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Minireview

Coastal microbial mats: the physiology of a small-scale ecosystem

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Coastal inter-tidal sandy sediments, salt marshes and mangrove forests often support the development of microbial mats. Microbial mats are complex associations of one or several functional groups of microorganisms and their formation usually starts with the growth of a cyanobacterial population on a solid substrate. They are considered as analogues of fossil Precambrian stromatolites. Primary production by the cyanobacteria fuels the metabolism of sulfate reducing bacteria and the sulfide that they produce is oxidised by anoxygenic phototrophic bacteria and by colorless sulfur bacteria. Growth and metabolism of these microorganisms result in markedly fluctuating vertical gradients of oxygen and sulfide that shift during a day-night cycle. This review discusses the metabolic contributions of the different functional groups of microorganisms and how their joint effort results in the formation of the mat.

Introduction

In their natural environment, microorganisms may occur essentially in one of the following 3 ways: free-living, associated with and often inside other organisms or forming multicellular, mono- or multispecies aggregates. In the latter form they may occur as colonies, biofilms or microbial mats. I will not attempt to give an all-embracing definition of any of the 3 forms of microbial aggregates, because this seems to be a nearly impossible task and also the borderlines between the different forms are in fact continuous. Nevertheless, it is important to indicate in a more general way of what is understood in the framework of this review by a microbial mat. Microorganisms that grow on a solid surface may eventually form microbial mats. Microbial mats have been the subject of 3 international meetings of which the results have been published (Cohen *et al.* 1984, Cohen and Rosenberg 1989, Stal and Caumette 1994). A mat differs from a biofilm mainly because of size and the coherent structure typical for the former. In its ultimate form, microbial mats resemble something like a doormat, which can be peeled from the surface as a whole. This explains the origin of the concept of a microbial mat. Before this term became common property, microbial mats were known as 'laminated microbial ecosystems' or recent or potential stromatolites (Krumbein 1983). Stromatolites are fossil laminated rock formations of biogenic origin. The oldest stromatolites date back to more than 3.5 Gyr B.P. and represent the earliest indications of life on earth. It is generally accepted that stromatolites have been built through the growth and metabolic activity of microbial mats that lithified through calcification and subsequently by silicification and other diagenetic processes (Walter 1976). The lamination in the rock represents the seasonal or erratic growth pattern of the microbial mats, comparable to the growth rings in the trunk of a tree. In stromatolites fossil remnants of microorganisms have been found that morphologically resemble modern cyanobacteria, which also today are common builders of microbial mats (Schopf 2000). Since there is little doubt that many of these stromatolites were formed through autotrophic metabolism, it is tempting to believe that they were built by cyanobacteria. However, the recent discovery that cyanobacteria are an evolutionary relatively young group within the Proteobacteria, argues against this hypothesis (Gupta 2000).

Modern microbial mats have been termed recent stromatolites in order to distinguish them from the fossil ones. However, the majority of the modern microbial mats do not lithify and therefore the term 'stromatolite' was considered not appropriate, except in the few examples that are known to calcify. The same is true for 'potential stromatolites' because it is uncertain whether non-calcifying microbial mats in fact possess the potential of lithification. Similar as stromatolites, modern microbial mats retain a lamination, representing older, partly degraded mats. However, the term 'laminated microbial ecosystems' usually does not refer to this historical lamination but rather to a vertical zonation of

different functional groups of microorganisms, which is often visible to the naked eye because of the different colors. Thus, the actual active microbial mat is laminated through different groups of organisms.

However, neither the historical nor the instantaneous lamination is the law of the Medes and Persians. Microbial mats, after growth has ceased, may be decomposed completely, not leaving a visible trace and the substrate is colonised every season anew. Likewise, a microbial mat may be composed of one species or different species may not be separated into different strata or they may not be distinguished as such. Such systems are obviously not 'laminated' but they do not principally differ from the 'laminated microbial ecosystems' and can all be embraced by the term 'microbial mat'.

Theoretically, microbial mats may be composed of one particular species. However, as a rule a complex microbial ecosystem forms, in which a variety of different functional groups of microorganisms represent a structural and physiological unit (Van Gemerden 1993). It has been proposed that microbial mats are structural and physiological equivalents of tissues (Wachendorfer 1991).

