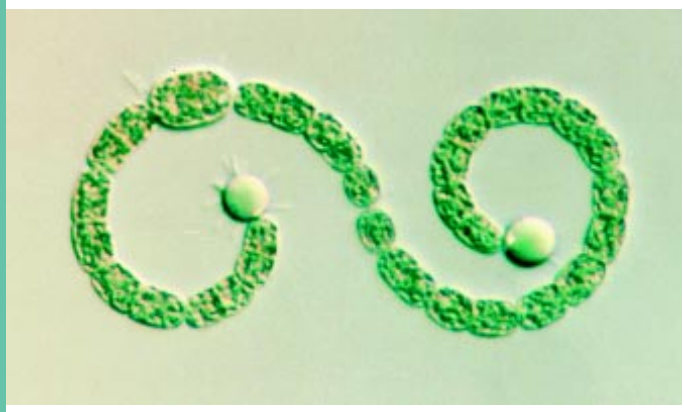
# Cyanobacterial Nitrogen Fixation (CYANOFIX)

**An ESF scientific programme**



*Anabaenopsis* sp. with terminal heterocysts. © Claudio Sili, CSMA-CNR.

The European Science Foundation acts as a catalyst for the development of science by bringing together leading scientists and funding agencies to debate, plan and implement pan-European initiatives.

**T***his ESF programme builds on the long-standing European research tradition in cyanobacterial nitrogen fixation. It is, however, the first coordinated European interdisciplinary programme in the field. The aim is to bring together researchers operating in a wide range of disciplines, from ecological to molecular and practical applications. This will allow gaps in knowledge to be bridged, synergism to be generated and scientific productivity to be thereby enhanced. Mobility of researchers among the various laboratories via fellowships and travel grants is therefore important, as are other activities such as training summer schools and topical workshops, all of which will be supported by CYANOFIX. At the end of the programme, one summarising symposium will be arranged.*

*After water, it is the availability of nitrogen that limits crop productivity. Consequently the developed world commits a large portion of its energy budget to the production of nitrogen-based fertilisers. This underpins the impressive efficiency of modern intensive agriculture. However, overuse of nitrogenous fertiliser has caused problems such as the pollution of drinking and recreational water supplies. Paradoxically, nitrogen is abundant in the Earth's atmosphere (78%; 3.9 x $10^{15}$ tonnes), but very few organisms, all of them prokaryotes, are able to make use of atmospheric nitrogen. These organisms are termed nitrogen-fixers or diazotrophs. Nonetheless, some diazotrophs have been exploited in agriculture since classical times.*

*Many strains of cyanobacteria can fix $N_2$ and these organisms make a major contribution to the global nitrogen cycle. Cyanobacteria generate all of their cellular carbon and nitrogen from atmospheric sources: they literally live on fresh air.*

*Cyanobacteria possess unique mechanisms for the protection of nitrogenase, the nitrogen-fixing enzyme, against $O_2$ and for nitrogen control of the expression of the $N_2$-fixing machinery and form symbioses with widely different organisms, and so have an important role in modern $N_2$ fixation research.*

## The ESF programme

**C**yanobacterial $N_2$ fixation has been exploited for fertilising purposes in agriculture since antiquity. Several diazotrophic cyanobacteria also form symbiotic associations with higher plants, and references to the use in rice culture of one such symbiosis, the water fern *Azolla*, go back 2000 years in the Chinese literature.

In Europe, there is also a long-standing tradition in the field of cyanobacterial $N_2$ fixation that goes back more than 100 years. And our aim now is to build on this and establish the first coordinated European inter-disciplinary programme on $N_2$-fixing cyanobacteria.

Cyanobacterial $N_2$ fixation is a field that attracts researchers from many disciplines, ranging from chemistry and chemical engineering, through biochemistry, physiology, genetics and ecology, to agronomy. Over 30 leading scientists from 11 different countries expressed a wish for their teams to be included in this programme and areas have been identified that are particularly suited to collaborative research. We predict that pooling the research investment currently being made by individual laboratories in Europe will have a synergistic effect on research progress. The various groups complement each other, bringing together key-laboratories and expertise in many different symbiotic and free-living model systems. Among the cyanobacterial-plant symbioses studied, expertise covers host plants from all major plant groups whilst groups that study free-living cyanobacteria cover all types of $N_2$-fixers. The groups also study different aspects of the progress of $N_2$ fixation: regulation, diversity, signal transduction and the involvement of specific genes and proteins in $N_2$ fixation.

