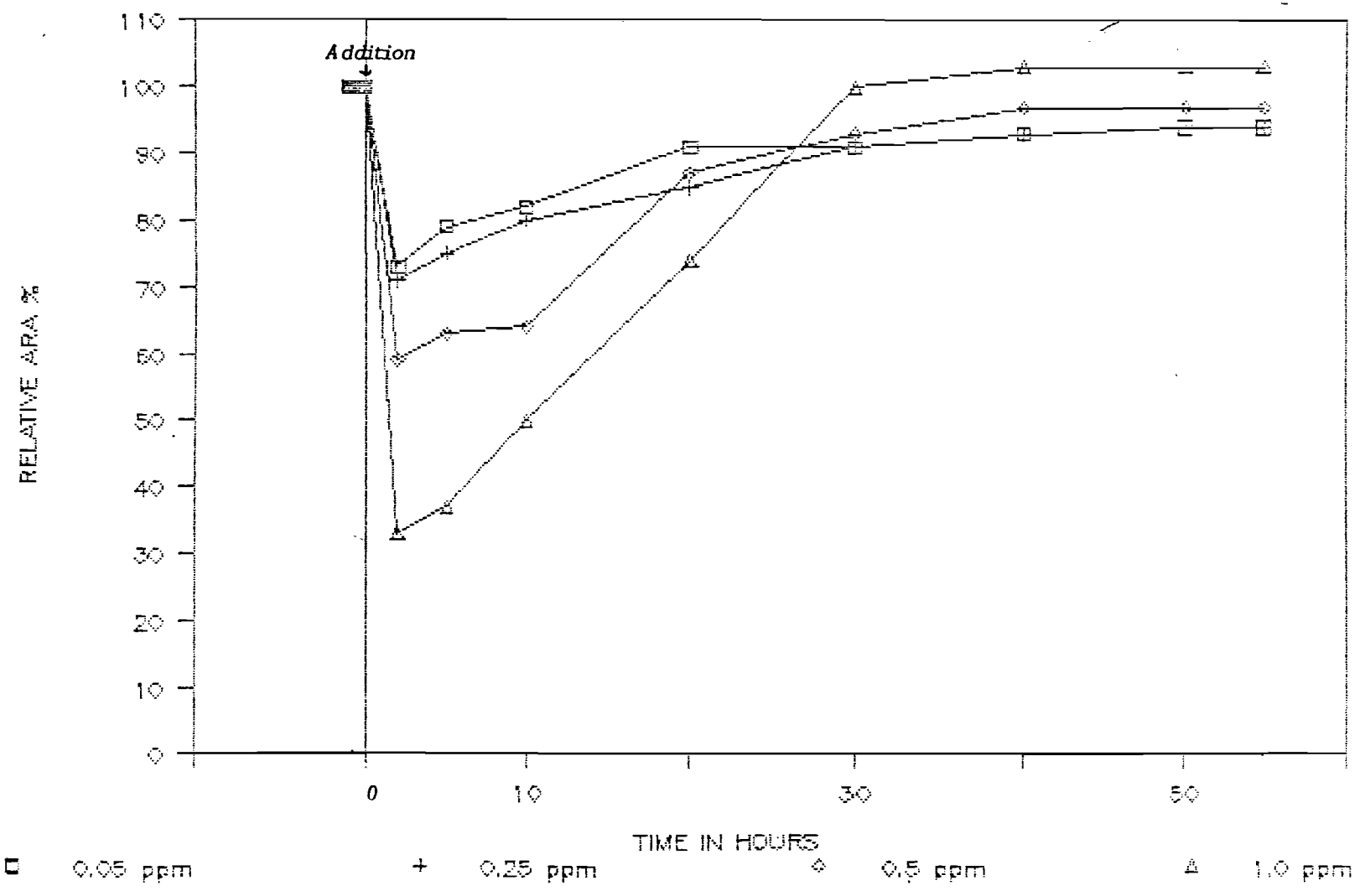

Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE A.R.A. %
0
10
30
50
TIME IN HOURS
0.05 ppm
0.25 ppm
0.5 ppm
1.0 ppm

*Table 14. Herbicide machete and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).*

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | Machete (ppm) | | | |
| | 0.05 | 0.25 | 0.5* | 1 |
| No addition | | | | |
| Control | 8.1 (100) | 8.1 (100) | 8.1 (100) | 8.1 (100) |
| After addition (Hrs) | | | | |
| 2 | 5.92 (73) | 5.8 (71) | 4.8 (59) | 2.7 (33) |
| 5 | 6.4 (79) | 6.1 (75) | 5.13 (63) | 2.07 (37) |
| 10 | 6.7 (82) | 6.5 (80) | 5.2 (64) | 4.1 (50) |
| 20 | 7.39 (91) | 6.9 (85) | 7.04 (87) | 6.01 (74) |
| 30 | 7.41 (91) | 7.42 (91) | 7.54 (93) | 8.10 (100) |
| 40 | 7.59 (93) | 7.59 (93) | 7.87 (97) | 8.39 (103) |
| 50 | 7.64 (94) | 7.62 (94) | 7.91 (97) | 8.41 (103) |
| 55 | 7.64 (94) | 7.64 (94) | 7.91 (97) | 8.34 (103) |

*Field dose

Fig.14. Effect of the herbicide Machete (0.05, 0.25, 0.5 and 1 ppm) on the nitrogenase activity (acetylene reduction) of Anabaena variabilis.


Addition
110
100
90
80
70
60
50
40
30
20
10
0
0
10
30
50
RELATIVE ARA %
TIME IN HOURS
0.05 ppm
0.25 ppm
0.5 ppm
1.0 ppm

in exposure time and the alga attained 100% activity at the end of 30 hrs. The initial inhibition in the activity at 2 hrs for both the 10% of field dose (0.05 ppm) and 50% of the field dose (0.25 ppm) was less compared to the field dose i.e. 27% for 0.05 ppm and 29% for 0.25 ppm. After two hours the activity increased to the maximum of 94% in both the concentrations at the end of 55 hrs. Machete in all the 4 concentrations resulted in initial reduction in the acetylene reduction activity to varying degrees but the alga overcame the inhibition within 30 hrs of the addition of the herbicide.

(m) Herbicide Diuron and the nitrogenase activity

Table 15 and Fig. 15 show the effect of Diuron on nitrogenase measured as acetylene reduction. In presence of 0.25 ppm (Field dose) Diuron there was a sharp decline in the activity to the level of 55% at 2 hrs and even at 55 hrs the recovery was marginal. With twice the field dose concentration (0.5 ppm) of Diuron the inhibition was much severe and there was no recovery in the activity even upto 55 hrs. 0.025 ppm equivalent to 10% of field dose and 0.125 ppm equivalent to 50% of field dose resulted in initial reduction to the extent of 47% and 49% at 2 hours respectively and 66% and 61% activity was present at the end of 55 hrs. The results indicate that recovery was possible only at lower doses.

(n) Herbicide Dalapon and the nitrogenase activity

Table 16 and Fig. 16 show the effect of the herbicide Dalapon on nitrogenase activity. In presence of the recommended field dose of 3 ppm, the acetylene reduction activity fell by 56% at 2 hrs but then there was a linear increase in activity till 86% activity was

Table 15. Herbicides diuron and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1} hr^{-1}$ | | | |
|---|---|---|---|---|
| | Diuron (ppm) | | | |
| | 0.025 | 0.125 | 0.25* | 0.5 |
| No addition | | | | |
| Control | 8.3 (100) | 8.3 (100) | 8.3 (100) | 8.3 (100) |
| After addition (Hrs) | | | | |
| 2 | 4.41 (53) | 4.3 (51) | 3.8 (45) | 1.7 (20) |
| 5 | 4.65 (56) | 4.51 (54) | 4.17 (50) | 2.25 (27) |
| 10 | 5.6 (67) | 5.1 (61) | 4.6 (55) | 2.1 (25) |
| 20 | 6.70 (80) | 6.01 (72) | 5.14 (62) | 1.8 (21) |
| 30 | 6.66 (80) | 6.04 (72) | 5.18 (62) | 1.52 (18) |
| 40 | 5.51 (66) | 5.61 (67) | 4.80 (57) | 1.17 (14) |
| 50 | 5.41 (65) | 5.19 (62) | 4.64 (55) | 1.17 (14) |
| 55 | 5.48 (66) | 5.1 (61) | 4.79 (57) | 1.24 (15) |

*Field dose

*Fig.15. Effect of the herbicide Diuron (0.025, 0.125, 0.25 and 0.5 ppm) on the acetylene reduction activity of Anabaena variabilis.*