In this review I will describe the processes and organisms that are involved in the formation of one type of microbial mat that is built by cyanobacteria that is frequently found in coastal inter-tidal sediments, mangrove forests and salt marshes all around the globe. Microbial mats developing in extreme environments such as hypersaline ponds, thermal springs or hot or cold deserts, nor non-phototrophic systems such as mats of the gliding sulfur bacterium *Beggiatoa* or *Thioploca* (Larkin and Strohl 1983) are not discussed here.

The development of a cyanobacterial mat: primary production is the motor of the ecosystem

Colonisation

Inter-tidal sandy sediments are high energy environments, exposed to strong hydrodynamic conditions. Sediment particles of small grain size such as silt and clays will not deposit here, or they will be eroded away. On the most exposed areas only the heavier quartz sand grains are deposited. Inter-tidal sand flats can be considered as extreme environments because besides the physical forces they are low in nutrients and the periodic inundation causes desiccation and strong variations in salinity and temperature. Few organisms are capable of colonising these environments.

Cyanobacteria have remarkably few nutritional requirements. Their main way of life is photoautotrophic, which means that they use light as the source of energy, water as the electron donor and CO_2 as the sole source of carbon (Stal 1995). Moreover, many species are capable of fixing atmospheric nitrogen, which makes them independent on sources of combined nitrogen such as nitrate or ammonium or organic nitrogen, which are generally in low supply in the marine environment (Paerl *et al.* 1996, Bergman *et al.* 1997). Hence, the only critical nutrient for these cyanobacteria is phosphate. Cyanobacteria are well-known for their high affinity towards this important nutrient, which they can store intracellularly as polyphosphate (Riegman and Mur 1985).

Cyanobacteria are therefore excellent candidates to colonise low-nutrient environments. In inter-tidal coastal sediments they prefer fine sandy sediments, which combine moderately strong hydrodynamics and low sedimentation rates (Yallop *et al.* 1994). Moreover, the quartz sand grains allow excellent transmission of light into the sediments (Stal *et al.* 1985, Kühl *et al.* 1994). Initial colonisation of the sediment by the usually filamentous cyanobacteria is through adhesion to sand grains probably by sticky extracellular polymers. This property allow cyanobacteria to colonise environments with relatively high energy, without being washed away. Some species are particularly well equipped to settle in high-energy environments. For instance, in the Bahama's *Schizothrix* spp. is known to colonise environments with strong wave currents where other organisms are unable to settle (Reid and Browne 1991). These organisms give rise to the formation of modern stromatolites.

Photorespiration

Once a successful colonisation has occurred, the cyanobacteria through growth and their photosynthetic activity enrich the sediment with organic matter, which becomes available to other microorganisms. There are a number of different mechanisms by which the photosynthetically fixed carbon is liberated into the environment. An important mechanism could be through photorespiration. The cyanobacterial mat is characterised by a high concentration of biomass. The oxygen that is produced through photosynthesis accumulates in the mat and can only leave it through diffusion. Although the diffusion coefficients of the polysaccharide matrix of the cyanobacterial mat is not much different from that of water, the medium is stagnant and no turbulence can aid the exchange of gas with the overlying water or air. Hence, the cyanobacterial mat may become supersaturated with oxygen (Revsbech *et al.* 1983). Two to three-fold oxygen supersaturation in cyanobacterial mats is not exceptional. At the same time the inverse is true for CO_2. Carbon dioxide is fixed during photosynthesis and depleted from the mat. It can only be replenished by diffusion from the overlying medium. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme of the reductive pentose phosphate cycle (Calvin) and the enzyme responsible for the fixation of CO_2, possesses also oxygenase activity, i.e. uses O_2 for the oxidative cleavage of ribulose-1,5-bisphosphate (Tabita 1994). In fact, Rubisco has a much better affinity for O_2 than for CO_2 and in order to be able to effectively fix CO_2, its concentration must be much higher than O_2. In an aerobic environment, many cyanobacteria (and other microalgae) achieve this requirement by concentrating CO_2 (Carbon Concentrating Mechanism, CCM) and Rubisco in carboxysomes (Price and Badger 1991, Reinhold *et al.* 1991). However, when both the ambient CO_2 concentration is low and the O_2 level is high, the CCM may not be able to prevent the oxygenation of ribulose-1,5-bisphosphate, which eventually will lead to the formation of glycolate that is excreted into the medium (Renstrom and Bergman 1989). Hence, the oxygenation reaction leads to a loss of fixed carbon. This photorespiration might be a major source of organic carbon for the microbial community associated with the cyanobacterial mat.

