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Physiological ecology of cyanobacteria in microbial mats and other communities

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CONTENTS

SUMMARY

In this review some aspects of the physiological ecology of cyanobacteria are discussed by taking a microbial mat as an example. The majority of microbial mats are built and dominated by cyanobacteria which are primary producers at the basis of the microbial foodweb in microbial mats. These micro-scale ecosystems are characterized by steep and fluctuating physico-chemical gradients of which those of light, oxygen and sulphide are the most conspicuous. Light is strongly attenuated in the sediment, and owing to constant sedimentation, the mat-forming cyanobacteria have to move upwards towards the light. However, at the sediment surface, light intensity, particularly in the u.v. part of the spectrum, is often deleterious. The gliding movement of the cyanobacteria, with photo- and chemotaxis, allows the organism to position itself in a thin layer at optimal conditions. The organic matter produced by cyanobacterial photosynthesis is decomposed by the microbial community. Sulphate-reducing bacteria are important in the end-oxidation of the organic matter. These organisms are obligate anaerobes and produce sulphide. Gradients of sulphide and oxygen move up and down in the sediment as a response to diurnal variations of light intensity. Cyanobacteria, therefore, are sometimes exposed to large concentrations of the extremely toxic sulphide. Some species are capable of sulphide-dependent anoxygenic photosynthesis. Other cyanobacteria show increased rates of oxygenic photosynthesis in the presence of sulphide and have mechanisms to oxidize sulphide while avoiding sulphide toxicity. Iron might play an important role in this process. Under anoxic conditions in the dark, mat-forming cyanobacteria switch to fermentative metabolism. Many species are also capable of fermentative reduction of elemental sulphur to sulphide. The gradients of sulphide and oxygen are of particular importance for nitrogen fixation. Very few microbial mats are formed by heterocystous cyanobacteria, which are best adapted to diazotrophic growth. However, these organisms probably cannot tolerate greater concentrations of sulphide or anoxic conditions or both. Under such conditions non-heterocystous cyanobacteria become dominant as diazotrophs. These organisms avoid conditions of oxygen supersaturation. In the ecosystem, nitrogen fixation and photosynthesis might be separated temporally as well as

spatially. In addition, non-heterocystous diazotrophic cyanobacteria have mechanisms at the subcellular level to protect the oxygen-sensitive nitrogenase from inactivation.

Key words: Photosynthesis, microbial mat, nitrogen fixation, cyanobacteria, metabolic versatility.

1. INTRODUCTION

Cyanobacteria (blue-green algae) are photoautotrophic prokaryotic organisms. They are unique since they are the only prokaryotes that perform a plant-like oxygenic photosynthesis in which two photosystems (PS II and PS I) are connected in series. A large number of species is known, and they differ considerably in their morphology (Castenholz & Waterbury, 1989). Unicellular species can occur as suspended single cells such as picoplanktonic species (e.g. *Synechococcus* spp.), or form aggregates of tens or hundreds of cells (e.g. *Microcystis aeruginosa*). Other unicellular species form more or less structured colonies with or without a defined multi-layered polysaccharide slime capsule or sheath (e.g. *Merismopedia* spp., *Gloeothece* spp., *Gloeocapsa* spp.). A wide variety of filamentous species is known, many of which possess a sheath. For instance, *Microcoleus chthonoplastes* forms bundles of trichomes that are enclosed by a common sheath. Some filamentous species are characterized by true cell differentiation. They form heterocysts which, unlike vegetative cells, lack an oxygenic photosystem and have an extraordinarily thick cell wall. These cells are the sites of nitrogen fixation, providing the vegetative cells with combined nitrogen, and are not viable when disconnected from the trichome. Many heterocystous cyanobacteria also form a second cell type, called an akinete. Akinetes are a kind of spore which can germinate when conditions become suitable for growth. Unlike bacterial endospores, akinetes are not heat-resistant.

Many cyanobacteria show a remarkably versatile metabolism (Stal, 1991*b*). The oxygenic photoautotrophic mode allows cyanobacteria to make use of basic growth requirements that are widely available, such as light, CO_2 and H_2O as, respectively, energy source, carbon source and electron donor. In addition, many species can also use N_2 as nitrogen source, which makes them independent of the often limited supply of combined nitrogen. Cyanobacteria are known to occur in oxic as well as anoxic environments (Castenholz, 1973*a*). Several species can switch to the typical bacterial anoxygenic photosynthesis using sulphide as electron donor. Other species assimilate sugars and other organic compounds in the light (photoheterotrophy) (Rippka, 1972). In the dark, cyanobacteria gain energy by respiring endogenous carbohydrates which are accumulated in the light. However, under anoxic conditions some species are able to cover maintenance requirements by fermentation. In a few cases chemoorganotrophy has been observed in cyanobacteria. Another important property of cyanobacteria is the variety of different storage compounds they can accumulate. These include glycogen (carbon and energy), poly-β-hydroxybutyrate (function uncertain), polyphosphate (phosphorus) and cyanophycin (nitrogen) (Kromkamp, 1987). Also important is the capacity of cyanobacteria to grow under a very low water potential. Such species can either resist dessication very well and grow in arid environments such as deserts, or tolerate high salinity and grow in hypersaline ponds.

Because of their versatile metabolism and their capacity to switch rapidly from one mode to another, cyanobacteria are successful in a wide range of environments. In many environments cyanobacteria are the primary producers at the basis of the food web of ecosystems. This is the case for example in many freshwater, (hyper)saline or soda lakes; in marine waters (including the oceans); in paddy fields; as benthic organisms in coastal sediments; in soils, in deserts, on cave walls and in many other environments. Cyanobacteria are also known as symbionts in a variety of other organisms (e.g. the marine diatom *Rhizosolenia*, roots of *Cycas* and leaves of *Azolla*) (Rai, 1990). In addition to their widespread geographic distribution, cyanobacteria have probably also played a major role throughout the biological history of the Earth (Schopf & Walter, 1982). Cyanobacteria are involved presently in the formation of stromatolites and laminated biogenic rocks (Reid & Browne, 1991) but fossil stromatolites are also known from the early Precambrian, 3500 Myr ago. Micro-fossils as well as carbon isotope data from these formations suggest the involvement of cyanobacteria (Des Marais & Canfield, 1994). It is also assumed that cyanobacterial photosynthesis was responsible for the oxygenation of the atmosphere (Ehrlich, 1990). This short summary indicates the tremendous importance cyanobacteria had and still have in the functioning of ecosystems on Earth.

The variety of environments in which cyanobacteria occur and the diverse metabolic capacities of these organisms cannot possibly be described in the framework of a review like this. Therefore, I will attempt to describe one particular ecosystem which is built and dominated by cyanobacteria, namely a microbial mat, and discuss some of the important metabolic capacities of cyanobacteria that are essential for their functioning in that community. Microbial mats are systems in which environmental conditions show extreme fluctuations and in which cyanobacteria show their full metabolic and

adaptational capacities (Cohen & Rosenberg, 1989). Some other ecosystems will also be described, in order to illustrate specific points.

II. MICROBIAL MATS

1. *General description*

Microbial mats are dense benthic communities of micro-organisms (Stal & Caumette, 1994). In many mats several populations or communities of micro-organisms occur vertically stratified. Often this lamination is readily recognized by the striking differences in colours that are caused by the pigments of the contributing organisms. This phenomenon has been given the German term 'Farbstreifensandwatt' (Hoffmann, 1942) which means colour-striped sand. In contrast to this 'instantaneous biological stratification' (Monty, 1976) a historical biomineralogical stratification might also be present. Laminations produced by the latter may be attributed to different growth periods, seasonal or periodic events, as well as to episodic or erratic events. Active microbial mats bind and trap sediment particles or precipitate minerals such as calcite. Organic matter in microbial mats is often incompletely mineralized. Distinct laminae of precipitated minerals and/or organic matter are characteristic of many microbial mats. Eventually, microbial mats might give rise to consolidated rocks. Such laminated rocks are known as stromatolites (Cohen, Castenholz & Halvorson, 1984). Stromatolites dating back to 3500 Myr are known and these are considered to be the first indications of life on Earth (Schopf & Walter, 1982). To date, true stromatolites (laminated lithified microbial mats) are only known to be formed in the Exuma Cays (Bahamas) and in Shark Bay (Western Australia) (Reid & Browne, 1991). Non-stromatolithic microbial mats are much more widespread and they can be found in a variety of environments. They are known from intertidal coastal sediments, marine salterns, hypersaline ponds and lakes, thermal springs, dry and hot deserts and Antarctic lakes. Most of these microbial mats are formed by cyanobacteria (Jørgensen, Revsbech & Cohen, 1983).

Many of the environments that harbour microbial mats can be considered extreme. Here, conditions are such that only few species, usually prokaryotes, are able to live and reproduce. Cyanobacteria are metabolically versatile organisms, possibly one of the reasons why they are often encountered in environments in which extreme fluctuations of conditions are normal (Stal, 1991*b*). In order to produce the macroscopic structure which is a microbial mat, cyanobacteria must be allowed to accumulate. Cyanobacteria are probably capable of growing in most environments. However, in the vast majority of situations, grazing pressure will prevent accumulation of cyanobacteria in numbers that are conspicuous as a microbial mat.

Cyanobacteria are photoautotrophic prokaryotic organisms (Stanier & Cohen-Bazire, 1977). They use light as source of energy, water as electron donor and CO_2 as carbon source. These basic requirements are usually in ample supply. In addition, many cyanobacteria are capable of fixing nitrogen (Fay, 1992), making them independent of sources of combined nitrogen, which are often in limited supply, particularly in marine environments. The capacity for diazotrophic growth of cyanobacteria can be regarded as crucial to the development of many microbial mats (Bebout *et al.*, 1987; Villbrandt, Stal & Krumbein, 1990).

Photosynthetic CO_2 fixation by cyanobacteria is responsible for the input of organic matter in the sediment ecosystem. Fixed CO_2 becomes available for other micro-organisms via a number of different mechanisms by which organic matter is liberated. The following processes must be considered: (i) death and lysis of the cyanobacteria; (ii) glycolate excretion; (iii) excretion of fermentation products; (iv) excretion of extracellular polymeric substances (eps). These processes are discussed below in more detail.

2. *Death and lysis of cyanobacteria*

The cyanobacterial layer of a microbial mat has a limited thickness. Self-shading of the cyanobacteria will decrease the light quality and intensity and eventually photosynthesis will cease (Jørgensen & Des Marais, 1988; Kühl & Jørgensen, 1992). The absence of light will eventually kill those cyanobacteria that find themselves permanently below the euphotic layer of the sediment and have exhausted their energy storage. Although chemotrophy is possible in a number of cyanobacteria, free-living species are not likely to compete successfully with other heterotrophic micro-organisms (Smith, 1982; Anderson & McIntosh, 1991). In temperate zones, the cyanobacterial community usually dies off after the growth season and subsequently the organic matter decomposes during the winter. However, the amount of organic matter that is liberated in this way is relatively small and cannot support metabolism at the rate commonly measured in microbial mats.

3. *Glycolate excretion*

Photosynthesis will increase the oxygen concentration in the cyanobacterial layer. Although the dimensions of the euphotic zone of a microbial mat are small (in the order of millimetres) the diffusion time of oxygen might be in the order of minutes or even hours (Jørgensen, 1994). These diffusion characteristics, in combination with the rapid dynamics of oxygen production and consumption,

might result in a supersaturation of oxygen of 200–300% (Revsbech *et al.*, 1983). Photosynthetic CO_2 fixation and the resulting increase of pH will lead to a depletion of CO_2 in the mat. The combination of oxygen supersaturation and CO_2 depletion will prevent efficient photosynthetic CO_2 fixation (Lorimer, 1981; Tabita, 1988). Although cyanobacteria are known to possess very efficient mechanisms of accumulating CO_2 and are capable of uptake of bicarbonate, it is thought that these mechanisms are not sufficient to cover metabolic demands during certain periods. Cyanobacteria might then switch to photorespiration, a process during which glycolate is excreted (Renström & Bergman, 1989). Although this process has not been studied in great detail, the reports of Bateson & Ward (1988) and of Fründ & Cohen (1992) emphasize the importance of glycolate in microbial mats.

4. *Excretion of fermentation products*

The rapid oxygen dynamics in microbial mats and the relatively slow diffusion of oxygen lead to depletion when darkness stops photosynthesis. Respiration of cyanobacteria and other microorganisms, as well as chemical processes, rapidly consumes all oxygen and the mat turns anoxic, often within minutes after photosynthesis ceases (Revsbech *et al.*, 1983). Cyanobacteria that normally provide themselves with energy by aerobic degradation of their endogenous storage compound glycogen then switch to fermentation (Stal, 1991*b*). These organisms are known to excrete a variety of simple organic compounds such as acetate, ethanol and lactate. It is possible that, together with glycolate excretion, these compounds provide the major source of organic carbon in the microbial mat. Fermentation in cyanobacteria is discussed in detail later in this review.

5. *Excretion of extracellular polymeric substances*

Many cyanobacteria excrete extracellular polymeric substances that are mainly composed of polysaccharides (Decho, 1990). Two types must be distinguished. These are the so-called capsular polysaccharides (cps) and the extracellular polysaccharides (eps) (Decho, 1990; Stal, 1994). The former are structural components that form capsules and sheaths which surround the cyanobacteria. The latter are usually regarded as material which is loosely associated with the cell or is freely present in the medium, and are often also known collectively as mucilage or slime. The question must be considered whether eps and cps, being extracellular compounds, are readily available to other organisms. Little information is available about the microbial degradation of these polysaccharides but some seem to be recalcitrant. This is particularly true of cps, although they might be used as a food source by larger eukaryotic grazers (meiobenthos) (Decho, 1990). Production of cps seems to be an intrinsic property of the cyanobacterium, and the polysaccharide sheath is a structural component of the organism (Drews & Weckesser, 1982), whereas production of eps is probably the result of unbalanced growth. This is the case under nutrient limitation which in microbial mats is often nitrogen limitation. Nitrogen limitation decreases the synthesis of structural cell components such as protein but does not affect photosynthetic CO_2 fixation which is then diverted to the production of carbohydrates. Another explanation for the production of mucilage is its possible involvement in gliding motility (see Section III.1). Excretion of extracellular polymeric substances contributes to the structure and stabilization of sediments. It has been shown that eps decrease the erosion of coastal areas (Paterson, Crawford & Little, 1990).

6. *Sulphate reduction*

As argued above, the most important sources of organic matter in the microbial mat probably originate from photorespiration (in the day-time) and fermentation (during the night). In microbial mats cyanobacteria are responsible for both processes. The low-molecular-mass compounds that are excreted by cyanobacteria are excellent substrates for sulphate-reducing bacteria. In anoxic marine environments dissimilatory sulphate reduction is considered to be the most important process during decomposition of organic matter. Sulphate-reducing bacteria are considered to be obligate anaerobes. Investigations from several researchers have shown that, notwithstanding their anaerobic nature, sulphate-reducing bacteria are found throughout the mat and are particularly abundant in the upper layer, where, apparently, they live close to the cyanobacteria (Visscher, Prins & van Gemerden, 1992; Stal, 1993). In addition, the highest rates of sulphate reduction were measured in the top layers of microbial mats (Cohen, 1989).

If it is considered that cyanobacteria fuel sulphate-reducing bacteria, their occurrence close to the source is perhaps not surprising. On the other hand, the fact that sulphate-reducing bacteria are obligate anaerobes seems in contradiction with their occurrence in the layer of cyanobacteria which is oxygenated during the day. At night when the mat is usually anoxic they might decompose the fermentation products excreted by the cyanobacteria. It is possible that the sulphate-reducing bacteria avoid the periods of oxygen supersaturation by moving down into the sediment into the permanently anoxic zone. However, such behaviour has not been demonstrated. It seems more likely that sulphate-reducing bacteria in microbial mats tolerate oxygen.

Several species can tolerate considerable concentrations of oxygen for prolonged periods of time (Cypionka, Widdel & Pfennig, 1985; Abdollahi & Wimpenny, 1990); some are even capable of aerobic respiration (Dilling & Cypionka, 1990; Dannenberg *et al.*, 1992). Recent reports claim the existence of aerobic sulphate reduction (Canfield & Des Marais, 1991; Fründ & Cohen, 1992) but this process has so far not been shown in culture (Marschall, Frenzel & Cypionka, 1993; Cypionka, 1994). However, if it exists, aerobic sulphate reduction could be fuelled by glycolate (Fründ & Cohen, 1992).

7. *Sulphide oxidation*

The activity of the sulphate-reducing bacteria results in the formation of sulphide. Reduction of elemental sulphur by a variety of other micro-organisms, including cyanobacteria (see Section IV.2), also leads to the formation of sulphide in the mat. In the presence of oxygen, sulphide in the mat is oxidized chemically or biologically. The latter process is carried out by aerobic chemolithotrophic sulphur-oxidizing bacteria, and is much faster than the chemical oxidation. In microbial mats large numbers of chemolithotrophic bacteria are often found (Visscher *et al.*, 1992). Therefore a sharp interface exists between oxygen and sulphide, which usually co-exist only at very small concentrations. Some chemolithotrophic sulphide-oxidizing bacteria are capable of denitrification, an anaerobic process, but anaerobic sulphide oxidation by these organisms is not important because in microbial mats nitrate is usually not available. Under anoxic conditions in the light, anoxygenic phototrophic bacteria use sulphide as an electron donor in anoxygenic photosynthesis. Frequently, a purple layer of these bacteria can be found underneath the layer of cyanobacteria. As will be shown below, cyanobacteria are also involved in sulphide oxidation (see Section V.1). Another important reaction in microbial mats is the oxidation of sulphide by ferric iron. Ferric iron in microbial mats might be formed by chemical or biological oxidation by oxygen or by the recently discovered anoxygenic photosynthesis with ferrous iron as electron donor (Widdel *et al.*, 1993).