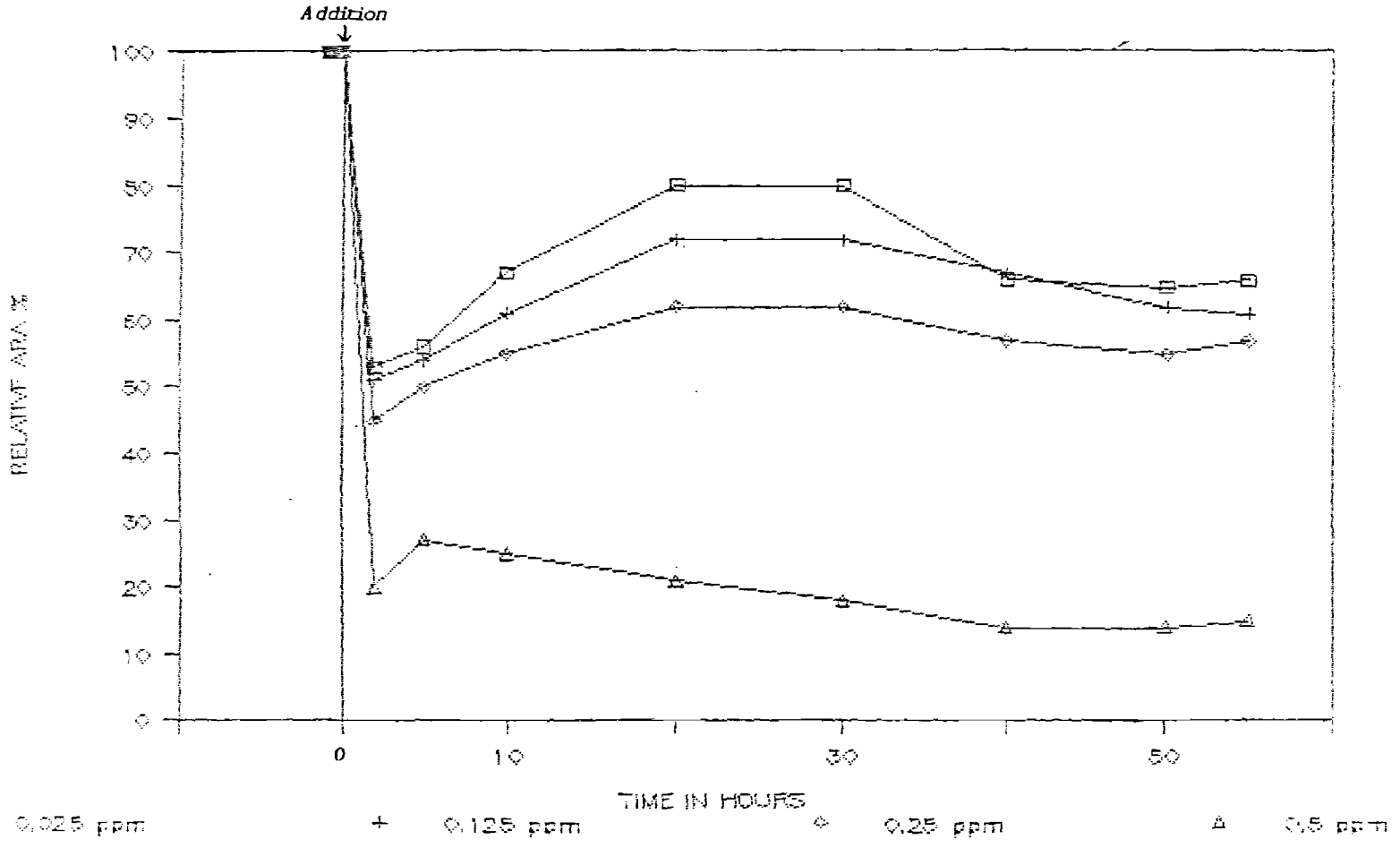
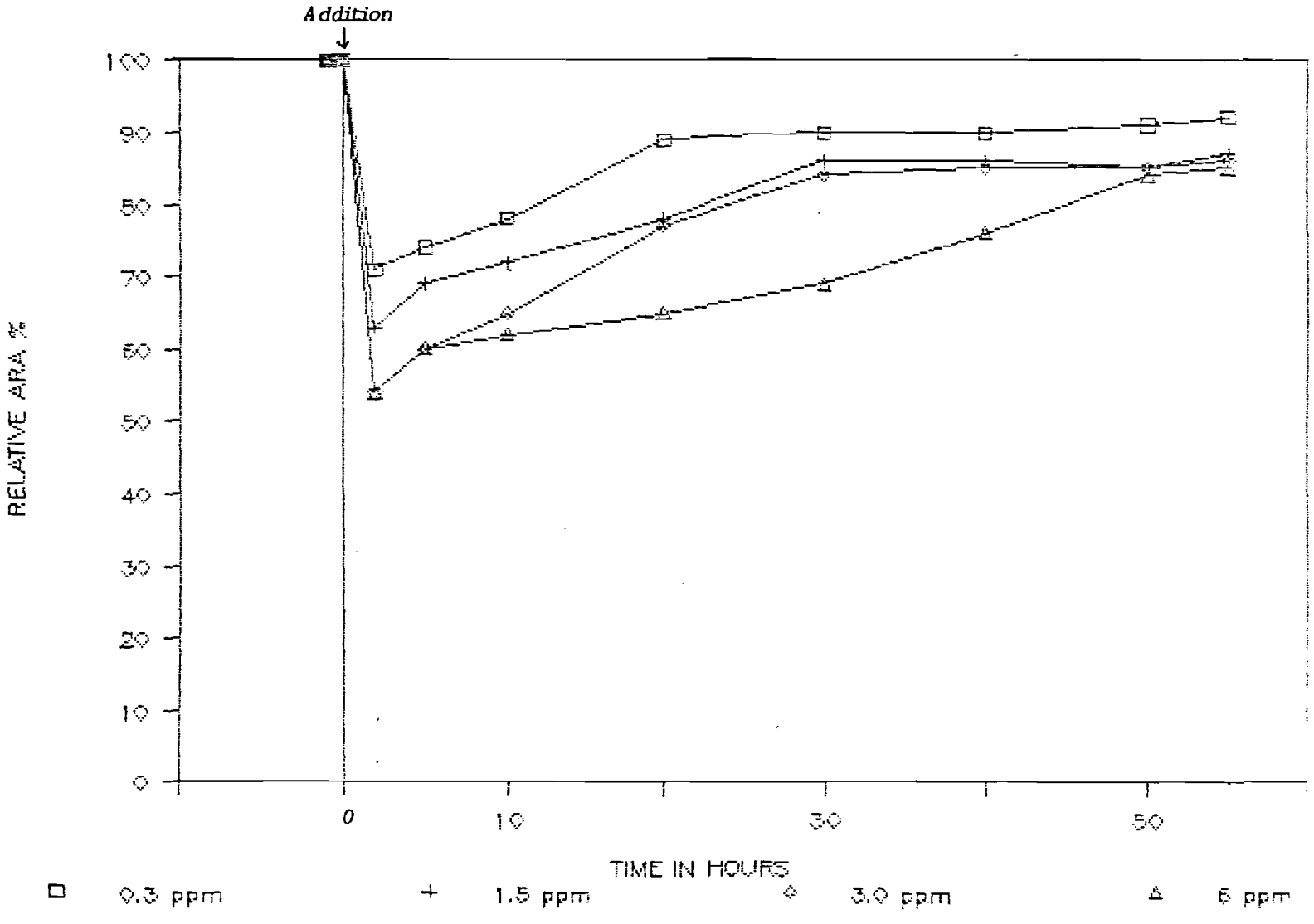

Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE APA %
0
10
30
50
TIME IN HOURS
0.025 ppm
+ 0.125 ppm
◇ 0.25 ppm
△ 0.5 ppm

*Table 16. Herbicide dalapon and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).*

| Incubation | $\mu$mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | (Dalapon (ppm) | | | |
| | 0.3 | 1.5 | 3* | 6 |
| No addition | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| After addition (Hrs) | | | | |
| 2 | 6.01 (71) | 5.3 (63) | 4.61 (54) | 4.60 (54) |
| 5 | 6.21 (74) | 5.8 (69) | 5.04 (60) | 5.04 (60) |
| 10 | 6.6 (78) | 6.1 (72) | 5.5 (65) | 5.2 (62) |
| 20 | 7.52 (89) | 6.6 (78) | 6.5 (77) | 5.48 (65) |
| 30 | 7.56 (90) | 7.3 (86) | 7.07 (84) | 5.8 (69) |
| 40 | 7.59 (90) | 7.3 (86) | 7.19 (85) | 6.4 (76) |
| 50 | 7.63 (91) | 7.2 (85) | 7.14 (85) | 7.07 (84) |
| 55 | 7.75 (92) | 7.34 (87) | 7.26 (86) | 7.17 (85) |