Fermentation

Another major source of organic matter is the fermentative metabolism of cyanobacteria. Whereas in the light the cyanobacterial mat may be supersaturated with oxygen, in the dark not only no oxygen is produced but also there is a high demand for oxygen. Initially, the cyanobacteria will mobilise their storage carbohydrate (glycogen) and respire it aerobically in order to generate biochemical energy. However, this will deplete the oxygen in the cyanobacterial mat quickly and diffusion from the overlying water or air is slow. This will result in the factual anoxic conditions in the mat. Measurements of oxygen dynamics in microbial mats have repeatedly shown that anoxic conditions are often established within minutes after darkening, meaning that the cyanobacteria in the mat are confronted with anoxia during virtually the whole night (Revsbech *et al.* 1983). In order to cover their energy demands during dark anoxic conditions these cyanobacteria switch to a fermentative metabolism (Stal and Moezelaar 1997). The reserve compound glycogen and sometimes also energy-rich compatible solutes (osmotica) are mobilised and fermented to a variety of low-molecular compounds that are excreted into the medium. Depending on the species, cyanobacteria have been shown to possess homo- and heterolactic fermentation, mixed acid fermentation and homoacetic fermentation. A considerable part of the glycogen is fermented and has to be replenished by photosynthetic CO_2 fixation during the subsequent day period. Hence, an important part of this fixed CO_2 ends up as low-molecular fermentation products in the medium and becomes available to other microorganisms.

Compatible solutes

Although fermentation and photorespiration are probably the major mechanisms by which organic matter becomes available in the cyanobacterial mat, their exist also other mechanisms, that may be important under certain conditions. Marine cyanobacteria accumulate compatible solutes that serve as osmoprotectants (Reed *et al.* 1986). The most common osmoprotectant in marine cyanobacteria is glycosyl glycerol, but also the disaccharides trehalose and to a lesser extent sucrose can be found. Glycine-betaine is more common as compatible solute in cyanobacteria in hypersaline environments. Particularly the disaccharides and glucosyl glycerol are energy rich compounds and it has been shown that they may be used in addition to the storage compounds as energy reserve in the dark, particularly under anoxic conditions. Cyanobacterial mats in inter-tidal sediments and on rocky shores may be exposed to dramatic changes in salinity. This is the case when during exposure the mats experience a salinity down-shock when it rains. The only way for cyanobacteria to protect them from such a sudden osmotic down shock is to quickly excrete the compatible solute into the environment where it subsequently becomes available to other microorganisms. This mechanism of transfer of organic matter to the community is rather erratic.

Extracellular polymeric substances

Mat-building cyanobacteria also excrete large amounts of polymeric substances (extracellular polymeric substances, EPS), which are largely composed of polysaccharides and with minor components of protein and lipids (Stal 1994, Decho 1994). One may distinguish roughly two types of EPS: one is more or less intimately associated with the organism (cells or trichomes) and is usually designated as the sheath (De Philippis and Vincenzini 1998). This is a more or less structural cell component, albeit that it is outside the cell wall. Depending on the organism this sheath may be thick and may in fact be wider than the trichome itself, or it may be vanishing thin or even absent. Some unicellular cyanobacteria such as *Gloeothece* spp. or *Gloeocapsa* spp. form colonies of which the cells are embedded in a polysaccharide sheath (Tease *et al.* 1991). The trichomes of the cosmopolitan mat-forming cyanobacterium *Microcoleus chthonoplastes* form bundles that are enclosed by a common sheath (Garcia-Pichel *et al.* 1996). Cyanobacteria such as *Lyngbya aestuarii* that produce very thick sheaths are usually not motile (Rippka *et al.* 1979). The sheath may serve different functions. The highly hydrated polysaccharides are an effective protection from desiccation that may occur when the mat is exposed to the air. The sheath is also effective as an adhesive that attaches the organism to the substrate. It may further scavenge rare micronutrients and protect the organism from a variety of external threats, including grazing. The sheath is always produced in the cyanobacteria that have one, but its properties may vary with the environmental conditions in which the organism thrives. The other type of EPS produced by cyanobacteria is not intimately associated with the organism and is excreted into the medium as mucilage. In many cases this type of EPS seems to be produced as the result of unbalanced growth. This occurs when growth of the organism is limited by a nutrient (often N) while its photosynthetic and CO_2-fixing capacity are not impaired. Under such conditions, cyanobacteria initially produce more of the intracellular storage compound glycogen, but the space for this inside the cells is limited (Lehmann and Wöber 1976). Cyanobacteria rarely produce more than 50% of dry weight as glycogen. Excess of fixed CO_2 is subsequently excreted as mucilage into the medium. This seems a waste but in this way, cyanobacteria dissipate the light energy that they harvest through their pigments. This EPS forms a gel matrix in which the cyanobacteria are embedded and it may have similar advantages for the community as a whole as the sheath has for individuals. In addition, EPS excretion may occur as part of the mechanism of gliding motility of some cyanobacteria (Castenholz 1982).