8. *Cyanobacterial species composition in microbial mats*

Although many different species of cyanobacteria have been found in microbial mats, most mats are dominated by one species. The type of cyanobacterium dominating a specific microbial mat might depend on several factors which are little understood.

Many microbial mats from all over the world and from very divergent environments are dominated by the cosmopolitan cyanobacterium *Microcoleus chthonoplastes*. Although some strains of this species can fix N_2, mats of *M. chthonoplastes* generally do not. *M. chthonoplastes* possesses a remarkable morphology. This filamentous species forms bundles of trichomes which are ensheathed. Sometimes several tens of trichomes are found within one common sheath. In a recent investigation by Prufert-Bebout & Garcia-Pichel (1994) it was found that different isolates of *M. chthonoplastes* from mats of diverging environments showed remarkable similarities. All isolates contained the pigment phycoerythrocyanin, which was thought to be restricted to certain groups of heterocystous cyanobacteria. All strains also contained a mycosporin-like compound which is thought to serve as a sunscreen pigment, and none of the strains showed catalase activity. It was hypothesized that this group of cyanobacteria is likely to be genetically homogeneous. An important property of *M. chthonoplastes* might be bundle formation. A bundle of trichomes enclosed by a polysaccharide sheath might provide a suitable micro-environment for the organism. For instance, the polysaccharide sheath has a high water-retaining capacity and might protect the organism from desiccation, which is frequently encountered by micro-organisms in microbial mats in different environments. Moreover, the polysaccharide sheath might also protect from other environmental stresses.

The capacity for nitrogen fixation is also an important selecting factor. Many microbial mats develop in environments in which combined nitrogen is not available. Where conditions allow, heterocystous cyanobacteria develop in mats that are nitrogen-limited. Often *Calothrix* sp. is found in full marine or hypersaline conditions, *Nodularia* is found in brackish systems and *Nostoc/Anabaena* are the typical freshwater organisms. *Scytonema* can also be found in mats of marine mangroves. Generally, heterocystous cyanobacteria are rare in marine systems. The reason for this is not clear but it has been suggested that the connection between the heterocyst and vegetative cells is weak and cannot resist the high turbulence that frequently occurs in marine waters (Howarth *et al.*, 1993).

The fact that cyanobacteria in sediment ecosystems must be highly motile in order to position themselves under optimal conditions might likewise be incompatible with the possession of labile cell-to-cell connections but experiments do not support this hypothesis (Howarth *et al.*, 1993). It has also been suggested that the high sulphate content of the marine environment excludes nitrogen-fixing organisms. It is believed that molybdate, a necessary co-factor of nitrogenase, is taken up by the same mechanism as sulphate and is competitively inhibited by it (Cole *et al.*, 1993). However, for this hypothesis also no direct evidence is available. Moreover, if such a mechanism were important, it would also affect the use of nitrate as nitrogen source,

since cyanobacterial nitrate reductase also uses molybdate as co-factor (Cole, 1988). Furthermore, nitrogen fixation is indeed important in the vast majority of microbial mats, including marine mats, in which the non-heterocystous cyanobacterium *Trichodesmium* spp. is well known as a diazotrophic organism. In most cases the filamentous non-heterocystous cyanobacteria *Oscillatoria* and *Lyngbya* occur in nitrogen-depleted marine microbial mats, and the unicellular cyanobacterium *Gloeothece* is found to form mats on walls of carbonate caves.

It is assumed that the polysaccharide sheath protects the organism against desiccation. This might be particularly important in coastal microbial mats and desert mats. Coastal microbial mats are exposed to tides which cause periodic exposure to the atmosphere, during which desiccation might occur. That desiccation occurs in desert communities is obvious. Here, such mats collect water mainly from early morning dew which might then be retained in the polysaccharide sheath.

Microbial mats are often formed in environments in which high rates of sedimentation occur. In order to cope with this sedimentation, mat-forming cyanobacteria must be motile, to allow them to move to the surface after deposition of sediment has buried them. The occurrence of microbial mats in very turbulent environments also forces cyanobacteria to attach to the surface. In other environments with lower energy, loose microbial aggregates are often found that are poorly attached to the sediment surface. Clearly, many different factors determine the success of a specific cyanobacterium. In the majority of microbial mats the factors involved are unknown.

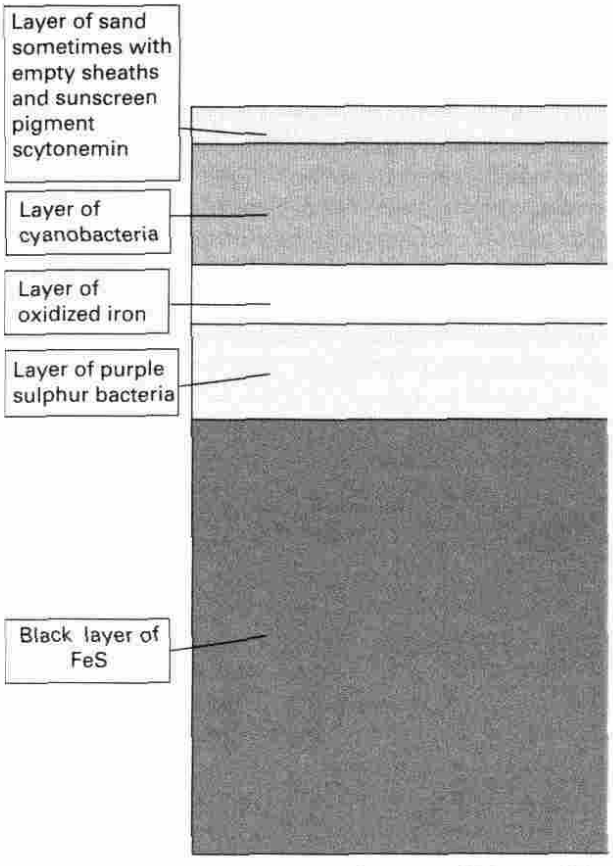

Figure 1. Schematic drawing of a microbial mat. The cyanobacteria form a layer on top of the sediment. They are positioned underneath a thin layer of sediment which often contains empty sheaths in which the sunscreen pigment scytonemin might be present. Often a layer of oxidized iron is seen below the cyanobacteria. Below the iron a layer of purple sulphur bacteria is often seen. These anaerobic bacteria perform anoxygenic photosynthesis using sulphide as electron donor. Below the purple sulphur bacteria an extensive zone of black FeS is present. For further details, see text.

9. *Structure and function of microbial mats*

The metabolic activities of cyanobacteria and of the sulphate-reducing bacteria are responsible for the steep and strongly fluctuating physico-chemical gradients that are characteristic of many microbial mats. Oxygen evolved by photosynthetic activity of the cyanobacteria accumulates during the day time and can often reach 2–3 fold supersaturation. The concomitant fixation of CO_2 results in dramatic increases of pH (which can sometimes reach values of over 10) in the euphotic layer of the mat (Revsbech *et al.*, 1983). The oxygen profile in the microbial mat follows the penetration of light, which is attenuated quickly in the mat (Jørgensen & Des Marais, 1988; Kühl, Lassen & Jørgensen, 1994). The cyanobacteria absorb particularly well those parts of the spectrum that support oxygenic photosynthesis. This has been shown by techniques using *in situ* light measurements by micro-probes (Kühl & Jørgensen, 1994). At a certain depth, light conditions are such that oxygenic photosynthesis is not possible and the sediment there is permanently anoxic. At night oxygen is consumed rapidly by the large biomass, and anoxic conditions usually prevail during most of the night. The sediment turns anoxic, often as far as the surface. During the night sulphide accumulates as the result of the activity of the sulphate-reducing bacteria.

Figure 1 shows a schematic drawing of a microbial mat. The cyanobacteria form a mat in the top layers of the sediment. Often these mats are covered by a layer of sand or dead organic matter. The layer covering the cyanobacteria might contain empty sheaths with the sunscreen pigment scytonemin (see next section). Hence that layer serves, like sunglasses, to protect the shade-adapted cyanobacteria from excess (u.v.-)light. Below the cyanobacteria a layer of oxidized iron is often observed. The origin of this layer is unknown but it is possible that it is the result of ferrous-iron-dependent anoxygenic photosynthesis (Widdel *et al.*, 1993). It is also possible that iron is oxidized chemically by oxygen produced by the cyanobacteria. Microbiological aerobic iron oxi-

dation at the pH normally encountered in microbial mats (neutral to alkaline) is unlikely (Ehrlich, 1990). Underneath the layer of oxidized iron the anoxygenic phototrophic purple sulphur bacteria might be present. These anaerobic bacteria perform anoxygenic photosynthesis with sulphide as the electron donor. The layer of oxidized iron probably serves as a barrier between the aerobic and anaerobic microbial communities. Sulphide is trapped by the reaction with ferric iron, resulting in the formation of elemental sulphur, ferrous iron and FeS (reactions 3–5, see Section V.2), whereas iron will react with oxygen. Below the layer of purple sulphur bacteria an extended layer of FeS is present. Often this is referred to as the layer of the sulphate-reducing bacteria but, as argued in the section on sulphate reduction, these organisms are likely to be most abundant in the euphotic zone of the mat, mixed with the oxygenic and anoxygenic phototrophs, where highest rates of sulphate-reduction have also been observed. The same is true of the chemolithotrophic sulphur-oxidizing bacteria.

III. CYANOBACTERIA IN LIGHT GRADIENTS

1. *Motility*

The predominant mode of growth of cyanobacteria is phototrophic and therefore these organisms depend on light. In microbial mats cyanobacteria suffer from self-shading caused by the extremely densely-packed organisms. Also, sedimentation might deprive the cyanobacteria of light. Hence, motility is of great importance to these organisms, enabling them to position themselves in optimal light. Other environmental factors might also control cyanobacterial motility.

Cyanobacteria are either non-motile or they glide. Only one report exists of a unicellular cyanobacterium capable of swimming motility (Waterbury *et al.*, 1985). Although this swimming resembled motility produced by a flagellum, no such structure was found and no explanation was offered. However, gliding motility is also still unexplained. To date two hypotheses have received close attention: (i) the secretion of slime and (ii) surface undulations produced by contractile elements.

The hypothesis of gliding by self-propulsion as a result of the secretion of slime finds its origin in some ultrastructural characteristics of a number of cyanobacteria. Several cyanobacteria possess small pores in the cell wall on both sides of the cross wall (Pankratz & Bowen, 1963). These pores were assumed to be the sites from which slime was secreted (Pringsheim, 1968). There are, however, problems with this hypothesis. *Phormidium uncinatum* is a highly motile cyanobacterium that produces slime yet does not have pores in the cell wall (Häder, 1987*b*). The slime secretion model also does not explain the fact that members of the *Oscillatoriaceae* rotate along their long axis, whereas members of the *Nostocaceae* simply move forward along the long axis (Burkholder, 1933), nor does it explain how cyanobacteria frequently change direction. Most important perhaps are theoretical considerations that show that the amount of slime needed for gliding movement would exceed the volume of the cells several times (Holton & Freeman, 1965).

Halfen & Castenholz (1971) have found microfibrils in the outer cell layers of *Oscillatoria princeps*. They suggested that these microfibrils were contractile and that these would generate surface undulations that could propel the cyanobacterium on a solid surface (Castenholz, 1973*b*). This solid surface might well be the polysaccharide sheath, through which the filament glides. *Oscillatoria* might go back and forward through this tube, or leave it while producing a new sheath. Trichomes might also use each other as a substratum on which to glide.

2. *Photoresponses*

Cyanobacteria show three different types of photoresponse: (i) phototaxis, (ii) photokinesis; (iii) photophobic (Häder, 1987*a*, *b*).

Phototaxis is a movement dependent on the light direction. Positive phototaxis is movement towards the light and negative phototaxis away from the light. Cyanobacteria are the only prokaryotes that are capable of phototactic orientation. They often show positive phototaxis at small irradiances. This response obviously brings the organism closer to its energy source. However, most cyanobacteria are shade-adapted organisms and at larger irradiances they may show negative phototaxis. Phototactic response will thus position the organism under optimal light intensity. The cyanobacteria may be split into two groups, which differ in phototactic response (Häder, 1987*b*). *Phormidium* sp. orients itself according to a trial-and-error mechanism. This organism frequently reverses its direction of gliding, but movement in the direction of the light is accompanied by lesser reversals than is movement away from the light. By contrast, *Anabaena* sp. is capable of sensing the light direction. This property resides in all cells of a trichome and either the tip moves in the direction of the light source or the trichome moves as a U-shape (Nultsch, Schuchart & Höhl, 1979). *Phormidium* sp. does not show negative phototaxis but movement of the organism stops above 10 klux (Nultsch, 1961). Action spectra have shown that the accessory photosynthetic pigments (carotenoids, phycobiliproteins), but not chlorophyll *a* are involved in phototaxis in this organism. In *Anabaena* sp. positive and negative phototaxis are mediated by phycobiliproteins as well as chlorophyll *a*. In addition, a photoreversible pigment is involved in negative phototaxis. It has been proposed that

singlet oxygen ($^1O_2$), which is produced by the photosynthetic apparatus at large irradiances, is the trigger of a sign reversal generator (Nultsch & Schuchart, 1985).

Photokinesis is the dependence of speed of movement on irradiance. Speed is higher at higher irradiances which causes the organism to accumulate in shaded areas. In *Phormidium* sp. positive photokinesis was observed at irradiances up to 30 klux, with a threshold of 0·02 lux and an optimum at about 2 klux. Irradiances above 30 klux resulted in negative photokinesis. In *Anabaena* sp. no negative photokinesis was observed even at irradiances as high as 90 klux (Nultsch & Hellmann, 1972). Photokinesis in cyanobacteria is the result of a greater supply of energy at greater irradiances. In *Phormidium* sp. this seems to be mediated by cyclic (PS I) electron transport, whereas in *Anabaena* PS II is involved (Häder, 1987*a*).

A photophobic response is a reversal of the direction of movement as a result of a sudden change in light intensity. This type of light-mediated response is probably most important in cyanobacteria. Both step-down and step-up photophobic responses are known (Häder, 1987*a*). Step-down photophobic responses will result in the accumulation of the organisms in the light. Every time the organism senses a decrease in light intensity it reverses. At very high irradiances a step-up response will keep the organism in shaded areas. The action spectrum of photophobic responses clearly shows the involvement of photosynthesis (Häder, 1987*a*). It is assumed that the first step in a photophobic response is the modulation of the proton gradient over the thylakoid membrane. Since the threshold value for photophobic response is very low, amplification of the signal must be assumed. Häder (1988) proposed a model in which the change of proton motive force triggers the influx of Ca^{2+} into the cell. The massive influx of Ca^{2+} leads to the collapse of the electrical potential in the front end of the trichome which eventually results in the reversal of movement. The precise mechanism of this reversal of movement is still unknown (Häder, 1988). The combination of step-down and step-up photophobic responses might allow the organism to position itself under optimal light conditions.

In an elegant study by Ramsing & Prufert-Bebout (1994), motility of the mat-forming cyanobacterium *Microcoleus chthonoplastes* was investigated. Referring to recent studies using fibre-optic light-microsensors these authors concluded that light fields within the mat are uniform, which means that virtually no directional incident light is present. This uniformity of illumination is caused by scattering. They argued that owing to the uniform light field phototactic responses are not likely. Also, because in the deeper layers of the mat light will change gradually rather than suddenly, photophobic responses are equally unlikely. Using image analysis, they concluded that *M. chthonoplastes* minimizes movement if conditions are favourable. It does not change its speed of movement, rather it moves less frequently. Another strategy which is used by this organism is to reverse more frequently its direction of movement. This is not to be confused with photophobic reactions, which occur only after a step-up or step-down of light intensity. As a long-term strategy to position itself in an optimal light field *M. chthonoplastes* uses bending, which was shown to occur more frequently at optimal light intensities. Bending eventually leads to curling of trichomes into bundles in which motility is much more restricted. It was envisaged that this curling would produce a dense intertangled mat thriving at optimal light conditions.

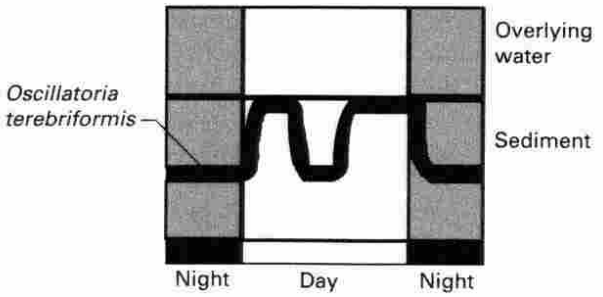

Figure 2. Pattern of movement of the filamentous cyanobacterium *Oscillatoria terebriformis* in a hot-spring microbial mat. During the night the organism accumulates in the deeper anoxic and sulphide-rich layers of the sediment. During the day it moves up to the sediment surface but around noon it might descend again to protect itself from high irradiances (re-drawn after Richardson & Castenholz, 1987*a*).