*Field dose

*Fig.16. Effect of the herbicide Dalapon (0.3, 1.5, 3 and 6 ppm) on nitrogenase activity (acetylene reduction) of Anabaena variabilis.*


Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
0.3 ppm
1.5 ppm
3.0 ppm
6 ppm

reached at the end of 55 hrs. Similarly twice the field dose (6 ppm) also resulted in an initial decline to the extent of 56% but the activity rose to 85% at the end of 55 hrs. Addition of 10% of field dose (0.3 ppm) resulted in only 29% reduction in activity at 2 hrs, while the inhibition was completely removed and 92% activity was achieved at the end of 55 hrs. In the same way 1.5 ppm Dalapon equivalent to 50% of the field dose caused a initial decline to the extent of 37% after 2 hrs, while the activity reached 87% after 55 hrs. Dalapon inhibited nitrogenase activity only to a maximum extent of 15% in all the concentrations tested.

(o) Insecticide BHC and the nitrogenase activity

Addition of the recommended field dose of 0.5 ppm BHC had a drastic reduction in the nitrogenase activity to the level of 59% at 2 hrs and the alga regained its activity only upto 61% after 55 hrs (See Fig. 17 and Table 17). In presence of twice the field dose (1 ppm) of BHC there was similar reduction in activity however, there was no recovery in activity even upto 55 hrs. Addition of 10% of field dose (0.05 ppm) and 50% of field dose (0.25 ppm) of BHC had similar initial reduction in acetylene reduction activity to the extent of 54% and 56% at 2 hrs respectively and then there was a linear increase in activity until 73% and 67% activity was present after 55 hrs (Table 17).

(p) Insecticide phorate and nitrogenase activity

Addition of the insecticide phorate at field dose of 0.5 ppm inhibited the nitrogenase activity initially severely to the extent of

*Table 17. Insecticide BHC and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).*

| Incubation | $\mu$ mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | BHC (ppm) | | | |
| | 0.05 | 0.25 | 0.5* | 1 |
| No addition | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| After addition (Hrs) | | | | |
| 2 | 3.9 (46) | 3.7 (44) | 3.5 (41) | 3.4 (40) |
| 5 | 4.2 (50) | 3.80 (45) | 3.76 (44) | 3.69 (44) |
| 10 | 5.7 (67) | 5.1 (60) | 4.01 (47) | 3.7 (44) |
| 20 | 7.05 (84) | 5.81 (69) | 4.57 (54) | 3.71 (44) |
| 30 | 7.1 (84) | 5.7 (67) | 5.17 (61) | 3.60 (42) |
| 40 | 6.5 (77) | 5.62 (67) | 5.01 (61) | 3.4 (40) |
| 50 | 6.10 (72) | 5.62 (67) | 5.1 (61) | 3.46 (41) |
| 55 | 6.17 (73) | 5.7 (67) | 5.13 (61) | 3.52 (41) |

*Field dose

*Fig.17. Effect of the insecticide BHC (0.05, 0.25, 0.5 and 1 ppm) on the nitrogenase activity (acetylene reduction) of Anabaena variabilis.*

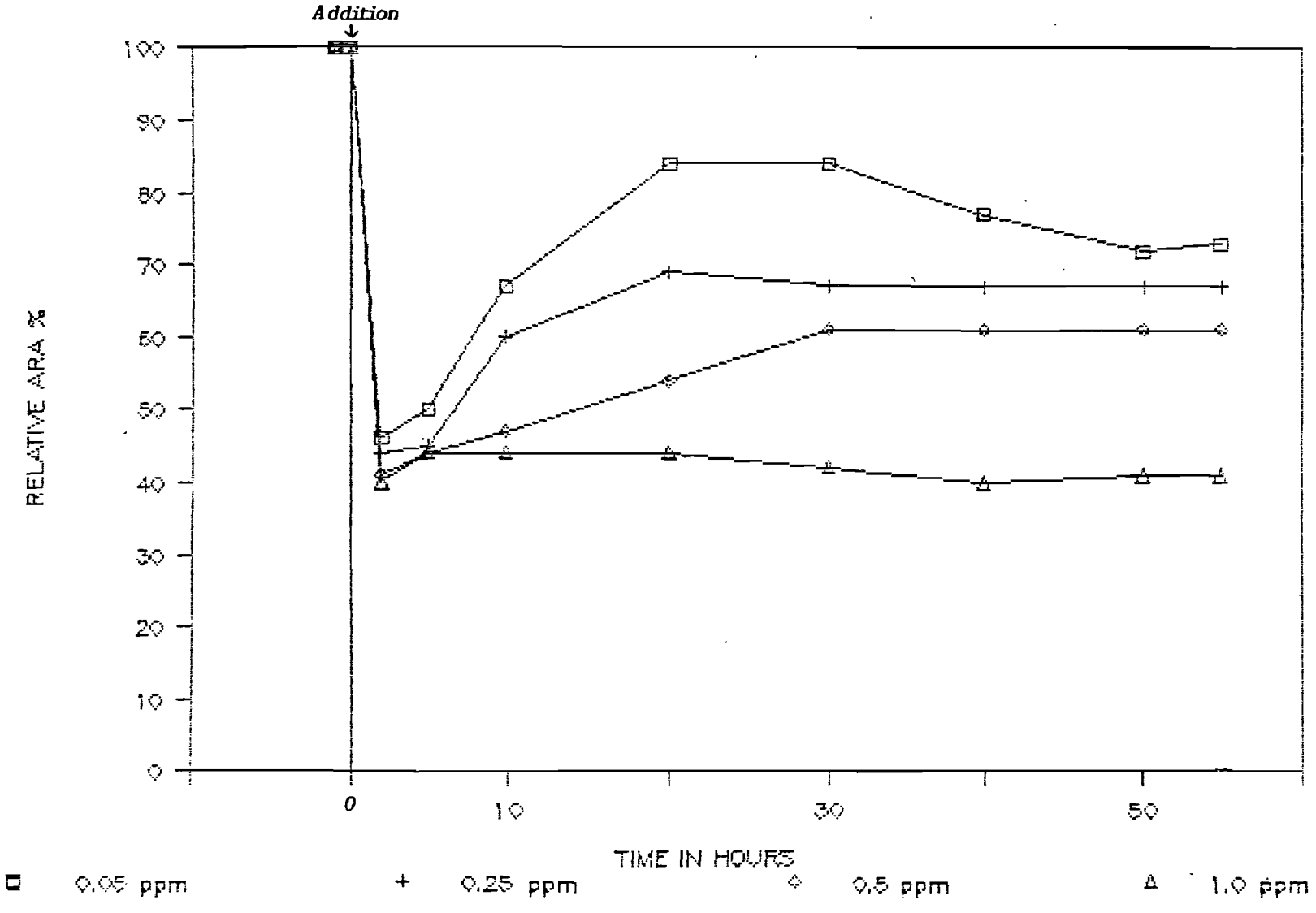
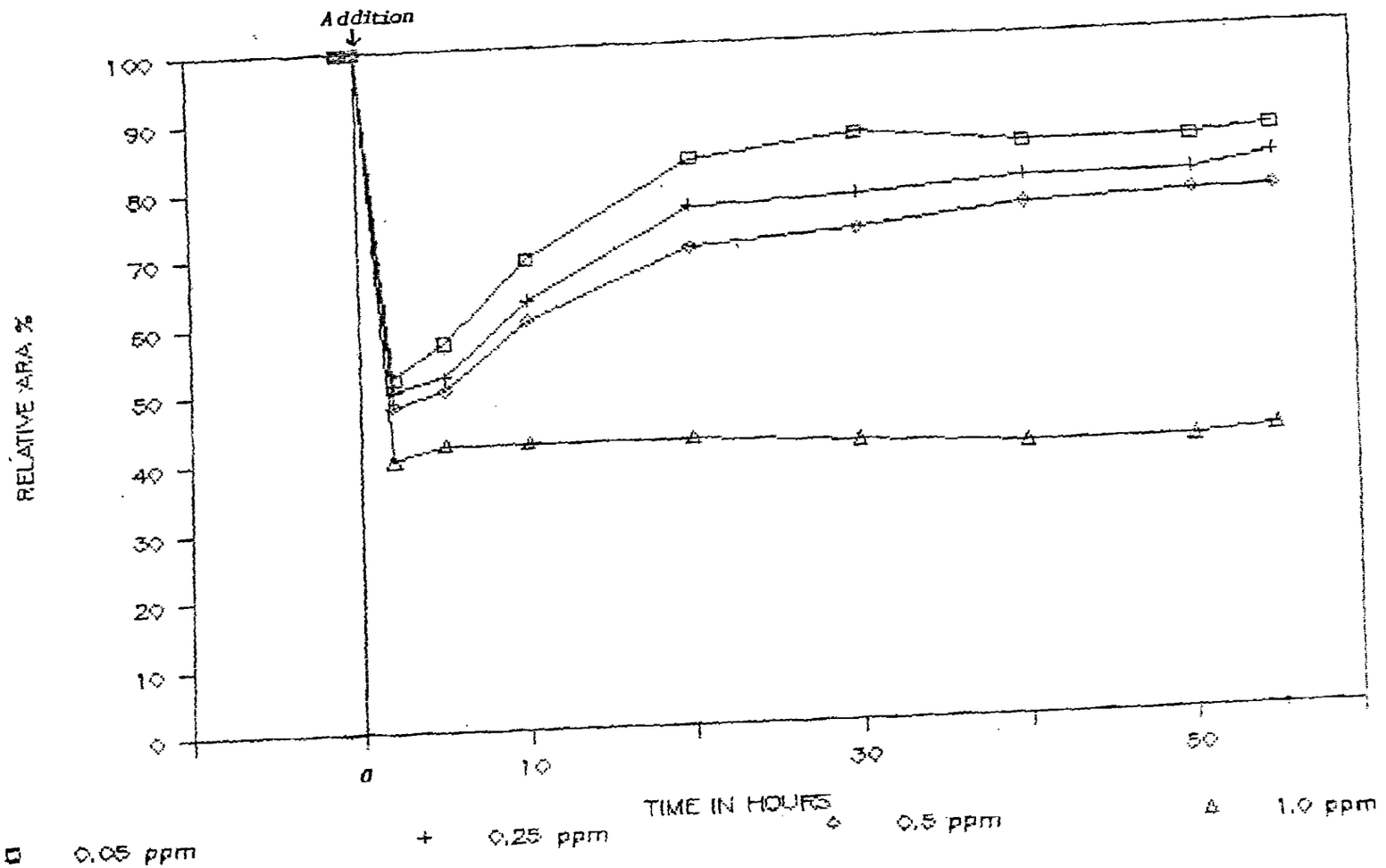