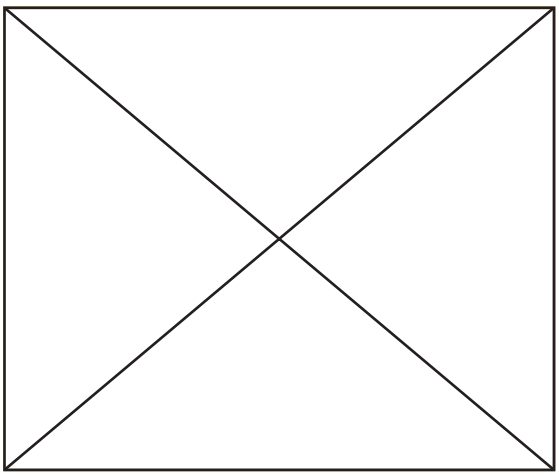
The five-year ESF programme (1998-2002) will focus on promoting mobility and transfer of knowledge among the European laboratories. To achieve these goals, a steering committee has been set up that has decided a series of actions that include short- and long-term travel grants/fellowships, three training summer schools, two workshops and one symposium.

## Aims and objectives

**T**he objectives of the collaborative research may be summarised under the following four headings:

**Physiology and molecular biology of cyanobacterial $N_2$ fixation.**

The interrelations between $N_2$ fixation and $O_2$ have fascinated



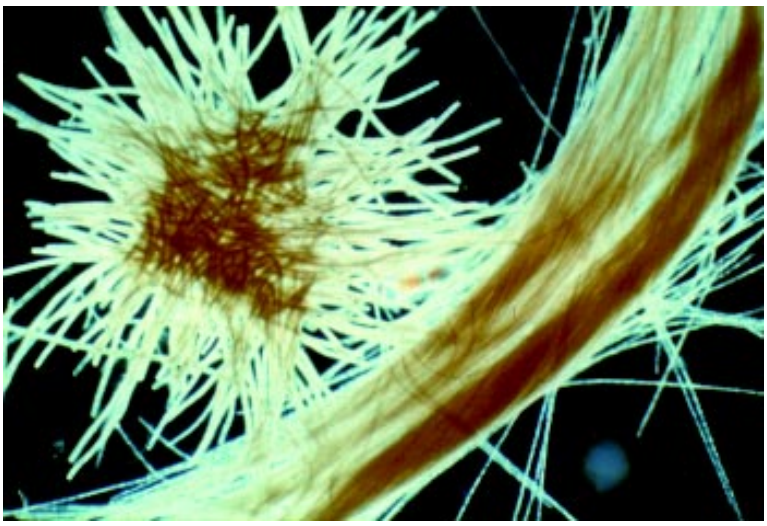
*Anabaena cylindrica*.
© Claudio Sili, CSMA-CNR.

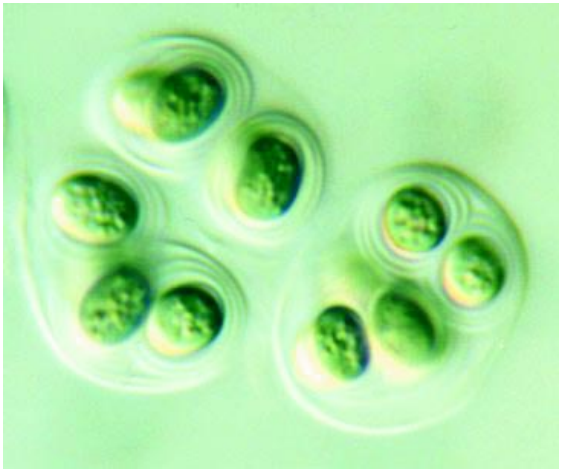
scientists ever since it was discovered that nitrogenase was inactivated by exposure to $O_2$. Since diazotrophic cyanobacteria have the ability simultaneously to generate $O_2$ and fix $N_2$, the mechanisms that $N_2$-fixers use in order to protect nitrogenase from $O_2$ may be particularly well-developed in cyanobacteria.