3. *Chemotaxis*

The mat-forming thermophilic *Oscillatoria terebriformis* exhibits unusual behaviour, as was elegantly shown by Richardson & Castenholz (1987*a*). During the night this organism moves down into the mat and moves up to the surface in the morning hours. At midday, when light intensities are very high it might move down in the mat again (Fig. 2). The explanation for this behaviour is the combination of phototaxis and an inhibition of motility by sulphide. When light is absent the organism moves randomly. However, when it reaches the layer containing sulphide, motility is inhibited. Under laboratory conditions it was shown that 0·7 mM of sulphide inhibited reversibly the motility of the trichomes both in the light and in the dark. Sulphide inhibition of motility in the light occurred only when the inhibiter of oxygenic photosynthesis, 3,-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was present (Richardson & Castenholz, 1987*a*). The rate of gliding movement

of *O. terebriformis* in the dark under oxic conditions is *c.* 0·3 mm min^{-1}, which is sufficiently fast to layer the trichomes in the subsurface. After a while this results in the trapping of all trichomes of *O. terebriformis* in the anoxic sulphide-rich layer. At dawn the sulphide interface moves down and the cyanobacteria move up to the surface. In the light sulphide does not inhibit motility and in addition the onset of oxygenic photosynthesis leads to the oxidation of sulphide. *O. terebriformis* is positively phototactic and shows a light-intensity-dependent gliding rate (photokinesis) (Castenholz, 1973*a*). The combination of these responses brings the organism back to the surface. Apparently, *O. terebriformis* avoids very high light intensities and at noon positions itself deeper into the sediment, probably by negative phototaxis, behaviour which appears to be of vital importance for this organism. When it experiences oxic conditions in the dark, respiration proceeds at a high rate, resulting in the fast depletion of endogenous carbon stores (glycogen). To survive the dark period, *O. terebriformis* avoids oxygen by switching to fermentative metabolism (Richardson & Castenholz, 1987*b*). Fermentation proceeds at a much slower rate but produces sufficient energy to meet maintenance requirements. It must be emphasized, however, that this cyanobacterium is unusual, since virtually all other cyanobacteria survive much better under oxic conditions in the dark than *O. terebriformis*.

The usual behaviour of motile cyanobacteria in microbial mats is to move up to the surface in the dark as well as under low light. The majority of species move deeper into the sediment at high light intensities (Pentecost, 1984; Whale & Walsby, 1984; Richardson & Castenholz, 1987*a*). Although responses to light are relatively well documented in cyanobacteria, dark responses are less understood. Geotactic or magnetotactic responses are not known for cyanobacteria (Whale & Walsby, 1984) and therefore it is likely that chemical gradients control the direction of movement of cyanobacteria in the dark. The movement of cyanobacteria to the surface of the mat during the dark might be triggered by sulphide. Castenholz (1982) proposed a chemophobic reaction in *O. terebriformis* triggered by sulphide, which would then lead to negative taxis. In many mats sulphide concentrations increase during the night and a gradient of sulphide is formed, declining in concentration towards the surface, causing the cyanobacteria to move in that direction. This mechanism has also been suggested by Whale & Walsby (1984) for *Microcoleus chthonoplastes*, a cyanobacterium that is known to move to the sediment surface during the dark.

Chemotactic responses controlled by sulphide are virtually uninvestigated, which is curious since sulphide is one of the most conspicuous chemical factors in microbial mats and is extremely toxic to all organisms. The inhibition of motility in *O. terebriformis* is the best described response to sulphide (Richardson & Castenholz, 1987*a*, 1989). Although it cannot be excluded, it seems unlikely that a chemophobic response towards sulphide, as suggested by Castenholz (1982), is involved in chemotaxis in *O. terebriformis*. Moreover, the movement of *M. chthonoplastes* to the mat surface in the dark could be equally well explained as a positive response to oxygen (aerotaxis) (Whale & Walsby, 1984). Chemotactic responses towards oxygen were shown in the cyanobacterium *Oscillatoria* sp. (Malin & Walsby, 1985), but it also responded to concentration gradients of CO_2 and bicarbonate. Chemotaxis was shown to be strictly light-dependent, advantageous, the authors suggested, for the organism's movement within the photic zone of the mat towards optimal CO_2 concentrations. On the other hand, movement towards large concentrations of oxygen in the light would have negative effects such as photooxidation (Eloff, Steinitz & Shilo, 1976) and less efficient CO_2 fixation (Lorimer, 1981; Reinhold, Kosloff & Kaplan, 1991). Oxygen-induced positive chemotaxis in the light is unexplained. Aerotaxis during the dark would be advantageous for cyanobacteria that usually carry out aerobic respiration using endogenous storage carbohydrate. However, Malin & Walsby (1985) were not able to demonstrate chemotaxis in *Oscillatoria* sp. in the dark, where it was capable of gliding for only a short period, after which it stopped because of exhaustion of energy reserves.

Chemotactic responses of cyanobacteria to organic compounds are also virtually unknown. A negative chemotactic response of *Oscillatoria* sp. to organic acids was reported by Fechner (1915). Richardson & Castenholz (1989) reported an inhibition of gliding motility of *O. terebriformis* by fructose. This inhibition was transient and the duration was proportional to the concentration of fructose. The gliding rate after resumption of motility was inversely proportional to fructose concentration. The effects of fructose were similar to those of sulphide, but when both were present the effect was stronger than the sum of the effect of each compound separately. *O. terebriformis* did not respond to glucose, acetate and lactate. Fructose, like glucose, can serve as an extracellular substrate for fermentation in *O. terebriformis* (Richardson & Castenholz, 1987*b*).

4. *Effects of u.v. light*

An important factor determining the position of cyanobacteria in microbial mats is u.v. light. Garcia-Pichel & Castenholz (1994) reported that cyanobacteria responded particularly well to u.v.-A light (315–400 nm) and that this was an order of magnitude more effective than visible light. Only

1·3 W m^{-2} of u.v.-A, which represents only 3–4 % of the maximum level at noon, would be sufficient to keep the cyanobacteria deep in the sediment. The same authors also showed that the cyanobacteria responded to u.v.-A by negative phototaxis rather than by impaired motility as shown for u.v.-B (280–315 nm) (Donkor & Häder, 1991; Donkor, Amewowor & Häder, 1993). Avoidance of u.v. light is important since it affects photosynthesis of cyanobacteria and other phototrophic microorganisms. Garcia-Pichel & Castenholz (1994) found considerable inhibition of photosynthesis in a mat of *Microcoleus chthonoplastes* as the result of the inclusion of u.v. light, with no recovery during the subsequent 3 h.

Many benthic cyanobacteria, particularly those exposed to high irradiations, synthesize a yellow-brown lipid-soluble pigment that occurs predominantly in their extracellular sheaths. This pigment, which absorbs strongly in the near u.v., is known as scytonemin. It has been shown that this pigment efficiently protects the organism against u.v. light. Interestingly, this pigment is very recalcitrant and can persist for long periods in the ecosystem. Often, in geographic areas where very high levels of irradiation occur, microbial mats can be covered by a layer of empty sheaths in which scytonemin is present. In this manner the whole ecosystem is protected against u.v. irradiation (sunglasses effect). Garcia-Pichel & Castenholz (1991) also showed that scytonemin was produced only at high light intensities and that only u.v.-A was capable of triggering its synthesis.

Another possibility for protection against u.v. irradiation is the production of mycosporine-like amino acids. These compounds absorb strongly in the u.v. spectrum between 230 and 400 nm. Garcia-Pichel & Castenholz (1993*a*) found that many cyanobacteria isolated from habitats exposed to strong insolation contained mycosporine-like compounds. On the other hand, the same authors (Garcia-Pichel & Castenholz, 1993*b*) concluded from a detailed study of the unicellular cyanobacterium *Gloeocapsa* sp. that, although the amount of mycosporine-like compounds in this organism was related to the u.v. irradiation that was received by the cell, little sunscreen effect could be attributed to these compounds. The adaptation of the physiology of *Gloeocapsa* sp. to increased levels of u.v. is apparently a result of another mechanism (Garcia-Pichel & Castenholz, 1993*b*). Recently, a brown *Nostoc* sp. was described which was isolated from soil (De Chazal & Smith, 1994). This organism does not produce scytonemin or mycosporin-like amino acids, yet it produces unknown compounds that absorb in the u.v. A, B and C regions. The compounds were produced only when the organism was grown under oxic conditions. Cells that contained the u.v.-absorbing compounds were particularly resistant to u.v.-C, and exposure to u.v.-C increased the resistance of this *Nostoc* sp. against u.v.-A and B. Since u.v.-C is absorbed by the Earth's atmosphere, the significance of this effect is not clear.

Garcia-Pichel & Castenholz (1994) concluded that an ecological correlation exists between the property of sunscreen synthesis and the occurrence of cyanobacteria in different types of microbial mats. They recognized three types of cyanobacteria: (i) those occurring in intertidal mats which have both the sheath pigment scytonemin and the intracellular, water-soluble mycosporine-like compounds; (ii) those occurring in Antarctic ponds which possess only scytonemin; (iii) those in thermal mats which do not contain sunscreen pigments. It was argued that in mats where conditions allow constant and balanced growth of the cyanobacteria, u.v.-repair mechanisms would be more efficient. In intertidal microbial mats desiccation or other unfavourable conditions might occur where growth is arrested yet light is still present. Under such conditions sunscreen pigments, which can account for 5–10 % of dry weight biomass, might be of great importance.

Iron has also been proposed as an effective u.v. screen (Pierson & Olson, 1989). Mat-forming cyanobacteria such as *M. chthonoplastes* are very efficient scavengers of iron. They bind large amounts of iron to the polysaccharide sheath (Stal, 1994). Whether the accumulation of iron in *M. chthonoplastes* indeed provides the organism with protection against u.v. is not known. The putative role of iron as u.v. screen has the same drawbacks as the unknown compound in *Nostoc* (De Chazal & Smith, 1994). Like this unknown compound, iron absorbs u.v. light mostly in the region 220 to 270 nm which does not reach the surface of the Earth.

IV. DARK METABOLISM

Cyanobacteria accumulate polyglucose (glycogen) as an energy and carbon store. In the light, part of the CO_2 assimilated is diverted to the synthesis of glycogen which is mobilized during the dark. Presumably all cyanobacteria degrade glycogen via the oxidative pentose phosphate pathway (Smith, 1982, 1983). This pathway results in the complete oxidation of glycogen to CO_2 while energy is obtained by transferring electrons, generated during glycogen catabolism to oxygen, through aerobic respiration. Energy obtained from aerobic degradation of endogenous glycogen is sufficient to allow synthesis of structural cell components in the planktonic cyanobacterium *Oscillatoria agardhii* (Van Liere *et al.*, 1979; Post, Loogman & Mur, 1986). In fact, it appeared possible to maintain growth of this organism at the same rate as in the light. Of course such growth will only last as long as glycogen is available. However, when the organism was grown under a light-dark regime, the rate of

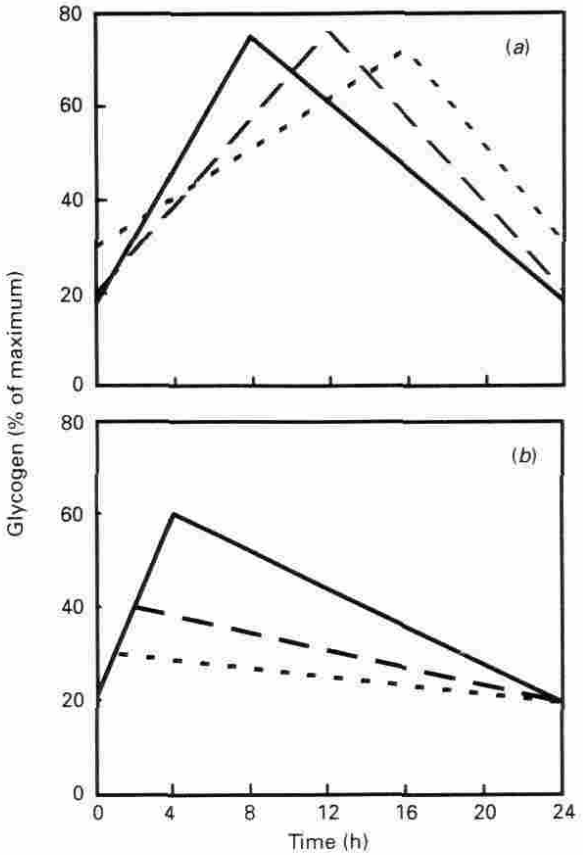

Figure 3. Dynamics of glycogen content of the filamentous cyanobacterium *Oscillatoria agardhii* grown under various alternating light–dark cycles. In (*a*) 8–16 (solid line), 12–12 (dashed line) and 16–8 (dotted line) h light–dark cycles are depicted. At the shorter light periods the rate of synthesis increases whereas the rate of mobilization in the following dark period decreases. The amount of glycogen accumulated at the end of the light period is independent of the period length. In (*b*) the light–dark cycles are of 4–20 (solid line), 2–22 (dashed line) and 1–23 h (dotted line). It is clear that during these short light periods the rate of synthesis of glycogen is maximal and that the amount of glycogen accumulated depends on the length of the light period. The rate of glycogen mobilization in the dark decreases with the increasing length of the dark period (re-drawn after Mur, 1983).

glycogen accumulation in the light varied with length of the dark period (Fig. 3) (Mur, 1983). Glycogen accumulation may also be controlled by the nutritional state of the organism. Unbalanced growth may occur when CO_2 assimilation exceeds the requirement for synthesis of structural cell components. This is the case for example when nitrogen limits growth. Under such conditions the synthesis of, among other things, proteins is limited and excess fixed carbon is diverted to glycogen synthesis (Lehmann & Wöber, 1976; De Philippis, Sili & Vincenzini, 1992). The amount of glycogen in N-limited cyanobacteria can account for up to 40% of dry weight which might allow the organism to survive for a considerable time in darkness, provided that the mobilization of glycogen is under metabolic control. It is well known that cyanobacteria sometimes have to cope with periods of prolonged darkness, for instance when an occasional high rate of sedimentation buries a cyanobacterial mat (Pentecost, 1984). In lakes, planktonic cyanobacteria can sink below the euphotic zone. The planktonic cyanobacterium *Microcystis aeruginosa* overwinters on lake-bottom sediments in complete darkness for 6–9 months of the year (Preston, Stewart & Reynolds, 1980; Reynolds *et al.*, 1981), and in surface scums of highly buoyant *M. aeruginosa*, many cells can thrive in darkness for prolonged periods of time (Ibelings & Mur, 1992). Since many microbial mats are nitrogen-limited it can be assumed that the mat cyanobacteria all have large intracellular concentrations of glycogen. However, anoxic conditions often prevail for the greater part of the dark period. This is the case in most microbial mats that turn completely anoxic during the night but also in surface scums and in lake bottom sediments, where *Microcystis* thrives under anoxic conditions. It has now been shown that a number of different cyanobacteria switch to fermentation in the absence of oxygen (Table 1).

The first cyanobacterium shown to be capable of fermentation was *Oscillatoria limnetica*, which thrives in the sulphide-rich hypolimnion of Solar Lake (Sinai). This organism switches to homolactic acid fermentation, producing two molecules of lactate for each molecule of glucose fermented (Oren & Shilo, 1979). No information on the metabolic pathway in this organism is available. However, homolactic acid fermentation probably involves the Embden–Meyerhof–Parnas pathway (glycolysis). Only very recently has the existence of the Embden–Meyerhof–Parnas pathway in a cyanobacterium been proved, by showing the presence of all enzymes of this pathway in *Microcystis aeruginosa* (Moezelaar & Stal, 1994*b*) and in *Microcoleus chthonoplastes* (Moezelaar & Stal, 1994*b*; Moezelaar, Bijvank & Stal, unpublished).

In the mat-forming *O. limosa* a very interesting fermentation occurs. This organism ferments glycogen to equimolar amounts of lactate and ethanol (Heyer, Stal & Krumbein, 1989). The enzymes of this heterofermentative lactic acid fermentation pathway have been identified. A major part of this route uses the same enzymes as the oxidative pentose phosphate pathway. However, *O. limosa* also fermented trehalose which serves as an osmoprotectant in this organism. On average five molecules of acetate were formed from each molecule of trehalose, which hinted at a homoacetate fermentation. Theoretically six molecules of acetate are expected from the degradation of one molecule of trehalose but in *O. limosa* the balance was made by CO_2 and H_2 (Heyer *et al.*, 1989). In addition, the key enzymes for homoacetate fermentation, CO-dehydrogenase, formate dehydrogenase, pyruvate:ferredoxin-oxidoreductase and acetate kinase, were present. The

Table 1. *Cyanobacteria capable of fermentation*

Organism	Origin, strain designation	Fermentation, products	Reference
Cyanothece sp.	PCC 7822 (Inst. Pasteur)	Mixed acid: H_2, ethanol, lactate, formate, acetate	Van der Oost *et al.* (1989)
Microcoleus chthonoplastes	Microbial mat	Mixed acid: H_2, ethanol, lactate, formate, acetate	Moezelaar & Stal (1994*a*)
Microcystis aeruginosa	PCC 7806 (Inst. Pasteur)	Mixed acid: H_2, ethanol, acetate	Moezelaar & Stal (1994*b*)
Nostoc sp.	Symbiont from *Cycas circinalis*	Homoacetic: acetate	Margheri & Allotta (1993)
Oscillatoria limnetica	Hypolimnion Solar Lake	Homolactic: lactate	Oren & Shilo (1979)
Oscillatoria limosa	Microbial mat	Heterolactic: lactate, ethanol Homoacetic acetate	Heyer *et al.* (1989)
Oscillatoria terebriformis	Hot spring microbial mat	Homolactic?	Richardson & Castenholz (1987*b*)

occurrence of homoacetate fermentation is unusual since this pathway has thus far only been found in a specialized group of obligately anaerobic acetic acid bacteria. Very recently, homoacetate fermentation was also reported in a symbiotic cyanobacterium, *Nostoc* (Margheri & Allotta, 1993). Homoacetate fermentation is energetically efficient, and it also implies the functioning of the Embden–Meyerhof–Parnas pathway.