Addition
100
90
80
70
60
50
40
30
20
10
0
RELATIVE ARA %
0
10
30
50
TIME IN HOURS
0.05 ppm
0.25 ppm
0.5 ppm
1.0 ppm

Table 18. Insecticide phorate and nitrogenase activity (Acetylene reduction activity) of Anabaena variabilis (Figures in parenthesis show the relative activity).

| Incubation | $\mu mol\ C_2H_4\ mg\ Chl^{-1}hr^{-1}$ | | | |
|---|---|---|---|---|
| | Phorate (ppm) | | | |
| | 0.05 | 0.25 | 0.5* | 1 |
| No addition | | | | |
| Control | 8.4 (100) | 8.4 (100) | 8.4 (100) | 8.4 (100) |
| After addition (Hrs) | | | | |
| 2 | 4.4 (52) | 4.2 (50) | 4.1 (48) | 3.4 (40) |
| 5 | 4.8 (57) | 4.41 (52) | 4.23 (50) | 3.52 (42) |
| 10 | 5.8 (69) | 5.3 (63) | 5.1 (60) | 3.52 (42) |
| 20 | 7.01 (83) | 6.4 (76) | 5.9 (70) | 3.51 (42) |
| 30 | 7.26 (86) | 6.51 (77) | 6.1 (72) | 3.48 (41) |
| 40 | 7.1 (84) | 6.66 (79) | 6.34 (75) | 3.42 (40) |
| 50 | 7.05 (84) | 6.7 (79) | 6.39 (76) | 3.44 (40) |
| 55 | 7.21 (85) | 6.84 (81) | 6.44 (76) | 3.46 (41) |

*Field dose

*Fig.18. Effect of the insecticide phorate (0.05, 0.25, 0.5 and 1 ppm) on nitrogenase activity (acetylene reduction) of Anabaena variabilis.*


Addition
100
90
80
70
60
50
40
30
20
10
0
0
10
30
50
RELATIVE A.R.A. %
TIME IN HOURS
0.05 ppm
0.25 ppm
0.5 ppm
1.0 ppm

52% at 2 hrs. However, the inhibition was reversed gradually with the increase in period of exposure and 76% of the activity was obtained after 55 hrs. When twice the concentration of the field dose ( 1 ppm) was added there was immediate reduction upto 60% in activity of 2 hrs and there was no recovery in the nitrogenase activity throughout the experimental period (55 hrs). At lower concentrations of 0.05 ppm and 0.25 ppm after an initial reduction in nitrogenase activity at 2 hrs, the activity increased to the level of 85% and 81% respectively after 55 hrs. The inhibition caused by the insecticide phorate is seen to the reversible in concentrations equivalent to field dose, 10% of field dose and 50% of field dose over a period of time (Fig. 18 and Table 18).