Although EPS represents a high quality and energy-rich substrate for microorganisms, it must be hydrolysed extracellularly before it can be taken up and utilised. Sheath EPS, but also the mucilage, can be considered as rather recalcitrant compounds. In many coastal microbial mats the amount of mucilage is low as compared to microbial mats in hypersaline environments. In the latter, the degradation of complex organic molecules appears to be very slow probably because of high salinity. In the well-investigated hyper-

saline microbial mats of Solar Lake (Sinai, Egypt) and the salterns of Guerrero Negro (Baja California, Mexico), the top layer of 1 and 0.1m, respectively, is purely organic in nature and formed through the accumulation of the successive active cyanobacterial mats. The mat (Pond 5) of Guerrero Negro grows at a rate of approximately 1cm y^{-1}, close to the rate of decomposition of the older layers, keeping the system more or less at a thickness of 10cm (Des Marais 1995). However, some net accumulation must have occurred in order to produce the layer of 10cm of organic matter. The Solar Lake microbial mat system has been estimated to be 2000 years old (Krumbein *et al.* 1977). An average yearly net accumulation rate of 0.5mm would have yielded the present day thickness. This is only a fraction of the yearly gross accumulation, which is in the same order of magnitude as in the Guerrero Negro mat. This means that 99.5% of the organic matter produced is degraded in these hypersaline mats and that only the most recalcitrant molecules remain as refractory matter in the system. In coastal inter-tidal microbial mats this net accumulation is usually not observed (Stal *et al.* 1985, Stal 1994). This may be attributed to the fact that the degradation of recalcitrant organic matter may be easier at lower salinity, but it is more likely that these systems are exposed to dynamic conditions, causing erosion or oxygenation. The occasionally introduction of oxygen would facilitate the degradation of recalcitrant organic molecules.

Grazing

After having discussed photorespiration, fermentation, osmotic down-shock and EPS exudation as major mechanisms by which photosynthetic fixed CO_2 is liberated as organic matter in the environment, a few other mechanisms should be mentioned here as well. Obviously, organisms and that includes cyanobacteria, have a limited life. Cells may stop dividing and eventually die and disintegrate. In coastal microbial mats grazing may be more important as a cause of cell death (Fenchel 1998, Fenchel and Kühl 2000). The possibility of viral or bacterial attack has a cause of cell lysis in microbial mats has received only little attention in literature (Margulis *et al.* 1990), but may prove to be an important process. However, considering the fact that the total amount of fixed CO_2 that is liberated as non-structural compounds may exceed 90%, the contribution of structural cyanobacterial cell material to the chemotrophic community is comparatively small.

Secondary metabolites

Other organic compounds that may be produced and excreted by mat-forming cyanobacteria are the result of secondary metabolism (Jüttner 1987, Carmichael 1992). The amounts of these compounds are usually small and do not contribute to the organic substrate that is available for the microbial community, but they may be of great importance for the ecosystem functioning. Examples of products of secondary metabolism include geosmines and other volatile organic odorous compounds. Not much is known of these compounds from microbial mats. The same is true for cyanobacterial toxins. They are well-known from certain planktonic, bloom-forming cyanobacteria, but benthic cyanobacteria have not been investigated in this respect. Recently, it has been suggested that cyanobacteria, including mat-forming species, may be produce compounds with antibiotic activities or substances that are involved in cell-to-cell signaling (Kreitlow *et al.* 1999). All these subjects are still in their infancy.