It is not known how two different fermentation pathways operate simultaneously in *O. limosa*. It is possible that a subcellular compartmentalization exists. Glycogen degradation probably occurs within the thylakoid space, whereas the osmoprotectant trehalose is dissolved in the cytoplasm, where it is presumably metabolized. The fact that *O. limosa* degrades its osmoprotectant is paradoxical. Not all trehalose is degraded and 10–25 % of the original amount remains. It is not known how this organism counterbalances the osmotic pressure but it is assumed that inorganic ions such as K^+ can temporarily serve as an osmoticum. On the other hand, trehalose represents an interesting energy store for the organism, on which it probably continues to draw during anoxic dark conditions. Likewise, *M. chthonoplastes* utilizes its osmoprotectant, glucosyl-glycerol (Moezelaar, Bijvank & Stal, unpublished). In this organism the osmoprotectant is used only in cells that contain small amounts of glycogen. Only the glucose part of the osmoprotectant is used: glycerol is excreted. Unlike *O. limosa*, this organism probably ferments glucose originating from the osmoprotectant via the same pathway as glucose derived from glycogen.

In all fermentative cyanobacteria investigated so far the enzymes of fermentation are constitutive. Cultures grown photoautotrophically start fermentation immediately upon transfer to dark anoxic conditions, even if chloramphenicol is added immediately before dark anoxic incubation. The enzymes involved in fermentative metabolism can be detected in photoautotrophically grown cells. Anoxic dark incubation does not usually change the specific activities of these enzymes. This property makes these cyanobacteria very responsive, which is of great importance in environments such as microbial mats where conditions can change quickly. Very little is known about the regulation of these pathways. However, the activities of enzymes of fermentative pathways are small compared with aerobic metabolism, and specific regulation of these pathways is perhaps not required. The low rates of fermentation invite consideration of its ecological relevance. Moezelaar (1995) calculated that the energy generation during fermentation is of the order of magnitude necessary for maintenance of cyanobacteria. Indeed, cyanobacteria capable of fermentation might survive very long periods of anoxic dark conditions. It has already been mentioned that *O. terebriformis* is not capable of surviving very long periods of darkness when oxygen is present, owing to the fast rate of glycogen degradation (Richardson & Castenholz, 1987*b*). Cyanobacteria that did not possess fermentative pathways lysed within 3 h after transfer to dark anoxic conditions (Stal, unpublished). This shows clearly the ecological relevance of fermentation. Although some cyanobacteria such as *O. terebriformis* and *M. aeruginosa* are capable of fermenting external substrates such as glucose and fructose, the concentrations of these substrates required for fermentation (5 mM) seem not to be of ecological relevance. Thus it seems that, in the dark, free-living cyanobacteria depend mainly on their endogenous energy storage.

Several cyanobacteria that are capable of fermentation of endogenous storage carbohydrate have been shown to use elemental sulphur as electron acceptor, resulting in the production of sulphide (Stal, 1991*a*). In microbial mats elemental sulphur is often present in vast amounts. Despite its attractiveness as an electron acceptor, true sulphur respiration (i.e. when elemental sulphur serves as terminal electron acceptor in a respiratory electron transport chain) has not been demonstrated in

cyanobacteria. The few studies that have been made on sulphur reduction in cyanobacteria showed that the fermentation pattern shifted to the production of more of the oxidized acetate, while excess electrons were used to reduce sulphur (Moezelaar & Stal, 1994*a*). This shift is energetically advantageous since the production of acetate results in the formation of ATP, which is not the case when more reduced fermentation products such as ethanol are formed. Cyanobacteria that normally produce H_2 as a fermentation product do not evolve it when sulphur is present.

V. INTERACTIONS WITH SULPHIDE

1. *Anoxygenic photosynthesis*

Cyanobacteria occupy an exceptional position in the world of phototrophic prokaryotes. With the closely related Prochlorophytes, they are the only organisms in the kingdom of Eubacteria that perform a plant-like oxygenic photosynthesis, using two photosystems in series and water as electron donor. However, some species of cyanobacteria can induce the typical bacterial anoxygenic photosynthesis in which only PS I is involved and with sulphide usually serving as electron donor. These cyanobacteria can be considered as the bridge between the eukaryotic algae and the anoxygenic phototrophic bacteria.

Anoxygenic photosynthesis takes place in cyanobacteria when PS II is inhibited by DCMU or when PS II is not activated, for example by using far red light (> 700 nm). Anoxygenic photosynthesis in cyanobacteria is defined as PS I-mediated CO_2 fixation with sulphide as the electron donor, and is induced after exposure of the organism to sulphide. Light is also required for induction (Cohen, Padan & Shilo, 1975). The concentration of sulphide necessary to induce anoxygenic photosynthesis differs between the different species (Garlick, Oren & Padan, 1977; Cohen *et al.* 1986). Sulphide inhibits oxygenic photosynthesis, probably at the level of the manganese-containing, water-splitting enzyme (Oren, Padan & Malkin, 1979). The capacity for anoxygenic photosynthesis is acquired by a process in which *de novo* protein synthesis is involved. It usually requires 2–3 h to induce fully the capacity for anoxygenic photosynthesis. Inhibition of protein synthesis by chloramphenicol has been shown in all strains investigated so far to prevent induction of anoxygenic photosynthesis (Oren & Padan, 1978; Castenholz & Utkilen 1984; Garcia-Pichel & Castenholz, 1990). This was the case even in some strains of the thermophilic *O. amphigranulata* that acquired the capacity for anoxygenic photosynthesis almost immediately (Garcia-Pichel & Castenholz, 1990). In *O. limnetica* it is likely that a single protein that accepts electrons from sulphide is synthesized.

Apparently, induction of anoxygenic photosynthesis can be bypassed when Na-dithionite is present (Belkin & Padan, 1983*a*). These authors assumed that, by imposing a low redox potential on *O. limnetica* by dithionite, a site that directly accepts electrons from sulphide would become available. In another cyanobacterium, *O. amphigranulata*, dithionite appeared to be very inhibitory to photosynthesis and therefore it is uncertain whether dithionite would bypass induction in all anoxygenic phototrophic cyanobacteria (Castenholz & Utkilen, 1984). There are also different sites of electron donation to the photosynthetic electron transport chain. Padan (1989) considered two patterns of sulphide oxidation in *O. limnetica*. On the basis of studies with uncouplers they concluded that sulphide oxidation is not linked to NAD reduction by reversed electron transport. It seems that sulphide donates electrons directly to the photosynthetic electron transport system, probably at or before the cyt b_6f complex. According to this model 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) inhibited anoxygenic photosynthesis (Belkin & Padan, 1983*b*). By contrast, in sulphide-adapted *O. amphigranulata*, DBMIB inhibition was circumvented, suggesting that electrons were donated via cyt *f* or plastocyanin (Castenholz & Utkilen, 1984). Donation of electrons from sulphide directly to the photosynthetic electron transport system also occurs in the green sulphur bacteria, *Chlorobium* spp., whereas, in the purple sulphur bacteria, sulphide oxidation is driven indirectly by photosynthetic electron transport.

During anoxygenic photosynthesis, cyanobacteria oxidize sulphide to elemental sulphur according to the following equation:

$$12H_2S + 6CO_2 \rightarrow C_6H_{12}O_6 + 12S^0 + 6H_2O \qquad (1)$$

Elemental sulphur is deposited outside the cell as finely dispersed particles. In one case thiosulphate was found as the product of sulphide oxidation in anoxygenic photosynthesis: *Microcoleus chthonoplastes* oxidized sulphide to thiosulphate by the following reaction (De Wit & Van Gemerden, 1987):

$$6H_2S + 3H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 3H_2S_2O_3 \qquad (2)$$

Oxidation of sulphide to thiosulphate yields four electrons per sulphide oxidized. Thus *M. chthonoplastes* used sulphide twice as efficiently as do other anoxygenic phototrophic cyanobacteria, which obtain only two electrons from the oxidation of sulphide to elemental sulphur. Many anoxygenic phototrophic bacteria oxidize sulphide completely to sulphate, which yields eight electrons. Oxidation of sulphide to elemental sulphur seems to be inefficient but results in a faster consumption of toxic sulphide: the elemental sulphur can be used as an electron acceptor in the dark (see dark metabolism) or be used as source of assimilatory sulphur, as was shown for *O. amphigranulata* (Castenholz & Utkilen, 1984).

Table 2. *Types of adaptation of cyanobacteria to sulphide (after Cohen* et al. *(1986))*

Group 1	Sulphide-sensitive oxygenic photosynthesis only
Group 2	Sulphide-resistant oxygenic photosynthesis only
Group 3	Sulphide-insensitive oxygenic photosynthesis concurrent with sulphide-dependent anoxygenic photosynthesis
Group 4	Sulphide-sensitive oxygenic photosynthesis replaced by sulphide-dependent anoxygenic photosynthesis

Although most anoxygenic phototrophic bacteria can use elemental sulphur or thiosulphate as an electron donor, cyanobacteria are limited to sulphide. Only in *Anacystis nidulans* can thiosulphate support low rates of CO_2 assimilation (Utkilen, 1976; Peschek, 1978).

Electrons obtained from sulphide oxidation in the course of anoxygenic photosynthesis are used either for CO_2 fixation or for N_2 fixation. *O. limnetica* is capable of diazotrophic growth under anoxygenic conditions (Padan & Cohen, 1982). Although it seems likely that sulphide-driven diazotrophic growth in cyanobacteria is rather common, very little has been published on this topic (Bebout, Fitzpatrick & Paerl, 1993). When no CO_2 is available or when CO_2 fixation is inhibited, electrons from sulphide are diverted to H_2. This reaction is mediated by a hydrogenase. In *O. limnetica* the induction of hydrogen evolution needs considerable time (46 h). The redox potential, which probably reflects the properties of the hydrogenase involved is also important for hydrogen evolution (Belkin & Padan, 1979).

Sulphide is extremely toxic to all living organisms, even to those that depend on it (such as anoxygenic phototrophic bacteria and chemolithotrophic bacteria) or produce it (sulphate-reducing bacteria). Sulphide reacts readily with cytochromes, haemoproteins and other iron-containing compounds. Hence, it interferes with processes in which these proteins are involved, notably inhibiting respiratory and photosynthetic electron transport. Photosystem II in many cyanobacteria is extremely sensitive to sulphide, which is assumed to inhibit the water-splitting complex. This inhibition is irreversible but in *O. amphigranulata* a recovery of oxygenic photosynthesis could occur at moderate sulphide concentrations and at high light intensities. This recovery depended on *de novo* protein synthesis since it could be prevented by addition of chloramphenicol (Castenholz & Utkilen, 1984). Garcia-Pichel & Castenholz (1990) also observed differing degrees of recovery from sulphide exposure. Even anoxygenic photosynthetic electron transport is affected by sulphide. Furthermore, low redox potential might have negative effects on growth and metabolic activity of cyanobacteria. The toxicity of sulphide to cyanobacteria might be the result of a combination of these effects. Therefore, the degree of sulphide toxicity is expected to differ greatly between different organisms.

Cyanobacteria can be divided in four groups with respect to their behaviour to sulphide (Cohen *et al.*, 1986) (Table 2). Group 1 consists of organisms for which sulphide is extremely toxic, even very small concentrations of free sulphide ($< 0{\cdot}1$ mM) irreversibly inhibiting oxygenic photosynthesis. They are likely to be found in environments, such as most freshwater lakes, in which free sulphide will never reach significant concentrations. Examples of these organisms are *Anacystis nidulans* and *Plectonema boryanum*, in which CO_2 assimilation was completely inhibited at sulphide concentrations of respectively 60 and 75 μM (Cohen *et al.*, 1986). Group 2 are cyanobacteria that show a stimulation of oxygenic photosynthesis at moderate concentrations of free sulphide (< 1 mM) but are not capable of anoxygenic photosynthesis. Cyanobacteria of this type are found in microbial mats in which free sulphide accumulates at regular intervals. These organisms are discussed below. Group 3 cyanobacteria can also be found in environments in which moderate and fluctuating levels of sulphide occur at regular intervals. In contrast to Group 2, this type of cyanobacterium is capable of simultaneous anoxygenic, sulphide-dependent photosynthesis and oxygenic photosynthesis. Group 4 consists of cyanobacteria in which oxygenic photosynthesis is inhibited at small concentrations of sulphide ($< 0{\cdot}1$ mM) but is switched to anoxygenic photosynthesis. In *O. limnetica*, for example, PS II is as sensitive to sulphide as in cyanobacteria of Group 1, and is completely inhibited at sulphide concentrations of 0·1 mM. However, anoxygenic photosynthesis is induced only at much greater concentrations of sulphide (3 mM). Some of the organisms in Group 4 can tolerate considerable concentrations of free sulphide (< 10 mM). In *O. limnetica*, which is the best studied cyanobacterium with respect to anoxygenic photosynthesis, anoxygenic photosynthesis is gradually inhibited at sulphide concentrations > 4 mM though it can tolerate up to 9·5 mM. The capacity for anoxygenic photosynthesis must be induced, which might take 2–3 h. These cyanobacteria can be found in environments in which large concentrations of sulphide persist for prolonged periods.

The fact that cyanobacteria can perform anoxygenic photosynthesis does not necessarily mean that they can also grow under such conditions. For instance, *M. chthonoplastes* has an obligate requirement for oxygen (De Wit, Van Boekel & Van Gemerden, 1988) and therefore does not grow under anoxic conditions. Anoxygenic growth at the expense of sulphide when DCMU is present was only

Table 3. *Specific growth rate of* Microcoleus chthonoplastes *at different sulphide concentrations and the contribution of anoxygenic photosynthesis to total photosynthesis*

Culture system	Sulphide (mM)	Specific growth rate	Anoxygenic photosynthesis (%)
Batch	0	0·027	0
Continuous	0·12	0·031	17
Continuous	0·23	0·020	46
Batch	0·35	0·012	> 95
Batch	0·70	0·011	> 95
Batch	0·75	0·004	> 95
Batch	1·00	0	—

Data from De Wit & Van Gemerden (1988).

Table 4. *Effect of sulphide on CO_2 fixation in the mat-forming cyanobacterium* Oscillatoria limosa

DCMU (10^{-5} M)	Sulphide (mM)	$^{14}CO_2$ fixation (%)
+	0	0
−	0	100
+	0·5	0
−	0·5	302
+	1·0	0
−	1·0	74
+	5·0	0
−	5·0	0

CO_2 fixation was measured after 2 d of incubation. $^{14}CO_2$ fixation under normal oxygenic conditions (no DCMU, no sulphide) was taken as 100 %.

possible when some air was sparged into the culture. Anoxygenic growth in the absence of DCMU was possible only at sulphide concentrations < 1 mM. At concentrations ⩾ 0·35 mM of sulphide, very low activities of PS II were still present but contributed less than 5 % to total photosynthesis (Table 3). This low activity of PS II was presumably necessary to provide the organism with oxygen. Oxygen might be required for the oxidation of fatty acids. In *Aphanothece halophytica* an oxygen-dependent mechanism for the desaturation of fatty acids exists (Padan & Cohen, 1982). Several cyanobacteria contain polyunsaturated fatty acids (Kenyon, Rippka & Stanier, 1972), and it has been suggested that such organisms are not capable of anaerobic growth (Padan & Cohen, 1982). On the other hand cyanobacteria such as *O. limnetica* and *O. amphigranulata* grow well under totally anoxic conditions. *O. limnetica* has been shown not to contain polyunsaturated fatty acids (Padan & Cohen, 1982; Oren *et al.*, 1985).