*Heterocystous cyanobacteria* effect a spatial separation between oxygenic photosynthesis (in vegetative cells) and $N_2$ fixation (in the non-photosynthetic heterocysts). Upon nitrogen starvation, some filamentous cyanobacteria start a programme of differentiation that leads to the formation of heterocysts. These cells appear at semi-regular intervals along the filaments and are the sites of $N_2$ fixation. Heterocysts protect nitrogenase from inactivation by $O_2$ by several mechanisms, including a high rate of respiration and decreased permeability to $O_2$. Heterocysts do not evolve $O_2$ and cannot fix $CO_2$, so they rely on adjacent vegetative cells for a source of carbon. Studies on heterocyst development are being greatly facilitated by recent advances in the genetics of *Anabaena.* Genes involved in heterocyst development can now be tagged by transposon mutagenesis. By characterising mutants physiologically, by sequence analyses of tagged genes, and by biochemical analyses of the gene products, the mechanisms that regulate heterocyst differentiation will be clarified.

*Non-heterocystous cyanobacteria* effect a temporal separation between $N_2$ fixation and photosynthetic $O_2$ production. Typically, they fix $N_2$ in the dark, and photosynthesise in the light. However, it is now emerging that different non-heterocystous cyanobacteria achieve this in different ways. In some, the nitrogenase proteins are turned over and can be detected only when cultures are fixing $N_2$ whilst, in others, nitrogenase persists, with activity coinciding with a transient activation of the Fe-protein of this enzyme. Similarly, in some, the pattern of $N_2$ fixation is endogenous in that, once established under alternating light and darkness, it persists under continuous illumination: it may even be confined to a specific phase in the cell cycle. The marine cyanobacterium *Trichodesmium* fixes $N_2$ during the light phase despite lacking heterocysts but it seems to confine nitrogenase to a subset of cells of its filaments. It is not clear whether or not these cells produce $O_2$, so a detailed examination of $N_2$-fixing cells is needed. The precise mechanism(s) should be amenable to investigation with mutants when proper genetics have been developed.

The colony forming marine cyanobacterium *Trichodesmium*.
© LM micrograph by P. Lundgren, Department of Botany, Stockholm University.





*Gloeothece membranacea*, a unicellular cyanobacterium that photosynthesises during the day and fixes nitrogen at night.
© Claudio Sili, CSMA-CNR.

*Nitrogenase* consists of two proteins, a MoFe-protein and an Fe-protein. In a few other diazotrophs, the Fe-protein of nitrogenase undergoes reversible covalent modification (attachment of ADP-ribose) rendering the Fe-protein inactive. Covalent modification (inactivation) of nitrogenase is stimulated by ammonium or sources, by transfer of cultures to the dark or exposure to $O_2$. In a number of cyanobacteria the Fe-protein can also be resolved into two components, but there is no evidence as yet that it can be covalently modified. Further investigation into this phenomenon will therefore be worthwhile.

*The fate of the fixed nitrogen* is another challenging aspect to examine. Many $N_2$-fixing cyanobacteria release fixed nitrogen. For example, under alternating light and darkness, the unicellular cyanobacterium *Gloeothece* fixes $N_2$ in the dark, during which some of the fixed nitrogen is released into its external slime capsule. This nitrogen is re-assimilated during the following light phase and the nitrogenase activity may escape inhibition by accumulated nitrogen. In many cases, the release of amino acids is quite specific and transport systems have been described and the corresponding genes identified.

In symbiotic cyanobacteria (typically *Nostoc*), release of nitrogen to the non-$N_2$-fixing host plant is fundamental for the survival of the symbiotic association. However, the ways in which the plant manages to obtain fixed nitrogen from the $N_2$-fixing *Nostoc* are largely unknown. Identification of such mechanisms, which may open ways for biotechnological applications, is a major goal.

*Cyanophycin* is a copolymer of arginine and aspartate that constitutes a nitrogen-rich reserve material in $N_2$-fixing cyanobacteria. Recent progress on the enzymology of cyanophycin biosynthesis should be accompanied by an understanding of the turnover dynamics of cyanophycin in relation to the available nitrogen source. Further investigation on this topic is of much interest.