Oxygen dynamics in microbial mats

Cyanobacteria are oxygenic phototrophic organisms. Therefore, highly dynamic vertical oxygen profiles are an important phenomenon in microbial mats (Jørgensen *et al.* 1979). These are in the first place produced as a result from the daily light curve. During the night oxygen is consumed by respiration. The oxygen demand is usually high so that the entire mat may become anoxic. Only the mat surface may receive oxygen, particularly when it is exposed directly to air. During the day oxygenic photosynthesis is only possible in the upper part of the mat in which sufficient light penetrates. The euphotic zone of the mat is defined as the layer in which gross oxygenic photosynthesis occurs. This is not the same as the depth at which oxygen may penetrate. In the light a typical oxygen profile shows a concentration maximum at a depth of 0.1–0.3mm. This peak usually coincides with the maximum photosynthetic activity or a concentration of photosynthetic biomass or both. Closer to the surface the higher irradiation may be sub-optimal, resulting in lower photosynthetic rates or even in negative values when photooxidation prevails. Below the oxygen optimum, irradiance levels are not saturating, likewise resulting in lower oxygen production rates. Obviously, the actual total oxygen production rate at any place in the mat is a function of the active photosynthetic biomass and the level of irradiance. The latter varies during a diurnal cycle and the oxygen maximum is therefore pushed from the surface down to reach its maximum depth at noon, when the level of irradiance has become maximal. On the other hand, in some microbial mats the standing stock of photosynthetic biomass is also not constant at a particular place during a diurnal cycle. Many mat-building cyanobacteria are motile by gliding and may constantly move in order to position themselves at optimum light conditions. Hence, the cyanobacterial mat optimizes its photosynthetic performance continuously and this is reflected in the oxygen profile in the mat. In addition, light impinges rarely as a pure sinus curve and may fluctuate tremendously during a diurnal cycle due to clouds or to water covering the mat. Photosynthesis responds immediately to any fluctuation in light intensity, consequently changing the oxygen profile.

The actual oxygen concentration at any location in the mat is the result of its production by photosynthesis, its consumption by respiratory and chemical oxidation processes and diffusion. This is the basis of a popular method to measure photosynthesis in microbial mats using micro-electrodes: the decrease of oxygen concentration in the first couple of seconds after the darkening of the mat equals the photosynthetic rate (changing the minus sign to positive) (Revsbech *et al.* 1983). Downward diffusion of oxygen may result in aerobic or micro-aerobic conditions below the euphotic zone. On the other hand respiration and chemical

oxidations may exceed the photosynthetic oxygen production, resulting in anoxic conditions within part of the euphotic zone. In this latter case, the method for measuring photosynthesis as described above, does not work.

Microbial mats: a joint venture of several functional groups of micro-organisms

As we have seen, much of the photosynthetic fixed CO_2 becomes readily available to the microbial community. A large portion of this organic matter is easily degradable low-molecular compounds. All of this material is produced in the photosynthetically active cyanobacterial mat and it can be anticipated that is also decomposed there. In the presence of oxygen it can be assumed that aerobic processes predominate in the decomposition of this organic matter. During the night, oxygen is depleted often within minutes, which is mostly attributed to the respiratory activity of the cyanobacteria themselves, subsequently switching to fermentation. The major fermentation products are acetate, formic acid, lactate, ethanol, H_2 and CO_2. These are excellent substrates for the obligate anaerobic sulfate-reducing bacteria (Hansen 1994). Methanogenic or acetogenic bacteria could also be involved in the degradation of these compounds but since sulfate-reducing bacteria are superior in their affinity for these substrates and sulfate reduction yields more energy, these bacteria do not play a role of importance in coastal microbial mats as long as sulfate is present (Raskin *et al.* 1996). Seawater contains 28mM of sulfate and its depletion in marine microbial mats is only envisaged at extraordinary high productivity or when the supply of seawater lags behind. In hypersaline microbial mats methanogenic bacteria may be important because of the presence of non-competitive substrates (Oremland and Polcin 1982, Cytryn *et al.* 2000).