2. *Sulphide oxidation*

Oscillatoria limosa is a cyanobacterium that is important in initial sediment colonization (Stal, Van Gemerden & Krumbein, 1985). Its capacity successfully to colonize intertidal sediments might result from its efficient N fixation (Stal & Krumbein, 1981). *O. limosa* is not capable of anoxygenic photosynthesis. In Table 4 it is shown that CO_2 fixation is completely inhibited by DCMU. However, without DCMU, 0·5 mM sulphide stimulated CO_2 fixation about three-fold. Therefore *O. limosa* is a typical example of a group 2 organism. *O. limosa* also showed a considerable sulphide tolerance. At a concentration of 1 mM of sulphide, CO_2 assimilation was still 74 % of the control. At 5 mM of sulphide, CO_2 fixation was completely inhibited. The stimulation of oxygenic photosynthesis was also demonstrated by measurements of oxygen production (Table 5). Addition of sulphide to *O. limosa* gradually decreased net oxygen production. At 0·4 mM sulphide, no net oxygen exchange was found whereas at greater sulphide concentrations net oxygen uptake was observed. This does not mean that oxygenic photosynthesis was inhibited by sulphide. In fact, 0·4 mM of sulphide stimulated oxygenic photosynthesis almost two-fold. Because oxygen uptake occurs simultaneously these processes counteract at 0·4 mM sulphide. Dark respiration was not affected by sulphide, which means that sulphide-mediated oxygen uptake is light-dependent. Increased oxygen evolution could detoxify sulphide. The physiological background of the stimulation of oxygenic photosynthesis by sulphide is not known. It can be speculated that sulphide reacts with oxygen either chemically or biologically, reducing drastically the prevailing concentration of oxygen. Small concentrations of oxygen might increase the efficiency of

Table 5. *Effect of sulphide on oxygen exchange in* Oscillatoria limosa

Sulphide (mM)	O_2 exchange (μmol O_2 h^{-1} mg^{-1} Chl *a*)
	In the light
0	11·8
0·1	5·7
0·2	1·7
0·4	0·0
0·5	−2·1
1·0	−12·0
	In the dark
0	−12·4
0·4	−12·0
	Gross, in the light
0	11·8
0·4	20·0

At 0·4 mM sulphide net oxygen exchange was zero. However, when 10^{-5} M DCMU was added, 20 μmol O_2 h^{-1} mg^{-1} Chl *a* were taken up. Since 0·4 mM sulphide did not affect dark respiration, it was concluded that the organism had a gross production of oxygen of 20 μmol O_2 h^{-1} mg^{-1} Chl *a*.

CO_2 fixation, which would then be stimulatory to photosynthetic electron transport.

Although *O. limosa* is clearly not capable of anoxygenic photosynthesis, it oxidizes sulphide. Oxidation of sulphide resulted in the formation of elemental sulphur. This process also occurred, although at a lower rate, under completely anoxic conditions and in the presence of DCMU. Since *O. limosa* accumulates considerable amounts of iron in the external sheath, this suggests that the oxidation of sulphide might be chemical rather than biological. Sulphide might react with ferric iron according to the following reactions:

$$2Fe^{3+} + H_2S \rightarrow 2Fe^{2+} + S^0 + 2H^+ \quad (3)$$

$$2Fe^{2+} + 2H_2S \rightarrow 2FeS + 4H^+ \quad (4)$$

$$2Fe^{3+} + 3H_2S \rightarrow 2FeS + S^0 + 6H^+ \quad (5)$$

Part of the sulphide would precipitate as FeS which, in *O. limosa*, is usually bound to the trichomes. These reactions show that the interaction of cyanobacteria with sulphide is clearly not limited to anoxygenic photosynthesis, but is probably of importance in the ecosystem.

VI. NITROGEN FIXATION

1. *Nitrogen-fixing cyanobacteria*

Cyanobacteria are capable of growing in a wide range of different environments. They depend on light as source of energy, water as source of electrons and CO_2 as source of carbon. In many environments these growth requirements are in ample supply. This is often not the case with nitrogen. On average, the element nitrogen forms about 10% of dry biomass. In cyanobacteria, nitrogen is present in polypeptides such as proteins and cyanonophycin, in the nucleic acids, in the cell wall as peptidoglycan and in chlorophyll. Cyanobacteria can take up nitrogen in different combined forms such as nitrate, ammonium, organic nitrogen (e.g. amino-acids), urea or as molecular dinitrogen (N_2). In many environments, however, combined forms of nitrogen are in low supply. Organic nitrogen is subject to very active microbiological transformations. Deamination, nitrification and denitrification result eventually in the conversion of any form of combined nitrogen to dinitrogen. Moreover, many macroalgae or macrophytes are known to perform luxury consumption of nitrogen and can scavenge it effectively from the environment (Fujita, Wheeler & Edwards, 1989; Smith & Horne, 1988). The largest pool of nitrogen on Earth is in the form of dinitrogen, which is practically inexhaustible. However, because of the very stable triple bond between the two nitrogen atoms, only certain specialized organisms (diazotrophs) are capable of using dinitrogen as nitrogen source. These are prokaryotes, capable of synthesizing nitrogenase, which catalyses the following reaction:

$$N_2 + 8Fd^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 8Fd + 16ADP + 16P_i \quad (6)$$

This equation shows that nitrogenase has a high requirement for energy and a low-potential electron donor (reduced ferredoxin). In order to be capable of living diazotrophically, organisms must provide sufficient energy. Although this presents a problem for most organisms in the natural environment, cyanobacteria as photoautotrophic organisms use light and water for this purpose. It is, therefore, not surprising that many cyanobacterial species possess the capacity for diazotrophic growth. But, since nitrogenase is extremely sensitive to oxygen and can only be active in a totally anoxic environment, its occurrence in cyanobacteria is paradoxical (Fay, 1992; Gallon, 1992; Gallon & Stal, 1992), since cyanobacteria not only usually live in oxic environments but also produce oxygen intracellularly as a product of photosynthesis. Different types of nitrogen-fixing cyanobacteria can be distinguished (Table 6).

Since fixation of nitrogen must occur within an oxygen-free environment, cyanobacteria have to fulfil two important conditions: (i) nitrogen fixation must be separated from oxygenic photosynthesis and (ii) the entrance of oxygen into the cells must be prevented or at least limited. Heterocystous cyanobacteria have solved this problem by differentiating special cells – the heterocysts (Haselkorn, 1978). These cells have lost the capacity for oxygenic photosynthesis but can transform light into biochemical energy via photosystem I (PS I) (cyclic photophosphorylation). The heterocyst has a thick cell wall that presents a diffusion barrier for gases (Walsby, 1985): this thickness can be altered by the ambient oxygen concentration (Kangatharalingam, Priscu & Paerl, 1992). As a consequence of respiratory activity, the interior of the heterocyst is virtually anoxic (Walsby, 1985). It has long been known that the heterocyst is the site of nitrogen fixation in heterocystous cyanobacteria but direct immunological evidence has been obtained to support this (Bergman, Lindblad & Rai, 1986). The heterocystous cyanobacteria thus separate oxygenic photosynthesis from nitrogen fixation spatially, and have mechanisms to eliminate oxygen from the site of nitrogen fixation. Although the heterocyst can provide nitrogenase with ATP, reducing equivalents have to be imported from the vegetative cells, which in return receive fixed nitrogen. The heterocyst is not viable when not attached to a vegetative cell.

Although there is no doubt that the heterocyst is the site of nitrogen fixation and that these cells are required for the effective protection of nitrogenase from oxygen, N fixation activity has been observed in cultures that lack heterocysts. Kurz & LaRue

Table 6. *Types and characteristics of nitrogen-fixing cyanobacteria*

Type I Heterocystous Cyanobacteria
- Exclusively filamentous species that differentiate special cells: heterocysts
- Strategy: spatial separation of N_2 fixation and oxygenic photosynthesis and protection of nitrogenase in the heterocyst
- Diazotrophic growth under fully oxic conditions
- Examples: *Anabaena*, *Nostoc*, *Aphanizomenon*, *Nodularia*, *Calothrix*, *Scytonema*
- Occurrence: waterblooms (freshwater lakes and brackish seas), paddy fields, microbial mats; symbiotic with a variety of different organisms

Type II Anaerobic N_2-Fixing Non-Heterocystous Cyanobacteria
- Filamentous and unicellular species
- Strategy: avoidance (of oxygen)
- Induction and maintenance of nitrogenase only under anoxia or low oxygen
- Examples: *Plectonema boryanum*, *Oscillatoria limnetica*, *Synechococcus* sp., many other cyanobacteria
- Occurrence: many different environments but unclear whether growing diazotrophically

Type III Aerobic N_2-Fixing Non-Heterocystous Cyanobacteria
- Filamentous and unicellular species
- Strategy not precisely known (possibly temporal separation of N_2 fixation and oxygenic photosynthesis in concert with other oxygen protection mechanisms)
- Diazotrophic growth possible under fully oxic conditions
- Examples: *Gloeothece*, *Oscillatoria*, *Trichodesmium*, *Lyngbya*, *Microcoleus*
- Occurrence: tropical ocean (*Trichodesmium*), carbonate cave walls and paddy fields (*Gloeothece*), microbial mats (*Oscillatoria*, *Lyngbya*, *Microcoleus*)

(1971) were able to grow *Anabaena flos-aquae* in continuous culture in the absence of combined nitrogen. These cultures were virtually devoid of heterocysts but nevertheless nitrogenase activity was detected. Rippka & Stanier (1978) also demonstrated nitrogenase activity in the vegetative cells of Nostocacean cyanobacteria, using an anaerobic induction technique. However, in *Anabaena* the *nif* genes are rearranged during heterocyst differentiation and this process is a prerequisite for nitrogen fixation in this organism (Golden, Robinson & Haselkorn, 1985). Table 6 shows some of the characteristics of heterocystous cyanobacteria and examples of species and their occurrence.

A large number of filamentous and unicellular non-heterocystous cyanobacteria are known that can synthesize nitrogenase (Rippka & Waterbury, 1977). Many cyanobacteria are able to fix nitrogen only under anoxic conditions when oxygenic photosynthesis is inhibited. Their principal strategy therefore is the avoidance of oxygen. Anaerobic N_2-fixing cyanobacteria are known from a wide range of different environments. In most cases it is unclear whether these cyanobacteria can perform diazotrophic growth in the natural environment. In only one environment (Solar Lake) has anaerobic nitrogen fixation by a cyanobacterium been reported (Padan & Cohen, 1982). Solar Lake is a sulphide-dominated ecosystem and can be classified as a sulphuretum. Sulphureta are anoxic environments with large concentrations of sulphide (Baas Becking, 1925). Sulphide is known to inhibit oxygenic photosynthesis and the cyanobacteria in such environments are therefore believed to carry out anoxygenic photosynthesis. Such environments would provide excellent conditions for anaerobic nitrogen fixation. The reason that this has rarely been reported might be that cyanobacteria are not often the most conspicuous organisms in sulphureta, and such environments might also not be nitrogen limited. Bebout *et al.* (1993) provided evidence that nitrogen fixation in a microbial mat might have been driven by sulphide. Table 6 summarizes the characteristics of anaerobic nitrogen-fixing cyanobacteria and their occurrence.

The number of reports of non-heterocystous cyanobacteria, unicellular as well as filamentous, that are capable of nitrogen fixation under fully oxic conditions while carrying out oxygenic photosynthesis is increasing (Fay, 1992; Gallon & Stal, 1992). The strategy by which such organisms protect nitrogenase against oxygen is not known precisely. It has been suggested that by analogy with the spatial separation of oxygenic photosynthesis and nitrogen fixation in the heterocystous cyanobacteria, these organisms separate the processes temporally (Kallas *et al.*, 1983; Mullineaux, Gallon & Chaplin, 1981; Stal & Krumbein, 1985*a*, 1987). However, this is certainly not the only mechanism for nitrogen-fixation under oxic conditions and additional mechanisms must be present in order to exclude atmospheric oxygen from the site of nitrogen fix-

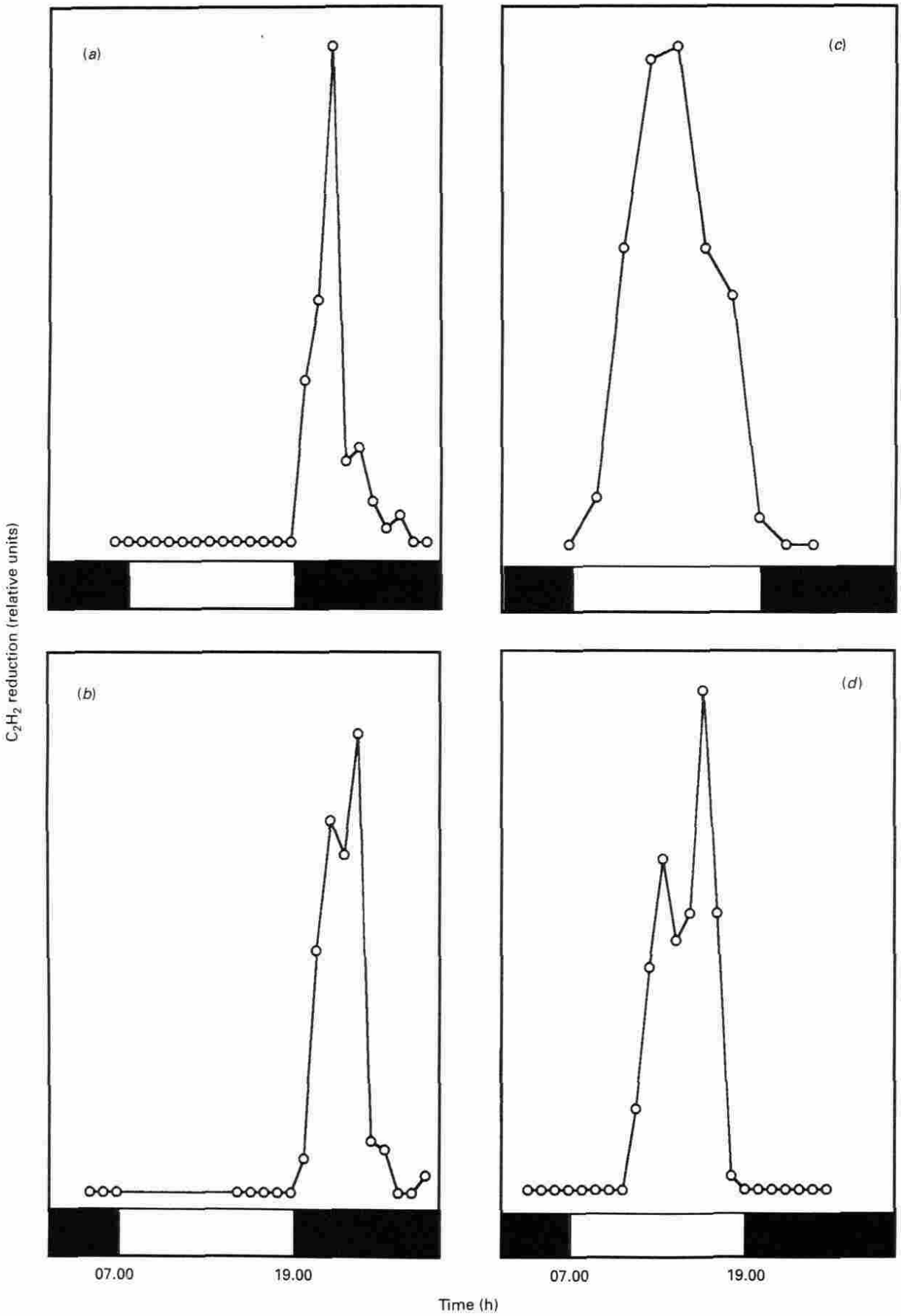

Figure 4. For legend see opposite.

ation (Fay, 1992; Gallon, 1992). It is highly possible that different mechanisms or combinations of mechanisms are used by different organisms. Table 6 lists the properties of aerobic nitrogen-fixing non-heterocystous cyanobacteria.

2. *Diel cycles of nitrogenase activity in natural communities of cyanobacteria*

Nitrogen fixation in natural communities of cyanobacteria shows distinct patterns, depending on the type of organism (Fig. 4). These patterns are the result of both the energy demand of nitrogenase and its oxygen sensitivity. In Fig. 4*d* the diel variation of nitrogenase activity of a mat of *Nostoc* sp. on a cave wall is depicted. Heterocystous cyanobacteria always show a clear light-dependence of nitrogenase activity both in natural communities and in cultures (Hübel & Hübel, 1974; Griffiths, Gallon & Chaplin, 1987). This is to be expected, considering that the heterocyst is capable of using light through photosystem I (PS I) and that these cells depend on vegetative cells for reducing equivalents and for acceptor molecules for the fixed nitrogen. A conflict with oxygenic photosynthesis does not exist since the heterocyst does not produce oxygen and is virtually anoxic. Heterocystous cyanobacteria usually show low nitrogenase activities in the dark although this might differ between different species (Stal & Krumbein, 1985*a*, *c*; Griffiths *et al.*, 1987) or cease almost completely (Mullineaux *et al.*, 1981). The limited diffusion of oxygen and the lack of endogenous storage carbohydrate is reflected in the inability of heterocysts to fix nitrogen in the dark.

The diel variation of nitrogen fixation in natural populations often shows a midday depression. This has been explained by a loss of buoyancy in planktonic organisms (Vanderhoef *et al.*, 1975) or, in microbial mats, by photorespiration which competes for reducing equivalents (Carpenter, Van Raalte & Valiela, 1978). Evidence for the latter was also obtained for cyanobacterial surface blooms (Storch, Saunders & Ostrofsky, 1990). In microbial mats as well as in surface blooms of cyanobacteria a combination of oxygen supersaturation and CO_2 depletion might occur. Such conditions promote photorespiration (Bergman, 1981) but the decreased fixation of CO_2 will also result in a lower supply of acceptor molecules for fixed nitrogen.