*The genetic regulation of nitrogen fixation* in cyanobacteria differs from that in other $N_2$-fixers. The presence of a source of combined nitrogen like nitrate or ammonium in the environment generally prevents cyanobacteria from fixing $N_2$. This behaviour makes bioenergetic sense, since assimilation of reduced forms of nitrogen is less expensive. Cyanobacteria fail to synthesise nitrogenase when grown in the presence of nitrate or ammonium, and it is clear that the genetic regulation of nitrogen metabolism in cyanobacteria is different. For example, expression of nitrogen assimilation genes – including genes involved in heterocyst differentiation and ammonia assimilation (e.g. *het*R and *gln*A) – is regulated by the recently discovered NtcA transcriptional regulator. Important aspects to study in relation to NtcA function are: a) how is NtcA activity modulated in response to the nitrogen status of the cell; b) which is the

earliest heterocyst differentiation gene that responds to NtcA; c) is NtcA function important for regulation of $N_2$ fixation in non-heterocystous and symbiotic cyanobacteria? The PII protein (encoded by *glnB*), involved in the co-ordination of C- and N-assimilation, has been characterised in some $N_2$-fixing cyanobacteria. The interesting hypothesis that PII may be involved in nitrogen signalling in cyanobacteria can therefore be raised. However, as PII can directly bind molecules relevant to conditions of nitrogen stress, it may not only act as a signal transducer but also as a sensor.

*Other stress responses* may be elicited by exposure of cyanobacteria to altered light, temperatures, desiccation (osmotic stress) and nutrient starvation. Synthesis of stress proteins, such as molecular chaperones, is already being studied in some laboratories, others study the effects of stresses on $N_2$ fixation under natural conditions (light, temperature, salinity, nutrients etc). In addition, many of the responses that cyanobacteria show to environmental stress are independent of the nature of the stress itself, though some responses are specific. The extent to which stress responses are interlocked is currently a fascinating area and one in which co-ordinated efforts will be valuable.

#### Genetics of cyanobacteria

One curious paradox concerning $N_2$-fixing cyanobacteria is that, whilst our understanding of the molecular genetics of some free-living heterocystous strains (notably *Anabaena*) has now outstripped our knowledge of their physiology and biochemistry, little is known about the genetics of other cyanobacteria. We therefore need to expand our understanding of the genetics of some hitherto rather neglected free-living and symbiotic organisms, (e.g. *Gloethece, Trichodesmium, Nostoc and Calothrix*) some of which have been resistant to isolation of DNA and to genetic transformation. Development of an appropriate genetic methodology in this type of cyanobacteria represents a high research priority in the programme.

Recent evidence suggests that some heterocystous cyanobacteria contains two molybdenum-based nitrogenase systems, *nif1* and *nif2*, with different expression patterns. The vegetative cell nitrogenase (Nif2) has its counterpart in non-heterocystous cyanobacteria that fix $N_2$ only anaerobically. Furthermore, some cyanobacteria may produce Mo-independent 'alternative' (V or Fe) nitrogenases, encoded by the *vnf* and *anf* genes. This is a topic amenable to combining genetic work with biochemical and ecological studies.

#### Symbiotic cyanobacteria

Symbiotically competent cyanobacteria have some excellent features that make them particularly significant in any attempt to extend the list of $N_2$-fixing symbioses to include plants of commercial interest, such as cereals. Unlike rhizobia (the symbionts of legume plants), most symbiotic cyanobacteria carry their own mechanism for protecting nitrogenase from inactivation by oxygen (heterocysts). They have an unmatched host range (fungi to angiosperms), are not restricted to roots but may form symbiosis with various plant parts, and do not need to be located intracellularly within the host plant. However, before we can establish new $N_2$-fixing symbioses we may need to expand our knowledge of those that already exist. It is becoming increasingly apparent that the symbionts communicate using chemical signals and during the establishment of a functional $N_2$-fixing cyanobacterial

Coralloid root of *Cycas revoluta;* cyanobiont zone is visible in the sectioned roots. © Maria Cristina Margheri, CSMA-CNR.

The bright red stem glands on *Gunnera* through which the cyanobacterium *Nostoc* enters when establishing the *Nostoc-Gunnera* symbiosis. © C. Johansson, Department of Botany, Stockholm University.

symbiosis, it is likely that genes are induced in both partners, the products of which would then assist in the establishment of the symbiosis. We will study the nature of such genes and their products as well as communication between the symbionts throughout symbiosis. Another aspect of vital biotechnological importance to study, is the transfer of fixed nitrogen to the host plant and the reciprocal flow of carbon to the cyanobacterium.