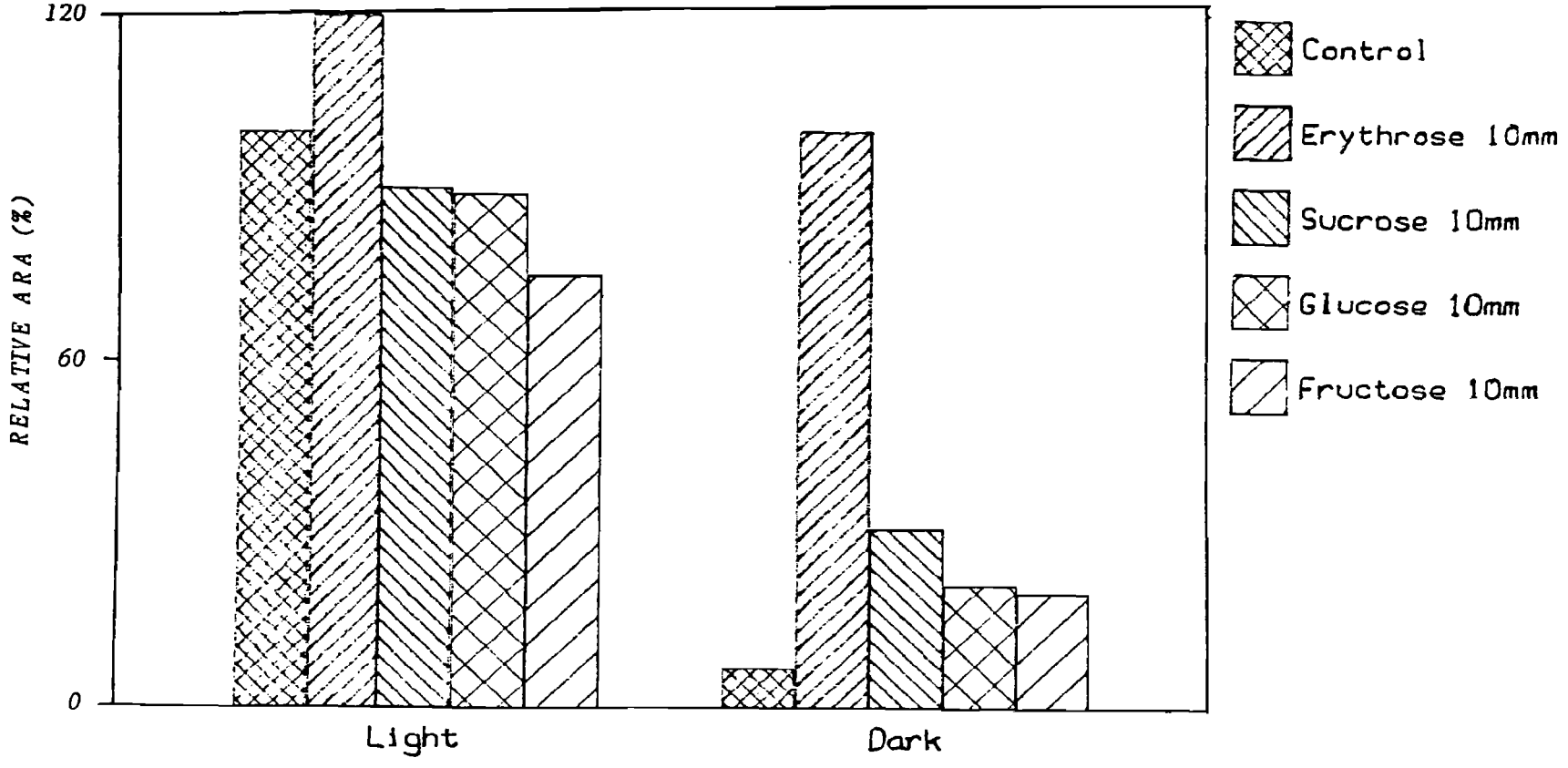
(4) Sugars and nitrogenase activity

Table 19 and Fig. 19 show the effect of different sugars on the acetylene reduction activity in dark as well as light. Acetylene reduction activity measured without any addition of sugars was 10 $\mu$ mole $C_2H_4$ mg $Chl^{-1}hr^{-1}$ in light and 0.7 $\mu$ mole $C_2H_4$ mg $Chl^{-1}hr^{-1}$ in dark. Erythrose, sucrose, glucose and fructose were examined at 10 mM concentrations on the acetylene reduction. In light, erythrose gave a slight stimulation in nitrogenase activity (120%) as compared to the control. Sucrose and glucose gave 90% activity and fructose 75%. However, when the filaments were incubated in dark only erythrose fully supported nitrogenase activity and was at par with light (control). Other sugars supported the activity to 20-31% only (Table 19).

Table 19. Sugars and nitrogenase activity (Acetylene reduction activity) of whole filaments of Anabaena variabilis (Figures in parenthesis show relative activity).

| Sugars (10 mM) | $\mu$mol $C_2H_4$ mg $Chl^{-1}hr^{-1}$ | |
|---|---|---|
| | Light | Dark |
| Control | 10.0 (100) | 0.7 (7) |
| Erythrose | 12 (120) | 10 (100) |
| Sucrose | 9 (90) | 3.1 (31) |
| Glucose | 8.9 (89) | 2.14 (21) |
| Fructose | 7.5 (75) | 2.01 (20) |

*Fig.19. Effect of different sugars at 10 m M concentration on the nitrogenase activity (acetylene reduction) of Anabaena variabilis.*


120
60
0
RELATIVE ARA (%)
Light
Dark
Control
Erythrose 10mm
Sucrose 10mm
Glucose 10mm
Fructose 10mm

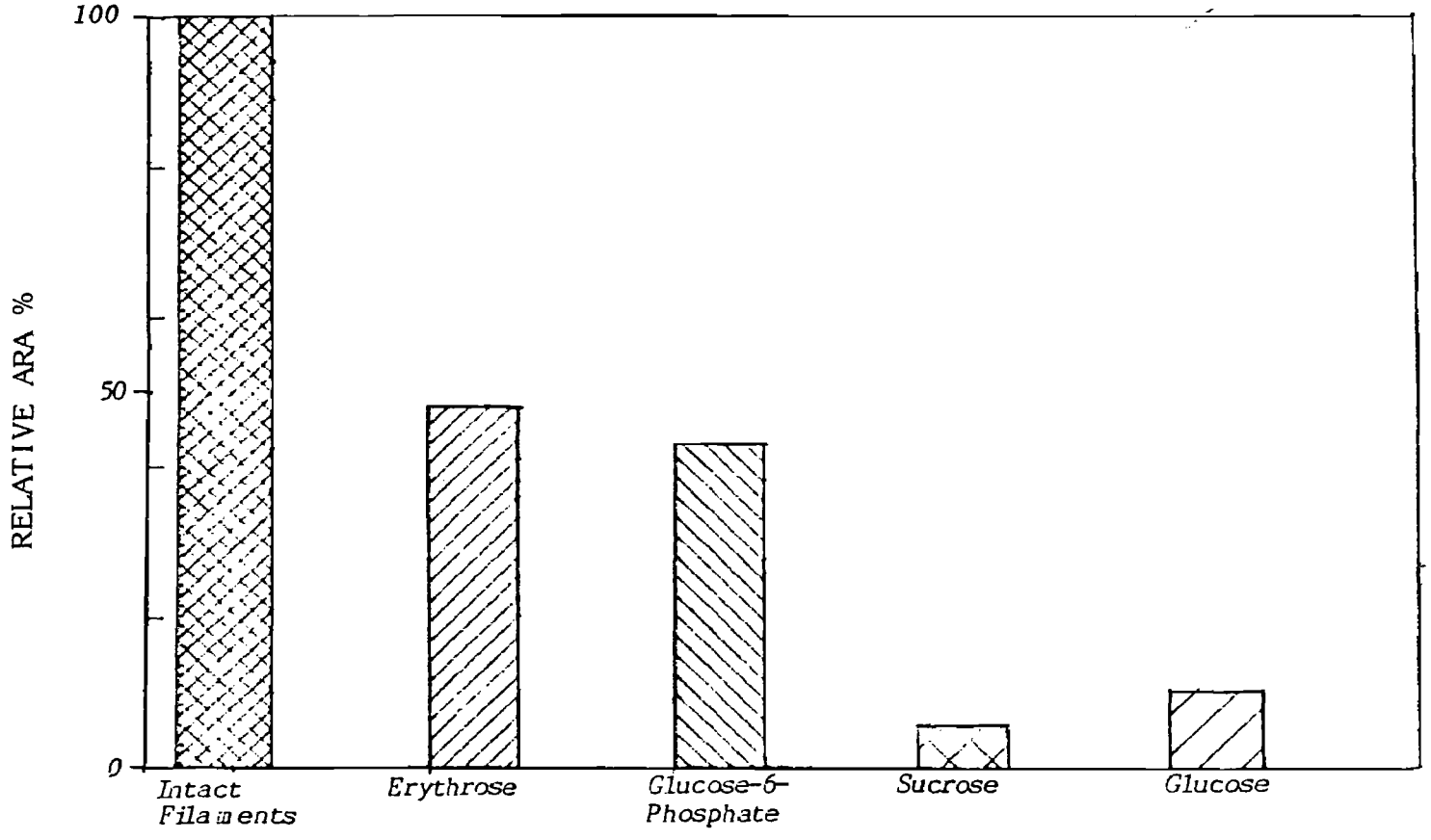
(r) Sugars and nitrogenase activity of isolated heterocysts

Table 20 and Fig. 20 show the effect of different sugars on the acetylene reduction activity of isolated heterocysts. Erythrose, glucose-6-phosphate, sucrose, and glucose at a concentrations of 10 m M were examined on the acetylene reduction activity compared with the intact filaments. In presence of glucose-6-phosphate isolated heterocysts gave 43% activity. While sucrose and glucose resulted only in 5 and 10% activity, respectively compared to the intact filments. Nearly 50% of the acetylene reduction activity by the isolated heterocysts was achieved due to erythrose at 10 m M supplementation when compared to whole filaments.

*Table 20.* *Sugars and nitrogenase activity (Acetylene reduction activity) of isolated heterocysts of Anabaena variabilis (Figures in parenthesis show relative activity).*

| Time (Hr) | $\mu$mol $C_2H_4$ ($10^6$ heterocysts/hour) | | | | |
|---|---|---|---|---|---|
| | Sugars (10 mM) | | | | |
| | Intact filaments | Erythrose | Glucose-6-phosphate | Sucrose | Glucose |
| 1 | 10.01 (100) | 4.8 (48) | 4.3 (43) | 0.5 (5) | 1.0 (10) |

Fig.20. Effect of various sugars at 10 m M concentration on the nitrogenase activity (acetylene reduction) of isolated heterocysts of *Anabaena variabilis*.