Many communities of non-heterocystous cyanobacteria show totally different patterns of nitrogenase activity. The unicellular cyanobacterium *Gloeothece* sp. forms mats on the inside of carbonate cave walls in dim light (Griffiths *et al.*, 1987). Nitrogenase activity was confined to the night and no activity was observed during daytime (Fig. 4*a*). The same pattern is known from several microbial mats dominated by the filamentous *Oscillatoria* sp. (Griffiths *et al.*, 1987), as shown in Fig. 4*c*. When grown under an alternating light-dark regime, many non-heterocystous cyanobacteria show the same pattern (Mullineaux *et al.*, 1981; Stal & Krumbein, 1985*a*). This pattern can best be explained by the mechanism of temporal separation of nitrogen fixation (at night) and photosynthesis (during day time). In contrast with heterocystous species, nitrogen fixation in the dark is possible because nitrogenase is located in photosynthetically fully active cells. Nitrogen fixation in the dark is fuelled by endogenous storage carbohydrate which is accumulated during the light period.

The fourth example of a diel variation of nitrogenase activity (Fig. 4*b*) concerns the marine planktonic cyanobacterium *Trichodesmium* sp. (Saino & Hattori, 1978). This organism is a filamentous non-heterocystous cyanobacterium but it nevertheless shows a diel variation of nitrogenase activity similar to that of heterocystous species. This clearly shows that temporal separation of nitrogen fixation and photosynthesis is not the only mechanism for nitrogen fixation in non-heterocystous cyanobacteria. The mechanism by which *Trichodesmium* sp. protects nitrogenase from oxygen inactivation is presently unknown. Because its behaviour is similar to that of heterocystous species, several authors have speculated about the possibility of a heterocyst-like cell differentiation morphologically indistinguishable from the vegetative cell (Carpenter & Price, 1976). Conclusive evidence to support this hypothesis is lacking (Carpenter *et al.*, 1990). Very recently Janson, Carpenter & Bergman (1994) showed, using immunological techniques, that nitrogenase was not uniformly distributed over the trichome, suggesting that some cells might have undergone differentiation. Earlier, these investigators found that nitrogenase was randomly distributed over the different trichomes within a colony, i.e. that nitrogenase was not confined to a certain part of the trichome (Bergman & Carpenter, 1991).

3. *Nitrogen fixation in* Gloeothece

Cultures of the unicellular cyanobacterium *Gloeothece* that are grown diazotrophically under

Figure 4. Four typical patterns of nitrogenase activity in natural populations of different types of cyanobacteria exposed to natural diel variations of light and darkness. In the non-heterocystous, unicellular cyanobacterium *Gloeothece* sp. nitrogenase activity is confined to the night (*a*). The same pattern is seen for the benthic non-heterocystous filamentous cyanobacterium *Oscillatoria* sp. (*b*). By contrast, the planktonic non-heterocystous cyanobacterium *Trichodesmium thiebautii* fixes nitrogen exclusively during the day (*c*). This pattern is very similar to that of the heterocystous *Nostoc* sp. (*d*). The data for (*a*), (*b*) and (*d*) were taken from Griffiths *et al.* (1987) and for (*c*) from Saino & Hattori (1978).

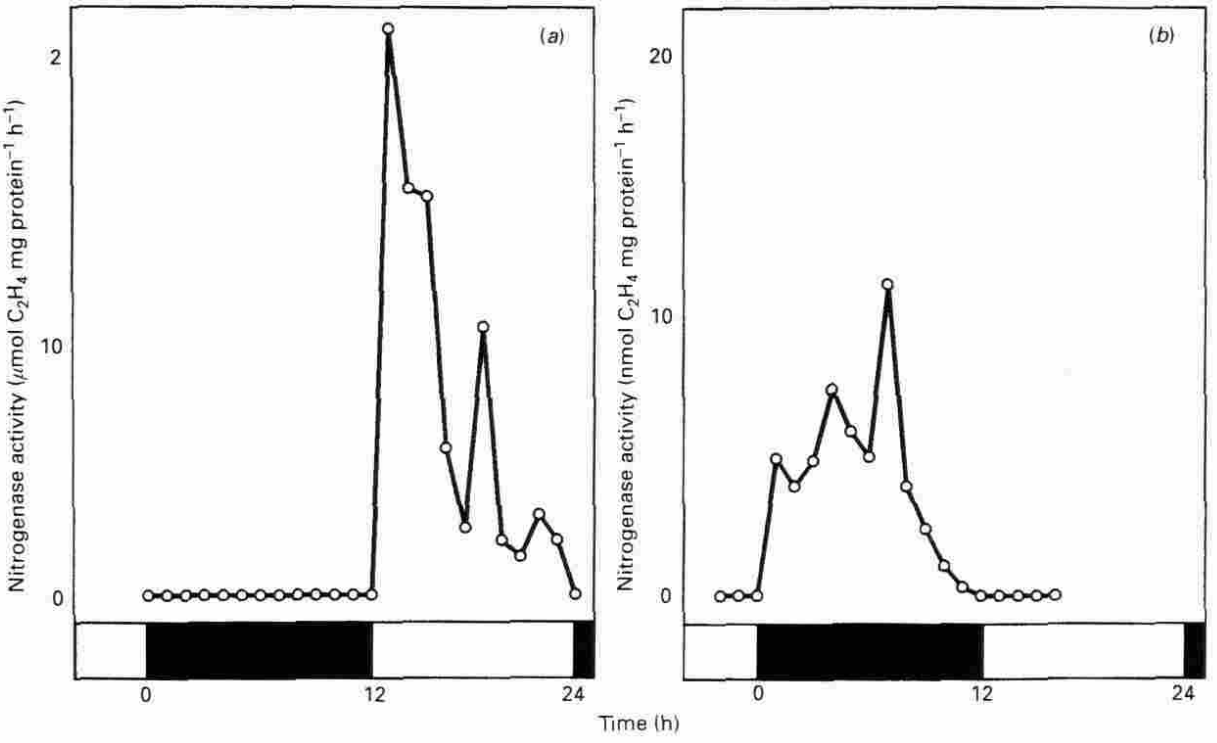

Figure 5. Patterns of nitrogenase activity in cultures of the unicellular cyanobacterium *Gloeothece* grown under 12–12 h light–dark cycle. (*a*) Continuous culture grown at D = 0·007 h^{-1} (data from Ortega-Calvo & Stal (1991)). (*b*) Batch culture (data from Stal *et al.* (1991)). Note the difference in scale which is three orders of magnitude larger in panel (*a*) (μmol) compared with panel (*b*) (nmol).

alternating light–dark cycles confine nitrogenase activity to the dark period. This pattern has also been found in a number of other unicellular and filamentous non-heterocystous species and it reflects the principle of temporal separation of nitrogen fixation and photosynthesis. However, *Gloeothece* and all other known non-heterocystous aerobic nitrogen-fixing cyanobacteria are capable of diazotrophic growth under continuous light. Moreover, the non-heterocystous cyanobacterium *Trichodesmium* sp. shows the opposite behaviour. Temporal separation of nitrogen fixation and photosynthesis could also occur under continuous light as was shown in several organisms, for instance *Plectonema boryanum* (Weare & Benemann, 1974; Giani & Krumbein, 1986), *Synechococcus* (Mitsui *et al.*, 1986), *Gloeothece* (Gallon, LaRue & Kurz, 1974) and *Oscillatoria limosa* (Stal & Krumbein, 1987). Nevertheless, the hypothesis of temporal separation of nitrogen fixation must be treated with caution.

Ortega-Calvo & Stal (1991) and Stal, Myint & Ortega-Calvo (1991) showed that continuous cultures of *Gloeothece* behaved differently from batch cultures of the same strain (Fig. 5). These authors noted two important differences between batch and continuous cultures of *Gloeothece*. In contrast to batch cultures, continuous cultures showed nitrogenase activity predominantly during the light period. This observation argues against the possibility of temporal separation of nitrogen fixation and photosynthesis in *Gloeothece*. Nitrogenase activity was up to three orders of magnitude higher in continuous cultures than in batch cultures. In fact, dark activity of nitrogenase in continuous culture was similar to that in batch cultures. The integrated nitrogen fixation over a 24 h period matched the requirements for growth in continuous culture (Table 7) fairly well. Continuous cultures also grew well under continuous light, but under alternating light–dark regime a minimum of 12 h light was necessary. In contrast, in batch cultures, *Gloeothece* grew even with as little as 4 h of light in a 24 h cycle. In order to explain these differences the culture conditions of batch and continuous cultures must be considered. The maximum growth rate of

Table 7. *Integrated rates of nitrogenase activity (acetylene reduction) and calculated rate of nitrogen fixation in continuous cultures of the unicellular cyanobacterium* Gloeothece *growing under an alternating light–dark cycle*

Dilution rate (h^{-1})	Acetylene reduction (nmol C_2H_4 mg^{-1} protein [24 h]$^{-1}$)	Nitrogen fixation (g N fixed g^{-1} N h^{-1})
0·005	1320	0·002
0·015	6130	0·008
0·020	2305	0·003

Data from Ortega-Calvo & Stal (1991).

Table 8. *Some properties of continuous cultures of the unicellular cyanobacterium* Gloeothece

Light*	Cycle†	Dilution‡	Medium§	A_{720}‖	Dry weight¶
22	16–8	0·007	−N	0·31	635
22	16–8	0·007	+N	0·31	880
28	16–8	0·015	−N	0·20	255
22	16–8	0·015	+N	0·30	715
18	CL	0·007	−N	0·29	730

* Light is the photon flux density (PFD) in the culture and expressed as $\mu E\ m^{-2}\ s^{-1}$.
† The light–dark cycle in hours. CL means continuous light.
‡ Dilution rate of the culture in h^{-1}.
§ Medium is either with nitrate (+N) or its nitrate-free variant (−N).
‖ A_{720} is the absorbance of the culture measured at 720 nm.
¶ Dry weight of the culture as $mg\ l^{-1}$.

Gloeothece in continuous culture was estimated as a doubling time of close to 24 h. The doubling time of growth in batch culture was always more than 100 h and was virtually independent of the light–dark regime imposed on the culture. The continuous cultures were well aerated in order to maintain optimum mixing, a prerequisite for this type of cultivation. The batch cultures used in that study were shaken in Erlenmeyer flasks in which mixing and gas exchange were much less efficient.

The explanation of the arrest of growth in continuous cultures at light periods of less than 12 h during a 24 h cycle may be as follows. During the light period, energy must be stored in order to continue metabolism during the dark period. When the light period is short, higher photosynthetic activity might be expected (Mur, 1983; Post *et al.*, 1986). Increased photosynthetic activity must result in a higher rate per cell of oxygen evolution which presumably exceeds the cell's ability to protect nitrogenase from oxygen inactivation. Also, in the well aerated continuous culture there is a higher flux of oxygen in the dark. Under such conditions the organism must spend a lot of effort to keep the intracellular concentration of oxygen sufficiently small. In addition, competition for electrons between nitrogenase and respiration might also limit seriously the fixation of nitrogen. Moreover, at high dilution rates ($D = 0{\cdot}020\ h^{-1}$) in continuous culture, nitrogenase activity in *Gloeothece* decreases dramatically (Table 7), a steady state cannot be achieved and the organism is washed out. The growth rate imposed would result in a rate of oxygen evolution per cell that is not compatible with nitrogenase activity.

Ortega-Calvo & Stal (1991) assumed that their continuous cultures were limited by light. However, they discovered recently that their cultures did not behave in all respects as light-limited cultures (Ortega-Calvo & Stal, 1994). From Table 8 the following conclusions can be drawn. (i) At a dilution rate (D) of $0{\cdot}007\ h^{-1}$ no difference in biomass between the culture grown with nitrate and the nitrogen-fixing culture was observed. This means that nitrogen fixation did not limit growth. (ii) There was also no difference in biomass between the nitrate grown cultures at $D = 0{\cdot}007$ and $D = 0{\cdot}015\ h^{-1}$ which means that in this case light was not limiting. If light was limiting one would expect higher biomass at the lower growth rate (D) (Mur, 1983). With a constant incident irradiance, the photon flux density (PFD, the mean irradiance as experienced by the cells) depends on the cell density (self shading). Thus, high biomass gives rise to low PFD, resulting in low growth rates and vice versa for low biomass. (iii) Therefore light limitation might have occurred only in the nitrogen-fixing culture grown at $D = 0{\cdot}015\ h^{-1}$ (compare the N_2-fixing cultures at $D = 0{\cdot}007$ and $D = 0{\cdot}015\ h^{-1}$).

Ortega-Calvo & Stal (1994) showed that sulphur was the factor limiting growth in these cultures of

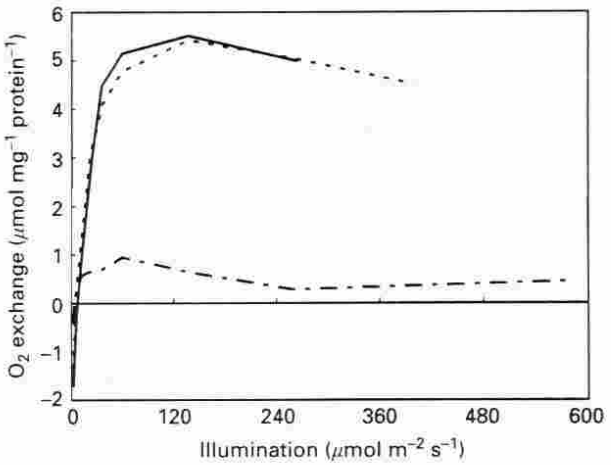

Figure 6. Photosynthesis versus light curves for the unicellular cyanobacterium *Gloeothece*. The solid line shows the curve for a continuous culture ($D = 0{\cdot}0007\ h^{-1}$, continuous light, nitrate-free medium), the dashed line the curve for the same culture after 7 d of batch cultivation without the addition of sulphate. The dotted line represents the curve 2 d after addition of 0·3 mM sulphate to the sulphate-depleted culture (data from Ortega-Calvo & Stal (1994)).

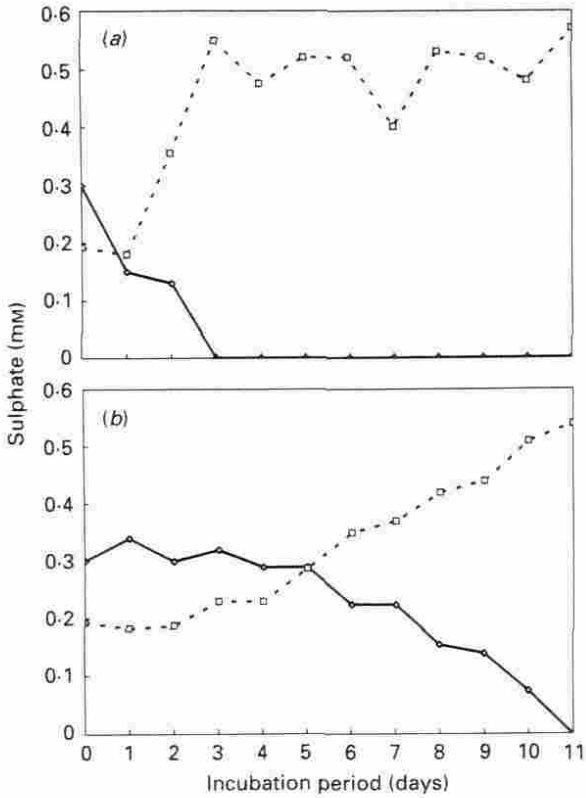

Figure 7. Fate of sulphate in the unicellular cyanobacterium *Gloeothece*. At day zero 0·3 mM sulphate was added to a culture and sulphate was monitored during the following days in the medium (solid line) and as associated with sheath material (sulphated sugars) (dotted line). The experiment was made under illumination at 70 (*a*) or 10 (*b*) μmol m^{-2} s^{-1} (data from Ortega-Calvo & Stal (1994)).

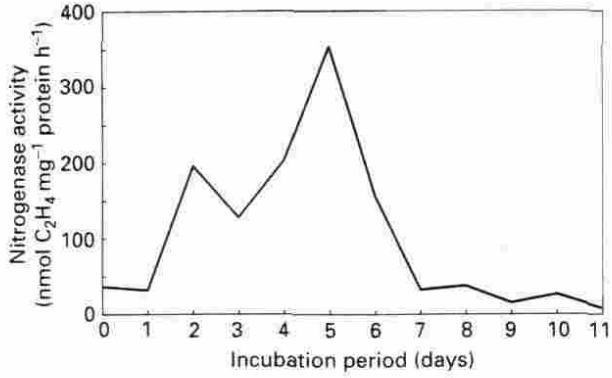

Figure 8. The pattern of nitrogenase activity (acetylene reduction) after addition of sulphate to a sulphate-depleted culture of the unicellular cyanobacterium *Gloeothece*.