Sulfate reduction

We may assume that a substantial part of the fermentation products excreted by the cyanobacteria is metabolised by sulfate-reducing bacteria. It should be noted that these bacteria should occur in the vicinity of where their substrate is produced, i.e. in the cyanobacterial mat. This is remarkable, since sulfate-reducing bacteria are considered as obligate anaerobic organisms and the cyanobacterial mat may be even supersaturated with oxygen during daytime. This seems contradictory. Although it could be conceived that either the sulfate-reducing bacteria move up and down in the mat with the shifting oxygen profiles or that the fermentation products diffuse into the lower permanent anoxic part of the mat, it has been clearly demonstrated that large numbers of sulfate-reducing bacteria are permanently present in the cyanobacterial mat and that the highest rates of sulfate reduction indeed are found in this layer (Visscher *et al.* 1992, Teske *et al.* 1998). More recent work has shown that many sulfate-reducing bacteria tolerate oxygen and that some are able even to perform limited aerobic respiration (Dilling and Cypionka 1990). The vertical distribution of sulfate-reducing bacteria reveals that typical oxygen-tolerant species dominate the top layer of the mat, whereas those that can not tolerate oxygen are found in the permanently anoxic layers (Risatti *et al.* 1994). Sulfate reduction has been demonstrated even in the light in the fully oxygenated cyanobacterial mat. Since a search for anoxic micro-niches in this mat was unsuccessful, it was conceived that sulfate-reduction would take place under fully aerobic conditions (Canfield and Des Marais 1991). Attempts to isolate sulfate-reducing bacteria that carry out sulfate reduction under aerobic conditions have failed so far. The substrate for aerobic sulfate reduction could be glycolate, excreted by the cyanobacteria in the light as the result of photorespiration. Glycolate has been shown to be an important substrate for chemotrophic bacteria in microbial mats and sulfate-reducing bacteria using glycolate have been isolated from cyanobacterial mats (Fründ and Cohen 1992, Nold and Ward 1996). These observations question the importance of aerobic degradation of the bulk of the organic matter in these microbial mats. Aerobic metabolism may be limited to the oxidative attack of complex, recalcitrant compounds.

Sulfide

Sulfate reduction is a form of anaerobic respiration, using sulfate as the terminal electron acceptor and producing sulfide. Next to oxygen, sulfide is a major compound determining microbial activities and the vertical stratification of different functional groups of microorganisms that is typical for marine microbial mats. It should be noted that sulfate reduction is not the only process that results in the formation of sulfide. For instance, cyanobacteria under anaerobic conditions may use zero-valence 'elemental' sulfur as electron sink for fermentation, which also results in the formation of sulfide (Stal 1991). A variety of other bacteria use zero-valence sulfur as electron acceptor for anaerobic respiration, but it is not known how important these organisms are in marine microbial mats.

Sulfide is chemically and biologically very reactive and in addition it is toxic to almost all organisms, including those who produce it or depend on it as substrate. In the first place, sulfide reacts instantaneously with iron. It is oxidised to zero-valence sulfur by ferric iron, which itself is reduced to ferrous iron. Sulfide precipitates with ferrous iron forming the virtually insoluble FeS, which produces the characteristic intense black sediment. FeS may subsequently react with zero-valence sulfur to form the very stable pyrite, which is a relatively slow process (Howarth and Jørgensen 1984, Thode-Andersen and Jørgensen 1989).

Sulfide also readily reacts chemically with oxygen and therefore both species can not co-exist at high concentrations. Co-existence of low concentrations of oxygen and sulfide has been shown to occur in microbial mats, but more frequently this is hardly measurable because the rate of biological oxidation of sulfide is much faster than the chemical oxidation (Krumbein *et al.* 1979, Revsbech *et al.* 1983). Two important functional groups of microorganisms are involved in sulfide oxidation in microbial mats. The colorless sulfur bacteria are chemosynthetic organisms that oxidise sulfide to sulfate using oxygen as electron acceptor. Many of these species can live autotrophic, i.e. are capable of fixing CO_2. Very high numbers (up to $10^9 cm^{-3}$) of these bacteria have been found in microbial mats (Visscher *et al.* 1992). Some

species are facultative anaerobic and capable of denitrification, using nitrate as the terminal electron acceptor, but since marine microbial mats are usually nitrogen-depleted, this mode of metabolism is considered unimportant. The colorless sulfur bacteria are more or less homogeneously distributed in the mat and are probably only active at the sulfide–oxygen interface, thereby generating an almost perfect separation between these two. Because the oxygen gradient is moving up and down during a diurnal cycle, the sulfide–oxygen interface follows it. It seems that the colorless sulfur bacteria would have only a very limited time to be metabolically active, unless they move with the sulfide–oxygen interface. It is not known which of these options apply in microbial mats.