100
50
0
RELATIVE ARA %
Intact Filaments
Erythrose
Glucose-6-Phosphate
Sucrose
Glucose

## V. DISCUSSION

Biological nitrogen fixation has gained a renewed interest due to fertilizer price increase during the last decade. However, biological nitrogen fixation requires energy generally obtained by the catabolism of photosynthetically fixed carbon. Only cyanobacteria are able to generate their own photosynthate from $CO_2$ and water and also fix dinitrogen. This trophic independence of cyanobacteria along with their ability to grow and withstand a variety of ecological stresses makes them attractive as a biofertilizer. Research on cyanobacteria has been focussed mainly on the paddy field ecosystem, since rice forms the staple diet of a high proportion of the world's population. Further, paddy field ecosystem provides a favourable environment for the growth of cyanobacteria with respect to their requirements for light, water, high temperature and nutrient availability. This could be the reason why cyanobacteria grow in higher abundance in paddy soils than in upland soils (Watanabe and Yamamoto 1971). The heterogenous and sometimes limited distribution of $N_2$ fixing cyanobacteria is still not well understood because no systematic analysis has correlated the presence or absence of cyanobacteria with environmental factors (Lowendorf, 1980). There is ample evidence at present that cyanobacteria contribute 20-30 KgN/ha per cropping reason and a 10-15% increase in rice yield. There are, however, many areas that need greater scientific research like ecology of cyanobacteria in rice fields and their action on plants, etc. Ecological studies of cyanobacteria in submerged soils are limited by problems in methodology, primarily in estimating algal biomass, quantitatively and qualitatively. In addition, problems in sampling techniques in relation to spatial distribution of cyanobacteria and their nitrogen fixing activity increase the inaccuracy of quantitative

measurement. The present investigation was undertaken to examine the effect of various ecological and environmental factors such as pH, temperature, light, combined nitrogen, phosphorous, sugars, fungicides, herbicides and insecticides on the nitrogenase activity of Anabaena variabilis. ARM 394.

Among the soil properties, pH is the most important factor in determining cyanobacterial composition, but it is difficult to distinguish the effects of pH that are due directly to $H^+$ concentrations from those that are due to other chemical factors such as solubility of trace elements like molybdenum and iron which are needed for the nitrogenase activity. In natural conditions, most cyanobacteria grow in environments that are neutral to alkaline. In submerged acidic soils, the supernatant water, in which most algae grow, always has a higher pH than the soil itself. In laboratory cultures, the optimal pH for growth of cyanobacteria seems to range from 7.5 to 10.0 and the lower limit is about 6.5 to 7.0. The beneficial influence of high pH on cyanobacteria is further demonstrated by the fact that the addition of lime increases the cyanobacteria growth and nitrogen fixation (Yamaguchi, 1980; Roger and Kulasooriya, 1980). However, the presence of certain strains of cyanobacteria in soils with pH values between 5 and 6 have been reported. Nostoc muscorum and Anabaena torulosa were reported to be growing in pH ranging from 5-7 (Durrel, 1964) and Aulosira fertilissima and Calothrix brevissima were reported to grow with pH from 3.5 to 6.5 (Aiyer, 1965). But whether these forms fix dinitrogen efficiently under these acidic conditions is not known. In the present investigation, pH 4 and pH 6 drastically reduced the acetylene reduction activity of Anabaena variabilis, which never improved in course of time. Nitrogenase activity was maximum in pH 7 and pH 8.

However, in pH8 there was an initial decline in activity but later the activity increased to 82% of the original activity. pH10 supported 73% of the activity after 30 hrs. Thus neutral and alkaline pH supported maximum nitrogen fixation by Anabaena variabilis. Stewart, however, reported that some tropical cyanobacteria exhibited ARA even at pH4 (Stewart et al., 1978). In the present form, acidic pH suppresses nitrogenase activity severely. Whether this reduction in acidic pH is directly due to $H^+$ ion concentration or due to non-availability of trace elements like Mo needed for nitrogenase activity is not clear. Possibly, both contribute to the inhibition in the nitrogenase activity. But in acidic soils, poor growth of nitrogen fixing cyanobacteria is probably due to the inability of cyanobacteria to compete with chlorophyceae, which are favoured by acidic conditions.

Under N-deficient conditions, $N_2$ fixing cyanobacteria are greatly favoured by a lack of competitiveness of the other algae and can develop profusely if the other environmental factors are not limiting. When nitrogenous fertilizers are applied, their nitrogen fixing activity is inhibited or at least reduced. They can utilize mineral nitrogen for their growth, but they have to compete with non-nitrogen fixing cyanobacteria and other eukaryotic algae (Roger et al., 1980). However, the effect seems to be transient as suggested by pot experiments (Reynaud, 1980) and preliminary field data. Very little is known about the competition between diazotrophic cyanobacteria and non-diazotrophic forms as affected by the nature and the concentration of inorganic nitrogen (Roger and Reynaud, 1979). However, it has been shown that nitrogenous fertilizers have selective and

inhibitory effect on diazotrophic cyanobacteria (Rinaudo, 1974). All the sources of combined nitrogen viz. $KNO_3$, $NH_4NO_3$, $NH_4Cl$ and urea examined in the present investigation inhibited nitrogenase activity to varying degrees and the inhibition was directly proportional to the concentration used. There was no recovery from the inhibition in any of the substances tested throughout the experimental period.

Nitrate has been reported to have variable and transitory inhibitory effect on nitrogen fixation by cyanobacteria (Haselkorn, 1978). Addition of 1mM, 2mM, 3mM $KNO_3$ to the nitrogen fixing cultures of Anabaena variabilis reduced the acetylene reduction activity. The reduction was proportional to the concentration used (see Fig. 6). However, the inhibition was less, compared to the inhibition caused by the $NH_4^+$ nitrogen (see Figs. 7 and 8). Addition of 1mM $NH_4NO_3$ and 1mM $NH_4Cl$ resulted in 89% and 78% reduction in activity at 48 hrs, while 1mM $KNO_3$ resulted only in 22% reduction in activity at 48 hrs. This is in accordance with the earlier findings that presence of high concentrations of $NH_4^+$ in the growth medium severely decreases the activity of nitrogenase (Dharmawardene et al., 1973; Meeks et al., 1977; Rowell et al., 1977; Stacey et al., 1977, 1979a,b; Tuli and Thomas, 1980) and glutamine synthatase (Sims et al., 1974; Milman et al., 1975; Quinto et al., 1977) and this has been shown to be mediated by a variety of mechanisms including repression (Woolfolk et al., 1966), degradation (Milman et al., 1975; Quinto et al., 1977) and disaggregation (Sims et al.,1974) of the enzyme. The marine Anabaena CA has been reported to partially express nitrogenase even in excess of $NH_4^+$, whereas the enzyme is not expressed in presence of $NO_3^-$ (Bottomley et al., 1979). This

anamoly could be due to a naturally occuring mutation in the regulatory mechanism.

$NH_4^+$ regulation of nitrogenase is mediated through glutamine synthetase (GS) (Streicher et al., 1974). Inhibition of GS causes K. pneumoniae, A. vinelandii (Gordon and Brill, 1974), Anabaena cylindrica (Stewart and Rowell, 1977) and Rhodospsendomonas Sphaeroides (Jones and Monty, 1979) to produce nitrogenase even in presence of excess $NH_4^+$ showing that a product of $NH_4^+$ assimilation is the repressor of nitrogenase. Besides repression, addition of $NH_4^+$ to nitrogen fixing cultures may cause inactivation of the existing nitrogenase as in Azotobacter (Shah et al., 1972), Klebsiella (Tubb and Postgate, 1973) and photosynthetic bacteria (Zumft and Castillo, 1978).

However, in Anabaena, nitrogenase is inactivated by excess $NH_4^+$ by a mechanism that does not require protein synthesis and $NH_4^+$ does not inactivate nitrogenase in cell-free extracts (Thomas and Tuli, 1980). The effect of different concentrations of $NO_3^-$ nitrogen on preformed nitrogenase is shown in Fig. 6. With 1 mM $KNO_3$, there was no inhibition of the activity upto 7 hrs, after which the activity decreased only by 22% at 48 hrs. But addition of 1 mM $NH_4^-$ nitrogen (see Figs. 7 and 8) has resulted in a decrease in nitrogenase activity over a 30 hr period and reached 11% (with $NH_3NO_3$ and 22% with $NH_4Cl_2$) of that obtained with $N_2$- grown cells. When $NH_4^+$ nitrogen was removed from the growth medium (results not shown) nitrogenase activity returned to its original level within 24 hrs. Thus it is clear that the effects of $NO_3^-$ and $NH_4^+$ -N on the preformed nitrogenase are quite different and that the inhibitory effect of $NO_3$-N on nitrogenase synthesis is less than that of $NH_4$-N.