Gloeothece. Cells of *Gloeothece* are ensheathed in a polysaccharide matrix which contains a large concentration of sulphated sugars. Figure 6 shows photosynthesis versus irradiance curves. When a N_2-fixing continuous culture was switched to batch mode the maximum rate of photosynthesis decreased dramatically. Addition of sulphate restored the original photosynthetic capacity of the culture within 2 d. The sulphate can be recovered completely from the polysaccharide sheath (Fig. 7). There is no difference between the nitrate and N_2 grown cultures but light intensity seemed to be important. From Figure 7 it is evident that the sulphate incorporation is much slower at low light intensity. This might very well explain the observation that *Gloeothece* grows very well only under low light intensities (Kallas *et al.*, 1983). The authors suggested that *Gloeothece* could not maintain nitrogenase activity at high light because of the resulting high production of oxygen. However, Myint (1991) observed that low light conditions were equally important for nitrate-grown cultures. High light rapidly depletes the medium of sulphate, and this arrests protein synthesis and growth in the same way as nitrogen depletion (Wanner *et al.*, 1986). The phycobiliproteins are degraded and the culture bleaches while the carbohydrate content of the cell increases. Addition of sulphate to such cultures results in the re-synthesis of phycobiliproteins and arrests the accumulation of carbohydrate. Subsequently, growth resumes, but only as long as free sulphate is present. The sulphate is again rapidly taken up and immobilized in the sheath.

Nitrogenase activity in sulphate-depleted batch cultures of *Gloeothece* is very low. Addition of sulphate to such cultures resulted in a dramatic increase of nitrogenase activity (Fig. 8). After a few days it reached a maximum and subsequently it decreased to the very low levels that are usually found in batch cultures. The sulphate-induced nitrogenase activity was similar to that observed in continuous cultures and occurred in the light. Activity was also clearly light-dependent, since under low light it was much smaller. Although in continuous cultures nitrogenase activity matched the requirements for growth (Ortega-Calvo & Stal, 1991), in these batch cultures it seemed that nitrogen fixation and growth were out of phase. In particular, under low light, activity of nitrogenase oscillated. It is suggested that, at the peaks of activity, nitrogen fixation exceeded the requirements for growth. A feed-back regulation therefore decreased activity until intracellular nitrogen became depleted again, resulting in a subsequent increase of activity. After a few oscillations, activity became increasingly damped, probably as a result of tuning the fixation of nitrogen to the requirements for growth. Another interesting phenomenon of nitrogen-fixing sulphate-repleted cultures was that the maximum rate of photosynthesis was much lower compared with nitrate-grown cultures, though the affinity for light (α) was not different. This indicates that growth on N_2 does not affect photosynthesis. A possible explanation for the lower maximum rate of photosynthetic oxygen evolution is a nitrogenase-associated oxygen uptake.

In experiments in which very high nitrogenase activities were induced, cultures were even showing net oxygen uptake in the light. This has been

shown for *Gloeothece* sp. (Stal & Myint, unpublished), *Oscillatoria limosa* (Stal & Krumbein, 1987) and for *Synechococcus* sp. (Mitsui *et al.*, 1986). Maryan *et al.* (1986) concluded that nitrogen fixation in *Gloeothece* is supported by respiration rather than photosynthesis. Peschek, Villgrater & Wastyn (1991) observed that cytochrome *c* oxidase activities in membranes of nitrogen-fixing cells were between 10- and 50-times higher than in membranes of nitrate-grown *Gloeothece*. They found very high rates of respiration in nitrogen-fixing cells and also observed that this respiration was much less inhibited by light than was the case in nitrate-grown cells. These results were taken as evidence for the respiratory protection of nitrogenase in non-heterocystous nitrogen-fixing organisms. Although none of these observations supports the hypothesis of the temporal separation of nitrogen fixation and oxygenic photosynthesis, this possibility cannot be excluded. It is still possible that the culture is composed of two populations of cells: some that fix nitrogen while photosynthesis is switched off and some that do the opposite. Depending on its physiological state, the cell could switch from one mode to the other. At the cellular level we would then see a temporal separation, whereas at the culture level a spatial separation is exerted. The possibility that cells differentiate in a sort of heterocyst seems unlikely, since immunological studies have shown that nitrogenase is present in all cells (Gallon & Bergman, unpublished), nor did fluorescence measurements of single cells reveal a different cell type (Stal, unpublished).

Sulphate depletion of the culture not only decreases the growth rate of the organism dramatically, and with it the rate of nitrogen fixation (which in steady-state cells is closely tuned to growth rate), but it might also interfere with oxygen protection of nitrogenase. The fact that nitrogenase is shifted to the dark might also be the result of the failure to synthesize glutathione which might be involved in oxygen protection in cyanobacteria (Bergman, personal communication). There is no doubt that *Gloeothece* shows a nitrogenase-associated oxygen uptake. In this context it is noteworthy that nitrogenase of *Azotobacter vinelandii* is capable of reducing oxygen (Thorneley & Ashby, 1989). The physiological importance of this so-called autoprotection of nitrogenase needs to be proved.

4. *Heterocystous versus non-heterocystous cyanobacteria*

Heterocystous cyanobacteria are obviously best adapted for diazotrophic growth. Because they make use of light to supply nitrogenase with energy, they can fix more nitrogen than can non-heterocystous species, which under natural conditions are forced to fix nitrogen in the night period when the possibilities for energy generation are much less. An exception is *Trichodesmium* in which nitrogenase is confined to daytime. Even so, nitrogen fixation in this organism is still much less efficient than in heterocystous species. *Trichodesmium* nitrogenase is irreversibly inactivated during the night and must be resynthesized every day (Capone *et al.*, 1990). This is not the case in heterocystous species, where nitrogenase is protected in the anoxic environment of the heterocyst and remains fully active.

The marine environment is usually considered to be nitrogen-limited (Dugdale, 1967). The capacity for diazotrophic growth would therefore present an important advantage for marine cyanobacteria. Notwithstanding, heterocystous cyanobacteria are rarely encountered in the marine environment (Stal *et al.*, 1994). Notably, marine microbial mats are known to be active nitrogen-fixing communities, yet the majority of these mats are formed by non-heterocystous species. We have investigated one of the few existing examples of a microbial mat formed by a heterocystous species. In intertidal flats near Guerrero Negro, Baja California, Mexico, two types of mats are present. They occur close to each other yet they occupy slightly different areas and are well separated (Fig. 9*a*). The smooth mat is composed of the non-heterocystous cyanobacterium *Lyngbya aestuarii* (Fig. 9*f*) whereas the pustular mat is formed by the heterocystous cyanobacterium *Calothrix* sp. (Fig. 9*g*). Both mats show high rates of nitrogenase activity. The *Calothrix* mat shows highest nitrogenase activities in the light (Fig. 10*a*). Dark activity is only a fraction of the activity in the light. DCMU, which inhibits PS II, has a slightly inhibitory effect. Because PS II is absent from heterocysts, DCMU would be expected to have no effect at all. However, the heterocyst depends on the import of reducing equivalents from the vegetative cells and this process is certainly affected by DCMU. In addition, DCMU might have some overall negative effects on cell metabolism.

The *Lyngbya* mat behaves differently. In this case DCMU greatly stimulates nitrogenase activity (Fig. 10*b*). This can only be interpreted as the elimination of the inactivating effect of photosynthetically evolved oxygen. Apparently, photosynthetic oxygen has such an inhibitory effect that the organism shows even higher nitrogenase activity in the dark. The diel variations of nitrogen fixation in these mats reflect this (Fig. 11). In the *Calothrix* mat, nitrogenase activity is totally confined to the light, whereas the *Lyngbya* mat fixes nitrogen only during the night. Since both mats occur very close to each other they would be expected to experience the same environmental conditions. The only difference that was noted was that the *Calothrix* mats occupied areas that were slightly higher in the intertidal flat than the mats of *Lyngbya*. This means that the *Lyngbya* mats are submersed more often and for longer periods of

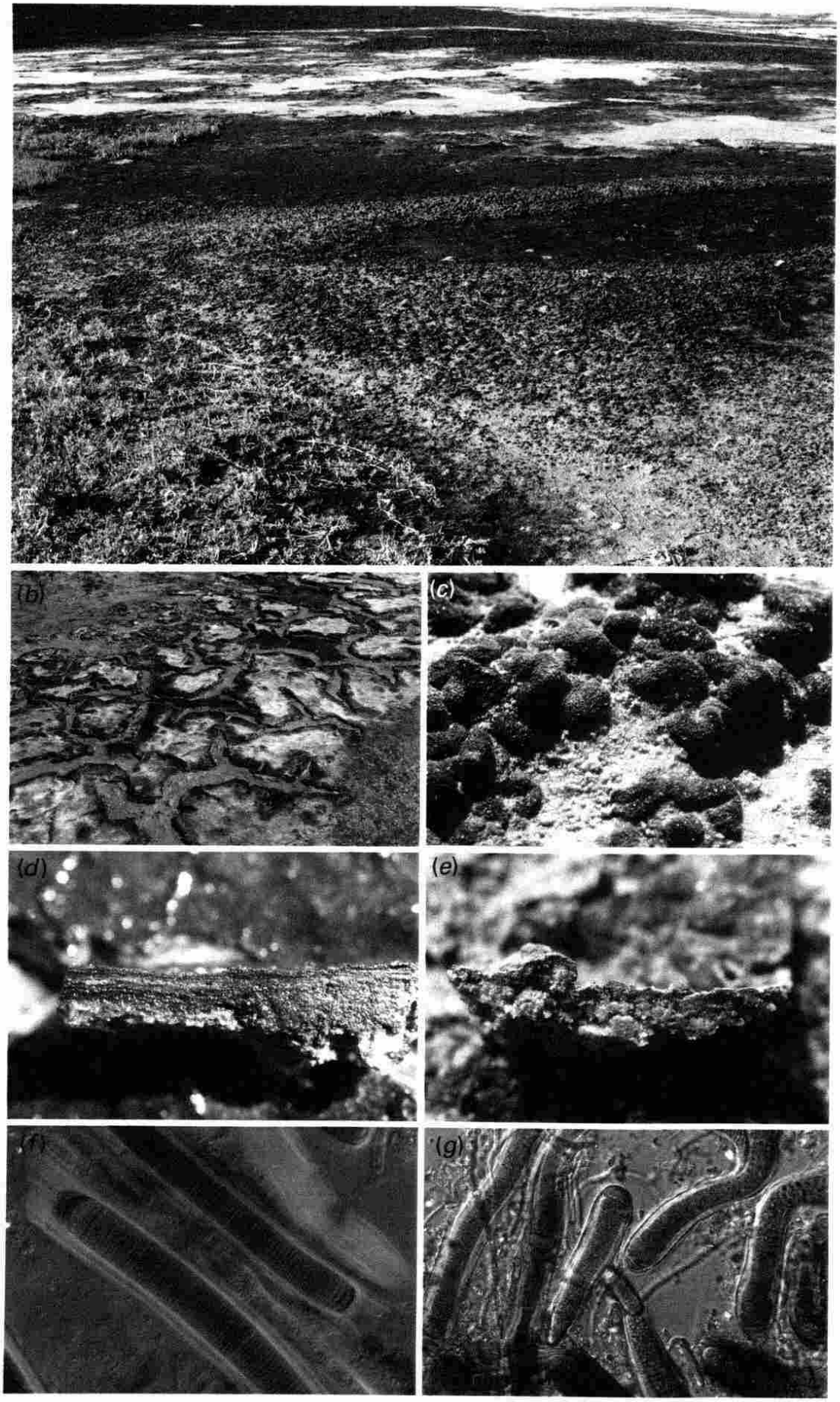

Figure 9. For legend see opposite.

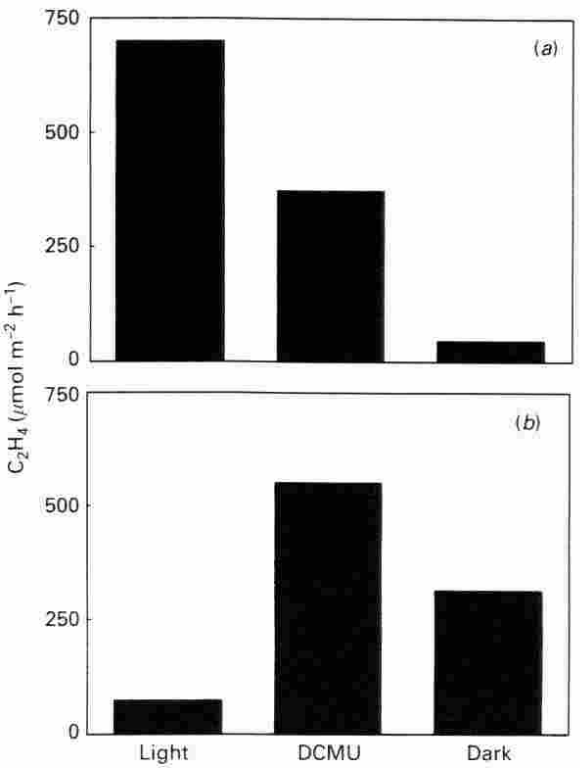

Figure 10. Effects of light and oxygenic photosynthesis on nitrogenase activity (acetylene reduction) in mats of *Calothrix* sp. (*a*) and *Lyngbya aestuarii* (*b*). Light and DCMU incubations were done under ambient light. DCMU (10^{-5} M) was added to inhibit oxygenic photosynthesis (PS II). Dark incubations were done on the same mat samples. (Data from Stal *et al.* (1994).)

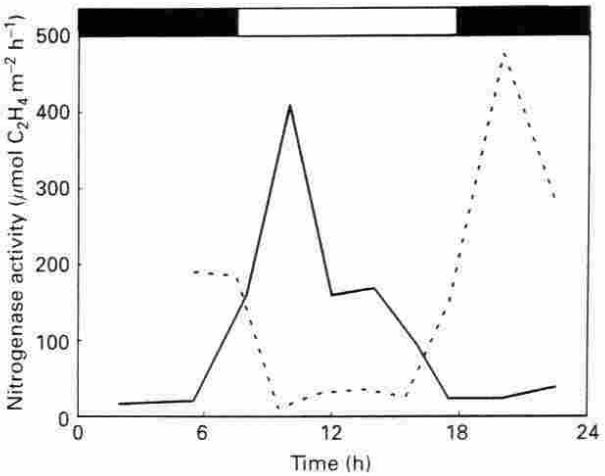

Figure 11. Diel variations of nitrogenase activity (acetylene reduction) in mats of *Calothrix* sp. (solid line) and of *Lyngbya aestuarii* (dotted line). The night is indicated with a black bar at the bottom of the panel. Nitrogen fixation by *Calothrix* sp. is clearly light-dependent whereas in the mat of *L. aestuarii* it is confined mostly to the dark period. (Data from Stal *et al.* (1994).)

time. Obviously, this might have a number of important consequences such as desiccation of the *Calothrix* mats.

Figures 9*d* and *e* show the cross sections of both mat types. From the cross sections it is apparent that the *Calothrix* mat lacks the black horizon of FeS. This mat is porous, which allows good gas exchange with the atmosphere and the penetration of oxygen into the sediment. The sulphide-producing sulphate-reducing bacteria are obligately anaerobic. Hence, the absence of the black layer can be interpreted as indicating the absence of anoxic conditions in this mat. By contrast, in the *Lyngbya* mat the black layer indicates the presence of an anoxic layer. Micro-electrode measurements supported these differences. Oxygen micro-profiles were measured in *Calothrix* and *Lyngbya* mats after reaching steady state in the light or in the dark. In addition, profiles were measured during inundation, which simulated high tide and during exposure to the atmosphere, which simulated low tide. As argued above, *Calothrix* mats experience only occasionally the situation of inundation. The profiles (Fig. 12) indicate that the dense *Lyngbya* mats turn anoxic during the dark, whether or not they are inundated. In the light, a supersaturation of oxygen was always observed. By contrast, *Calothrix* mats exposed to the atmosphere were always more or less air-saturated with oxygen. High levels of supersaturation with oxygen were not observed during daytime, nor were anoxic conditions established during the night. Such conditions would occur only if the mat was inundated

Figure 9. (*a*) Microbial mats in intertidal flats near Guerrero Negro, Baja California, Mexico. Two different mat types occur close to each other. The dark areas represent the smooth mats and the lighter areas the pustular mats. Note the difference in height. The pustular mats are located higher in the intertidal region than the smooth mats. (*b*) Detail of the smooth mat type. The mats show cracks which are typical of this type of mat and are caused by desiccation at low tide when the mat is exposed to the atmosphere and solar irradiation. (*c*) Detail of the pustular mat. (*d*) Cross-section of the smooth mat. The thin dense layer of cyanobacteria is clearly visible. This layer is about 1–2 mm thick. Underneath the cyanobacterial layer, a layer of purple sulphur bacteria is present, above a black layer of FeS. This mat is clearly very reduced. The presence of the intense black layer and the purple sulphur bacteria demonstrates that free sulphide must be present. (*e*) The cross-section of the pustular mat shows a layer of cyanobacteria that is only 2–4 mm thick and much less dense than that of the smooth mat. It is mixed with some sediment particles. Underneath the layer of cyanobacteria, white sand is visible. This mat is clearly not reduced. There is no black layer of FeS and there is no layer of purple sulphur bacteria. (*f*) The smooth mat is composed of the filamentous, non-heterocystous cyanobacterium *Lyngbya aestuarii*. This organism forms a thick polysaccharide sheath. The trichomes are embedded in a dense matrix of mucilage. (*g*) The pustular mat is formed by the heterocystous cyanobacterium *Calothrix* sp. The terminal heterocysts which are characteristic of this genus are clearly visible.