### Natural communities of $N_2$-fixing cyanobacteria

$N_2$-fixing cyanobacteria can be subdivided into three groups: a) filamentous cyanobacteria with heterocysts; b) non-heterocystous filamentous or unicellular cyanobacteria, capable of $N_2$ fixation under fully aerobic conditions; and c) non-heterocystous filamentous or unicellular cyanobacteria that induce nitrogenase activity only anaerobically. Representatives of all of these groups (free-living and symbiotic) are found in a wide range of environments ranging from terrestrial to aquatic environments, the latter including limnic (lakes and streams), brackish (e.g. the Baltic Sea) and fully marine habitats (oceans), and cyanobacteria may be found from cold polar regions (e.g. Svalbard) to the warmer Mediterranean and tropical areas. Heterocystous cyanobacteria are clearly best adapted for diazotrophic growth, but cyanobacteria in group b) also manage well without heterocysts. However, cyanobacteria in group c), constituting 30-40% of all non-heterocystous cyanobacteria, lack one or more $O_2$ protective mechanisms.

*Sulfureta* are environments characterised by high concentrations of sulphide and extended periods of anoxia. Some anaerobic $N_2$-fixing cyanobacteria occur in such environments. Sulphide is an inhibitor of oxygenic photosynthesis and, at high concentrations, cannot co-exist with $O_2$. In some systems, cyanobacteria therefore perform anoxygenic photosynthesis whilst, in others, photosynthesis is temporarily switched off by sulphide, thereby allowing $N_2$ fixation to flourish. Evidence has also been obtained for a spatial separation of oxygenic photosynthesis and $N_2$ fixation within lamined benthic mats. Either fixed nitrogen is transported or organisms move through the system, changing from $N_2$ fixation to photosynthesis.

*Microbial mats* are typically built by non-heterocystous cyanobacteria (e.g. *Oscillatoria*, *Lyngbya, Symploca*) though heterocystous species may occasionally dominate. Many mats are strongly diffusion limited and accumulate photosynthetic $O_2$ during the day, while at night the system turns anaerobic, probably permitting $N_2$ fixation by some non-heterocystous strains. Much more work is needed to understand such phenomena in natural systems.

Planktonic cyanobacteria are common in lakes as well as in open oceans and in the Baltic Sea. Recent calculations indicate that marine $N_2$ fixation is in fact of major global importance. Interestingly, the major $N_2$-fixing cyanobacterium in oceans is an

aerobic, non-heterocystous species, *Trichodesmium*. In contrast, freshwater blooms of diazotrophic cyanobacteria consist of heterocystous species (e.g. *Anabaena*), and these are also dominant in brackish systems such as the Baltic Sea with blooms of *Nodularia* and *Aphanizomenon*. Heterocystous species are also found as symbionts in marine plankton (diatoms), so the absence of free-living representatives in the open ocean remains a mystery. There is a great need to fully understand the behaviour of these bloom forming cyanobacteria in their natural environment.

In combination with classical measurements of $N_2$ fixation there is also a need to study *nif* gene expression and the occurrence of nitrogenase in natural systems. The regulation of nitrogenase activity by environmental factors (e.g. N, P, Fe) and the transport of fixed nitrogen within the ecosystem, are other important areas to be addressed. Also, effects on $N_2$ fixation of changes in climate and in anthropogenic activities are of immediate interest, also in a European perspective. Cyanobacteria growing in terrestrial locations (including culturally important archaeological sites) and in streams and rivers need to be studied. The latter are particularly interesting because they differ from their counterparts in lakes by being

*Calothrix*. © Arnaud Taton

perennials. There is also an urgent need to identify the ecological niches that are occupied by the different $N_2$-fixing cyanobacteria.

Cyanobacterial systematics using various genetic techniques will in addition be an extremely valuable complement to studies on $N_2$ fixation. A precise taxonomic position of cyanobacteria living in different environments will allow comparisons of data collected in different ecological niches and cultivated in different laboratories. This is also required in order to evaluate the diversity of the $N_2$-fixing machinery in cyanobacteria. A knowledge of the biodiversity of symbiotic cyanobacteria in relation to host species, habitat and geographical location will contribute to an understanding of the specificity of cyanobacterial symbioses and will form a valuable basis for the future elaboration of artificial symbioses.

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