Urea, the commonly used nitrogenous fertilizer inhibits nitrogenase activity in all the three concentrations used (see Fig. 9 and Table 9). Only 23%, 12% and 6% activity remained after 48 hrs of exposure at 1,2 and 3 mM urea respectively. The mode of inhibition of urea is similar to $NH_4$ because urea is broken down to $NH_4^+$. In fields, in situ ARA measurements confirm the inhibitory effect of N fertilizers on diazotrophic cyanobacteria. Venkataraman (1979), however, reported that ARA was not depressed in soil-rice BGA system with less than 40 ppm ammonium-N in stagnant paddy water. This inhibition is further influenced by the nature and the quantity of fertilizers as well as mode of fertilizer application. Surface application of NPK generally results in a profuse growth of algae which may cause seedlings to lodge. To prevent such a growth, basal dressing of fertilizers is practiced in Senegal (Roger and Reynaud 1979). Similarly, deep placement of urea supergranules not only prevents dense green algae growth, observed with surface broadcast of urea but also does not inhibit diazotrophic cyanobacterial growth and nitrogen fixation (Roger et al., 1980). In stagnant paddy water, within mixed algal masses, combined N may diffuse at a slower rate than the readily available dissolved $N_2$ and a local depletion in combined N may favour diazotrophic cyanobacteria. Thus in the fields the lack of competitiveness of $N_2$- fixing cyanobacteria in the presence of mineral nitrogen may not be as clear cut as it was first thought (Roger and Reynaud, 1979). Genetic engineering of diazotrophic strains that can fix nitrogen even in presence of high levels of exogenous combined nitrogen will be agronomically useful, as it will facilitate the combined use of biological and chemical sources. Similarly, induction of mutations derepressed for $N_2$ fixation will

cause the strains to leak out ammonia into the surrounding medium instead of the ammonia getting into the metabolic pool and subsequently mineralized during autolysis.

Arora (1972) reported that additions of phosphates in either soluble ($KH_2PO_4$) or insoluble ($Ca_3(PO_4)_2$) form stimulates the $N_2$-fixation of Anabaena and Tolypothrix in phosphate sets containing basic slag than in unphosphated sets. Similarly, in paddy soils, P-supplying manure enhances algal growth and ARA (Srinivasan, 1978). Okuda and Yamaguchi (1952) incubated 117 submerged soils in a green house and noted that cyanobacterial growth seemed to be closely related to the available P content of the soil; algal growth was poor in moist soils at 0 to 5 ppm, but vigorous above 6 ppm. The population of cyanobacteria, Azotobacter and Clostridum butyricum were also reported to show a tendency to correspond to total and available P in the plow-sole (Araragi, et al., 1978).

In the present investigation also addition of 1 mM phosphate stimulates nitrogen fixation to a very great extent although a small degree of inhibition to the extent of 23% in 10 mM concentration was observed (Fig. 10 and Table 10). This confirms the earlier findings that when other external factors are not limiting phosphorous increases the nitrogen fixation. Cyanobacteria are known to accumulate more phosphorous than they require and store the excess as polyphosphate, which can be used under P-deficient conditions (Roger and Reynaud, 1979) further the intracellular phosphorous pools widely fluctuates depending on whether or not the cyanobacteria are growing under P limited conditions.

In paddy fields the growth and activity of diazotrophic cyanobacteria is most commonly limited by low pH and P deficiency. Application of P together with lime has frequently produced positive results (Ishizawa et al., 1975).

Pesticides depending on their nature and concentration, and the cyanobacterial strain could have inhibitory, selective or even stimulatory effects. Experiments mainly with flask cultures suggest that cyanobacteria are generally more resistant to pesticides than other algae and are capable of tolerating pesticide levels recommended for field application (Venkataraman, 1972). In the present investigation it was found that, in general, pesticides have an initial depressive effect on the nitrogenase activity followed by either an increase or decrease in activity. Fungicide, ceresan at the recommended field dose of 5 ppm caused an initial depression in the activity but it regained 72% to its original activity (see Fig. 11 and Table 11). Even in twice this field dose concentration the cyanobacteria regained 71% of the activity in 55 hrs. In growth experiments it was found that 9 out of 10 strains of Anabaena tolerated 100 ppm of ceresan, only 1 strain was inhibited by concentration higher than 1 ppm (Venkataraman and Rajyalakshmi, 1971b) Fungicide, Dithane M45 at the field dose concentration did not cause any reduction in acetylene reduction activity until 2 hrs but later there was a gradual decrease in activity until activity reached to 58% after 55 hrs (see Fig. 12 and Table 12). Similar trend was observed for other concentration also. Little is known about the biochemical interaction between Dithane M45 and cyanobacteria in field conditions.

It has been reported that at recommended field doses 2,4-D and MCPA inhibited $N_2$-fixation of Nostoc muscorum, Nostoc punctiforme and Cylindrospermum (Inger, 1970). However, in the present studies at the recommended field dose concentration of 2,4-D, Anabaena variabilis regained 88% of its activity in 55 hrs after a slight initial reduction. Only twice the field dose depressed nitrogenase activity severely to the extent of 77% after 55 hrs (see Fig. 13 and Table 13).

With the herbicide Machete (Fig. 14 and Table 14) after a slight initial decline in nitrogenase activity the activity rapidly increased to the same level as that of control at the recommended field dose. Slight stimulation in activity was noted at a concentration twice the field dose. This may be due to the utilisation of pesticide or their intermediary degradable products as carbon source.

Fig. 15 and Table 15 show the effect of Diuron on nitrogenase activity. There was a initial steep decline in all the four concentrations and the recovery of nitrogenase activity was also not high. Even at the recommended field dose only 57% of the original activity was present after 55 hrs. Clearly Diuron inhibited nitrogen fixing activity of Anabaena variabilis.

Approximately there was only 15% reduction with the herbicide Dalapon in all the four concentrations (Fig. 16 and Table 16). The initial inhibition was followed by gradual increase in nitrogen fixing activity. Even in twice the field dose concentration the initial inhibition was to the extent of 46% and the cyanobacteria regained 85% of its original activity in 55 hrs. Dalapon was found to interfere with glucose utilisation and it was reported that for Dalapon, the

most sensitive metabolic site of inhibition is RNA synthesis (Hill 1978). However, in the present case, at the recommended field dose and at twice the field dose concentration the RNA synthesis is not affected as the cyanobacteria regained most of the nitrogenase activity.

Among the different groups of pesticides, insecticides have been shown to have stimulatory effect on algal growth. This is primarily an indirect effect due to a decreasing population of algal grazers. It has been observed that application of gamma BHC for the control of rice stem borer caused the development of a dense algal bloom at the surface of the flood water (Raghu and MacRae, 1967) in spite of the fact that BHC was observed to be more toxic to the algal than other insecticides (Singh, 1973). At the recommended field doses two insecticides BHC and Phorate used in the present investigation reduced the nitrogenase activity by 39% and 24% respectively after 55 hrs. At lower concentrations higher activity was observed. At twice the field dose concentration, however there was drastic reduction to the extent of 60% in BHC and Phorate. The toxicity caused by BHC is partially neutralized by the cyanobacteria within 20 hrs as seen in the Fig. 17. This is in accordance with the earlier observation that by repeatedly growth and removing cyanobacteria from a BHC containing medium, there was a gradual loss in the toxicity of the pesticide, suggesting detoxication by the cyanobacteria (Das and Singh, 1977). Effect of pesticides on nitrogen fixing activity has been generally studied with flask cultures including the present investigation. When extrapolating such results to field conditions, caution must be exercised because of the following reasons.

(1) The rate of degradation of pesticides in the field is likely to be more rapid than in flask experiments.

(2) In the field, toxicity also depends on the initial microbial population, the nutrient status (Das and Singh, 1977; Singh, 1978) and the mode of application of the pesticides.

(3) In the soil, many ecological factors will affect the ability of the pesticide to interact with the algae, like the biological degradation of the pesticide by other members of the soil microflora, non-biological degradation, leaching, volatization and/or absorption to the soil. However, consensus of opinion suggests that most of the diazotrophic cyanobacteria are capable of tolerating pesticide levels recommended for field application and their nitrogen fixing ability remains unaffected to a large extent in field conditions depending on several other factors.