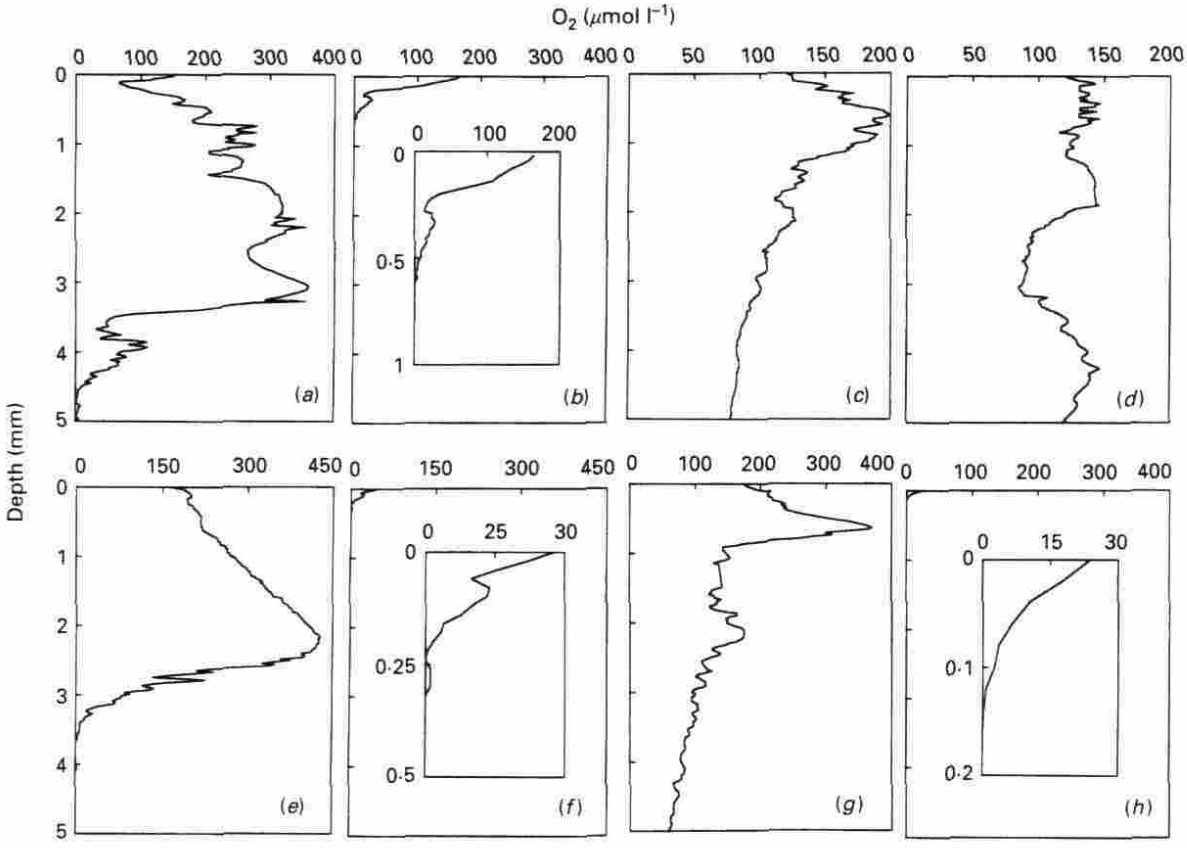

Figure 12. Profiles of oxygen in mats of *Lyngbya aestuarii* (left 4 panels; *a*, *b*, *e* and *f*) and of *Calothrix* sp. (right 4 panels; *c*, *d*, *g* and *h*). The upper 4 panels (*a–d*) show the situation of the mats when exposed to the atmosphere (simulating low tide) and the lower 4 panels (*e–h*) when inundated (simulating high tide). The panels *a*, *c*, *e* and *g* show the situation in the light whereas the panels *b*, *d*, *f* and *h* represent the situation in the dark. The inserts in the panels *b*, *f* and *h* show the oxygen profile in the upper few μm of the mats. The oxygen profiles show that the *L. aestuarii* mats are supersaturated with oxygen in the light and virtually anoxic during the night and that there is no difference between the high or low tide situations. In the mat of *Calothrix* sp., supersaturation in the light and anoxia at night occurs only during the high tide situation which is exceptional for this mat. When the mat is exposed to the atmosphere it stays oxygenated throughout the day and the night whereas some supersaturation is observed in the light. (Data from Stal *et al.* (1994).)

which, as argued above, occurs only rarely. From these measurements and observations it can be concluded that if conditions are suitable, heterocystous cyanobacteria will be the organisms most likely to form the mat. However, when anoxia is established and/or when free sulphide is present, heterocystous species are apparently excluded. Howsley & Pearson (1979) showed that heterocystous cyanobacteria might be more susceptible to sulphide toxicity. Oxygen supersaturation forces non-heterocystous cyanobacteria to confine nitrogen fixation to the dark period. Anoxic conditions during the dark promote nitrogenase activity although energy generation will be minimal under such circumstances.

5. *Nitrogen fixation in* Oscillatoria *mats*

Microbial mats in the intertidal sediments of the southern North Sea are formed by the cyanobacterium *Microcoleus chthonoplastes*. However, such mats often contain considerable numbers of another species, *Oscillatoria limosa*, which has been shown to be the pioneer organism in such sediments. This filamentous, non-heterocystous cyanobacterium was shown to be capable of aerobic nitrogen fixation in pure culture (Stal & Krumbein, 1981). It was also noted that these microbial mats possessed high nitrogenase activity and that this correlated positively with the number of *O. limosa*. Freshly colonized sediment was often dominated by this cyanobacterium. Seasonal changes were also noted. Annual mats showed greater numbers of *O. limosa* at the beginning of the growth season when highest nitrogenase activities were also detected.

Nitrogenase in the mats of *O. limosa* shows a clear sensitivity towards atmospheric oxygen and activity is stimulated when oxygenic photosynthesis is inhibited by DCMU. Also, nitrogenase activity shows a marked variation with depth. Specific nitrogenase activity, when related to chlorophyll *a*, shows a marked increase with depth of the sediment. These results suggest a spatial separation of nitrogen fixation and photosynthesis in the ecosystem. In the

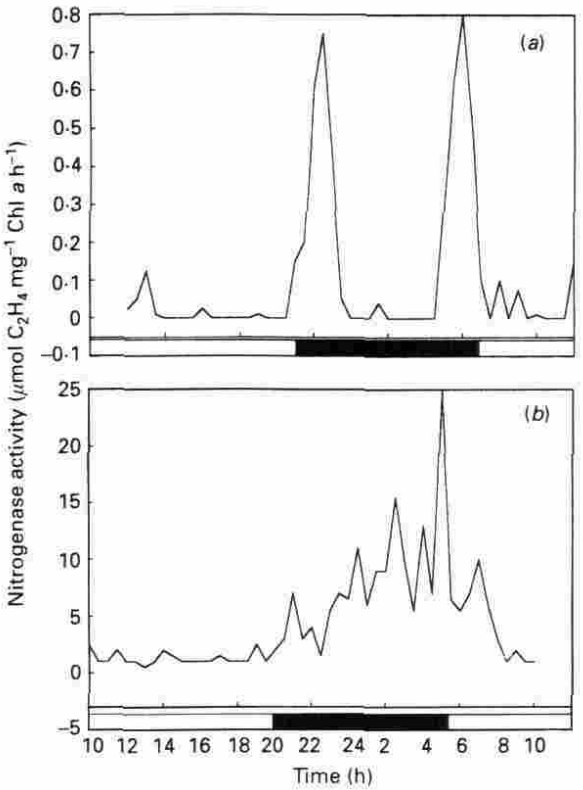

Figure 13. Diel patterns of nitrogenase activity (acetylene reduction) in mats of the non-heterocystous cyanobacterium *Oscillatoria limosa*. (*a*) Established microbial mats in which, besides *O. limosa*, the cyanobacterium *Microcoleus chthonoplastes* dominates. These mats show strong oxygen dynamics and turn completely anoxic during the night. (*b*) An early stage of development of the same mat in which *O. limosa* is almost exclusively present. This mat shows decreased concentrations of oxygen during the night but often does not become totally anoxic. During day time very high oxygen supersaturation may occur. (Data from Villbrandt *et al.* (1990).)

deeper layers, light that penetrates the sediment will not support oxygenic photosynthesis. Far red light, which penetrates the sediment fairly well (Stal *et al.*, 1985; Jørgensen & Des Marais, 1988), is used by PS I and might mediate anoxygenic photosynthesis. It supports nitrogenase activity in *O. limosa* very well (Stal & Krumbein, 1985*b*). Although Bebout *et al.* (1993) obtained evidence that in a similar microbial mat sulphide might have served as the electron donor for nitrogen fixation, *O. limosa* is not capable of sulphide-dependent anoxygenic photosynthesis. This means that nitrogen fixation in the deeper layers of the sediment depends on other electron donors. Hydrogen could serve as such (Stal, unpublished) but it is uncertain whether this electron donor is available in sufficient amounts in microbial mats. Several cyanobacteria are capable of utilizing organic compounds (Rippka, 1972), which might serve as electron donors for nitrogenase (Reddy *et al.*, 1993). This mode of metabolism has not been investigated in *O. limosa*. It is also uncertain whether organic compounds are available for free-living cyanobacteria in the natural environment owing to the poor affinity these organisms have for these substances (Smith, 1982). A possibility could be the use of endogenous storage carbohydrate, but this would allow nitrogen fixation for only a limited period. Many cyanobacteria that inhabit microbial mats are motile. It would be interesting to investigate whether cyanobacteria such as *O. limosa* move up and down in the mat to fix nitrogen below the euphotic depth or to photosynthesize at the sediment surface.

Mats of *O. limosa* show marked diel variations of nitrogen fixation. In freshly colonized sediment, in which the mat is formed almost exclusively by *O. limosa*, the typical pattern of nitrogenase activity for non-heterocystous cyanobacteria is seen. Nitrogenase activity is confined almost exclusively to the dark. However, in these mats activity usually reaches a maximum at sunrise (Fig. 13*b*) (Villbrandt *et al.*, 1990). Such mats contain relatively little biomass, show high specific rates of photosynthesis, accumulate large concentrations of oxygen in the light and often do not turn anoxic during the night. By contrast, established mats of a mixed community of *M. chthonoplastes* and *O. limosa* turn anoxic at night. In such mats nitrogenase activity is often confined to the transitions of light to dark and vice versa (sunset and sunrise) (Fig. 13*a*). At sunrise, activity is often greater (Stal, Grossberger & Krumbein, 1984), because light is available but the sediment is still anoxic. Such conditions seem to support nitrogen fixation best. During day time, oxygenic photosynthesis and oxygen supersaturation of the sediment would prevent nitrogen fixation in non-heterocystous cyanobacteria, whereas at night anoxia would impose energetically unsuitable conditions. This pattern of nitrogenase was shown in cultures of *O. limosa* grown under an alternating light–dark regime with anoxia during the dark (Fig. 14) (Stal & Heyer, 1987). Microbial mats often show a variety of different patterns that are probably the result of a combination of factors (Bebout *et al.*, 1987; Paerl, Bebout & Prufert, 1989; Paerl *et al.*, 1994). In addition, it cannot be discounted that other microorganisms contribute to nitrogen fixation in the ecosystem.

The mechanisms by which *O. limosa* is capable of diazotrophic photoautotrophic growth and by which it protects nitrogenase from oxygen inactivation are not completely understood. Immunological investigations have shown that nitrogenase is present in all cells of a trichome, presumably in all trichomes of a N_2-fixing culture, and that it is distributed homogeneously in the cell (Stal & Bergman, 1990). The density of antigen in cells of *O. limosa* was similar to that found in heterocysts. Western blotting revealed the existence of two bands of the Fe protein of nitrogenase. The appearance of a double band of the Fe protein has been found also in other cyano-

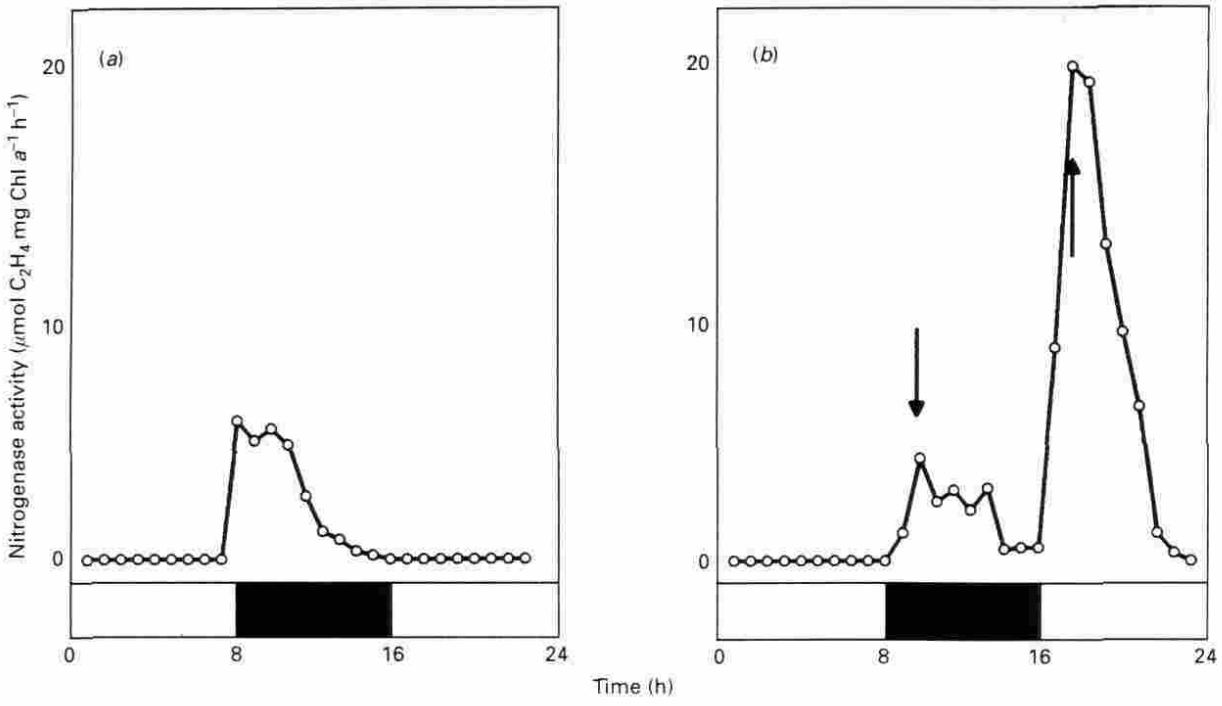

Figure 14. Pattern of nitrogenase activity in cultures of the non-heterocystous cyanobacterium *Oscillatoria limosa*. (*a*) Culture grown under air and a 16–8 light–dark cycle. (*b*) As (*a*) but the culture was transferred to an anoxic atmosphere of N_2 1 h after the onset of the dark period (arrow down) and back to air 1 h after the light turned on (arrow up).

bacteria, for example *Anabaena* (Ernst, Reich & Böger, 1990), and it has been suggested that the band of higher molecular weight might represent a modified Fe protein, as in the anoxygenic phototrophic bacterium *Rhodospirillum rubrum* (Kanemoto & Ludden, 1984). In this organism the Fe-protein can be modified by ADP-ribosylation (Lowery, Saari & Ludden, 1986). The modified protein is inactive, but insensitive to oxygen inactivation. When grown under an alternating light–dark regime, nitrogenase in *O. limosa* is synthesized *de novo* before the onset of each dark period, and is apparently irreversibly inactivated in the light (Stal & Krumbein, 1985*a*, *b*). The amount of antigen does not vary significantly during a light–dark cycle. However, whereas the amount of the heavier (37 kDa) form of the Fe protein does not vary, the lighter (36 kDa) form is present in significant amount only when nitrogenase activity is detected (i.e. at the end of the light period and in the dark) (Villbrandt *et al.*, 1992). It was concluded that the high molecular weight form of the Fe protein probably represents inactivated enzyme. Durner *et al.* (1994) showed that, in *Anabaena*, ADP-ribosylation of the Fe-protein of nitrogenase of the kind known in *R. rubrum* does not occur. Earlier, Stal & Krumbein (1985*b*) demonstrated that in *O. limosa* high rates of *de novo* synthesis of nitrogenase counterbalance the continuous inactivation of the enzyme when grown under continuous light. These authors concluded, from experiments in which *O. limosa* was alternatingly exposed to an oxygen-free atmosphere and to air, that this organism switched off nitrogenase when suddenly exposed to oxygen. However, instead of explaining this observation in terms of a reversible switch-off/on of nitrogenase, it could equally well be explained by the diversion of electrons to oxygen. In *O. limosa*, nitrogenase activity is accompanied by oxygen uptake. Oxygen exchange in an N_2-fixing culture of *O. limosa* in a light–dark cycle follows nitrogenase activity (Stal & Krumbein, 1987). When a culture is transferred from a light–dark regime to continuous light, nitrogenase is induced precisely as if it were dark but with much greater activity, which is the result of the availability of light energy. Such cultures also show high rates of oxygen uptake. Similar observations have been made of *Gloeothece* (Myint, 1991), which emphasize the importance of nitrogenase-associated oxygen uptake. This oxygen uptake is likely to be an important component of the mechanism of protection of nitrogenase from oxygen inactivation.

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