The optimal temperature for the growth and nitrogen fixation of cyanobacteria is about 30-35$^{o}$C and is higher than that of other eukaryotic algae. Temperature extremes inhibiting their growth are beyond the range within which rice grows; thus temperature is rarely a limiting factor for diazotrophic cyanobacteria in paddy fields (Roger and Reynaud, 1979). Temperature influences both algal biomass composition and productivity. Low temperature decreases cyanobacterial productivity and favour eukaryotic algae. In the present studies, low temperature of 5$^{o}$C cause an immediate drastic reduction in acetylene reduction activity to the extent of 97% within 2 hrs (see Fig. 3 and Table 3). Low temperature decreases the overall metabolic rate of cyanobacteria which may result in very low nitrogenase activity. In India, both field and pot experiments indicated growth reduction of diazotrophic cyanobacteria during the cold season

(Subramanyan et al., 1965). It is interesting to note that in arctic, lichen Solorina dinitrogen fixation has been recorded at -5°C (Kallio et al., 1977). Temperature requirement for nitrogen fixation seems to vary from species to species. In the present form, Anabaena variabilis, there is increase in nitrogenase activity as the temperature increases until 30°C (see Fig. 3). Temperature 25°C and 30°C are optimal for this form and supports continuous high nitrogenase activity However, at 35°C there is a 57% reduction in activity within 2 hrs and only 17% activity remained after 52 hrs (Fig. 3). This is surprising because for many tropical species, acetylene reduction activity is optimum between 30-35°C. However a Nostoc sp. isolated from the algal crust on a sandy soil in Senegal exhibited significant ARA at 65°C (Roger & Reynaud, 1977) Temperatures more than 35°C reduces daytime $CO_2$ assimilation which in turn may decrease the nitrogenase activity due to the lack of reductant and ATP. But forms like Aulosira fertilissima in Indian paddy water whose temperature varied between 34-39°C showed maximum growth and nitrogen fixing activity (Singh, 1976). It is quite clear that temperature requirements for optimum nitrogen fixation varied with species. In natural ecosystems high temperatures are frequently associated with high light intensities. It is important to segregate the effect of these two factors in ecological studies, especially ARA measurements where a "green house" effect may occur. In the present investigation since the experiments were carried out in an illuminated BOD incubator with constant light intensity this problem was overcome and nitrogenase activity measured directly reflected the effect of temperature only.

When the filaments of Anabaena variabilis were incubated in total darkness there was an immediate fall in the nitrogenase activity within 2 hrs (see Fig. 4 and Table 4) and only 1% activity was present after 52 hrs. This is largely because of the exhaustion of the photosynthetically generated energy reserves. In the present alga as the light intensity increased there was corresponding increase in the nitrogenase activity until 4000 Lux (see Fig. 4). It is clear that the requirement of ATP for nitrogenase activity is derived mainly from cyclic photophosphorylation in light. It has been found in Anabaena cylindrica that if adequate C-reserves are available, cyclic photophosphorylation can meet the whole ATP demand in the light (Benemann and Weare, 1974) and oxidative phosphorylation can do so in the dark (supporting about 50%) of the light rate of nitrogenase activity (Fay, 1976). However, in the present alga, Anabaena variabilis, incubation of the filaments in dark immediately inhibits 95% of the activity within 2 hrs suggesting that the supply of ATP by oxidative phosphorylation may be poor in this organism. Further the ability to store excess photosynthates may be the limiting factor in darkness. Another important reason for low nitrogenase activity in dark could be the reductant supply. Under aerobic conditions, $N_2$ fixation is believed to be confined to the heterocyst, since it lacks the Mn-containing component of the $O_2$-evolving system of photosystem II (Tel-Or and Stewart, 1977) and is unable to directly reduce ferredoxin with water derived electrons. Further, heterocysts lack ribulose-1-6-bisphosphate carboxylase and consequently are largely incapable of $CO_2$ fixation. Therefore, the reductant derived from C-metabolism must be provided by C-compound(s) imported from the vegetative cells. Since in dark, there is reduction of transport of C-compounds from vegetative cell there is a decrease in nitrogenase

activity for want of the reductant. Thus lack of ATP and reductant in dark is the key reason for low rates of nitrogenase activity. The nature of the carbon compound imported from vegetative cell into the heterocyst is somewhat uncertain. Of a variety of sugars tested (see Fig. 19 and Table 19), only erythrose supported 100% activity in dark-treated intact filaments. Sucrose supported 31%, glucose 21% and furctose 20% of the activity. Similarly, in isolated heterocysts also erythrose supported approximately 50% of the activity of the intact filaments. Glucose 6-phosphate supported 43% of the activity and sucrose supported 30% and glucose 20%. This is in accordance with the earlier report that D-erythrose supports high rates of nitrogen fixation in Anabaena sp. strain 7120 whole filaments and isolated heterocysts (Privalle, 1984, Privalle & Burris, 1984). After a short incubation of whole filaments with $^{14}CO_2$, heterocyst accumulated labelled quantities of alanine, suggesting that transport of alanine, glutamate and sugars into heterocyst occurs. (Juttner, 1983). If the imported alanine is oxidized in the heterocysts then several reducing equivalents would be produced offering a possible explanation for high activities of alanine dehydrogenase. Other possible reductant generating enzymatic pathways have been reported to be present in the heterocyst including glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase whose activities in the heterocyst fraction are five to ten times than of vegetative cells (Apte et al., 1978). This could be the reason for glucose-6-phosphate supporting 43% of the activity in isolated heterocyst in the present form.

From an ecological point, cyanobacteria can be regarded as low light species as they are sensitive to high light intensities (Roger and Reynaud, 1979). Nitrogenase activity in Anabaena variabilis is also sensitive to high light intensity. At 10,000 Lux there was a steep fall in nitrogenase activity immediately, and only 1% activity was present after 52 hrs. A positive correlation was observed between light intensity and nitrogenase activity from 500 Lux to 4000 Lux (see Table 4 and Fig. 4). 3000 and 4000 Lux supported maximum nitrogenase activity in the present form under laboratory conditions. In cultivated soils, the screening effect of a growing crop canopy appears to cause a rapid decrease of light reaching the algae. Thus the canopy of transplanted rice decreased light by 50% when plants were 15 days old, 85% after one month and 95% after two months. In Senegal, diatoms and unicellular green algae developed first and cyanobacteria developed later when the plant cover was dense enough to protect them from excessive light intensities, higher than 80 klux at 1300 hrs. The diazotrophic cyanobacterial biomass and density of plant cover were positively correlated (Roger & Reynaud, 1976). However, some strains of cyanobacteria are more resistant to high light intensities. Cylindrospermum sp. developed large biomasses in a harvested paddy field in Mali where light intensity was higher than 100 klux at 1300 hrs (Traore et al., 1978). As a future prospect it will be highly beneficial if diazotrophic cyanobacteria are engineered for enhanced photosynthesis and greater ability to grow in high light intensities and to store the excess of photosynthate which could then support diazotrophy in light as well as in dark.

## VI. SUMMARY

The effect of various environmental factors like pH, combined nitrogen, phosphorous, fungicides, herbicides, insecticides, temperature and light on the nitrogenase activity of the whole cells of a heterocystous cyanobacteria Anabaena variabilis was examined.

Neutral to slightly alkaline pH supported maximum nitrogenase activity. Acidic pH drastically reduced the nitrogenase activity.

All the combined nitrogen sources viz., $KNO_3$, $NH_4NO_3$, $NH_4Cl$ and urea examined, inhibited nitrogenase activity to varying degrees. As the concentration increased, there was proportionate decrease in the nitrogenase activity. The inhibition caused by $NO_3^-$-N was less as compared with $NH_4^+$-N. The possible regulatory role of $NH_4^+$ has been discussed. There was no recovery of the depressed nitrogenase activity during the course of experimental period.

Phosphate greatly stimulated the nitrogenase activity. The role of phosphorous in increasing the growth and nitrogen fixation of cyanobacteria in natural ecosystems is discussed.

In general, pesticides, depending on their nature and concentration have an initial depressive effect on the nitrogenase activity followed by either increase or decrease in activity. Of the two fungicides examined, ceresan inhibited the activity to a lesser extent than Dithane M45.

With all the four herbicides studied viz., 2,4-D, machete, diuron and dalapon, there was an increase in activity after an initial depressive effect. At the recommended field doses, the degree of

inhibition was greater in diuron, followed by dalapon and 2,4-D. There was total recovery from the initial depressive effect on nitrogenase activity by machete.

Both the insecticides, BHC and phorate inhibited nitrogenase activity at the recommended field doses, the former more than the later. Temperature 25°C and 30°C supported maximum nitrogenase activity. Low temperatures (5°C) inhibited nitrogenase activity completely.

Light intensity of 3000 and 4000 Lux supported maximum nitrogenase activity. Total darkness inhibited nitrogenase activity completely. The possible reasons for this drastic reduction in darkness has been discussed. Similarly, high light intensity inhibits nitrogenase activity drastically. The ecological importance of light with reference to diazotrophic cyanobacteria is discussed.

Of a variety of sugars examined, erythrose supported high rates of nitrogen fixation by dark-treated intact filaments and isolated heterocyst.

In conclusion, low light intensity, moderate temperatures, neutral to alkaline pH, high availability of phosphorous and the use of pesticides at the recommended field dose have been found to favour cyanobacterial nitrogen fixation.

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