Anoxygenic, purple sulfur, phototrophic bacteria

The other important functional group of microorganisms in microbial mats that are involved in the oxidation of sulfide are the anoxygenic phototrophic bacteria (De Wit and Van Gemerden 1988). These are essentially obligate anaerobic organisms that perform photosynthesis and fix CO_2. In stead of water they use sulfide as the electron donor. Most commonly found in marine microbial mats are the purple sulfur bacteria. Because they need light, they are found immediately below the cyanobacterial mat. Although the cyanobacteria filter out most of the visible light, purple sulfur bacteria use a different part of the spectrum, particularly in the infrared, which penetrates the mat extremely well. Moreover, purple sulfur bacteria are low-light adapted organisms that perform photosynthesis at light levels as low as 0.1% of full sunlight (Overmann *et al.* 1992). Another important reason why purple sulfur bacteria are found in microbial mats is that these organisms are also very oxygen-tolerant or are even capable of aerobic metabolism (De Wit and Van Gemerden 1990). This is important, because of their occurrence immediately below the cyanobacterial mat, where oxygen may be present. In the presence of oxygen, purple sulfur bacteria continue photosynthesis but they are unable to synthesise the major light-harvesting pigment: bacteriochlorophyll a. After prolonged exposure to oxygen, they will eventually end up as colorless organisms, not capable anymore of photosynthesis. Although these cells are capable of a chemosynthetic mode of metabolism, oxidising sulfide with oxygen, identical to the colorless sulfur bacteria, they can not compete with the latter organisms because of poor substrate affinity.

Purple sulfur bacteria oxidise sulfide in two major steps. They first oxidise it to zero-valence sulfur, which is stored intra cellularly (in fact outside the cytoplasm membrane). This reaction is relatively quick, by which the sulfide available is rapidly depleted. The oxidation of sulfide to sulfur produces only 2 electrons with which only ½ CO_2 can be fixed. The subsequent oxidation of sulfur to sulfate occurs after the sulfide has been depleted, is slower, and because it yields 6 electrons it allows for the fixation of 3 times as much CO_2. This strategy is ecologically advantageous when different species compete for sulfide. Once the sulfur is stored inside the cells, it is not available to other organisms (Van Gemerden 1983).

The intracellular stored sulfur serves another important function in purple sulfur bacteria. As is the case with cyanobacteria, the purple sulfur bacteria switch to fermentation in the dark, using elemental sulfur as electron sink. In this way, purple sulfur bacteria also contribute to the production of sulfide in the microbial mat (Van Gemerden 1968).

Whereas the anaerobic purple sulfur bacteria display aerobic metabolism when exposed to oxygen, most mat-forming cyanobacteria are capable of anoxygenic photosynthesis when exposed to sulfide (Cohen *et al.* 1986). Sulfide is a potent inhibitor of oxygenic photosynthesis, but it may donate electrons to photosystem I and in this manner allow CO_2 fixation. In some species this property must be induced and requires de novo protein synthesis. This is for example the case with the cyanobacterium *Oscillatoria limnetica* from the sulfide-rich hypolimnion of the hypersaline Solar Lake (Sinai) (Arieli *et al.* 1989). However, oxygen and sulfide gradients in microbial mats are strongly fluctuating and therefore mat-forming cyanobacteria such as *Microcoleus chthonoplastes* usually possess the capacity of anoxygenic photosynthesis constitutively and perform oxygenic and anoxygenic photosynthesis simultaneously at sulfide concentrations lower than 1mM, with the relative importance of oxygenic photosynthesis decreasing with increasing sulfide concentration (De Wit *et al.* 1988). Above 1mM sulfide oxygenic photosynthesis is completely inhibited. It is likely that in this case growth of the cyanobacterium becomes impossible, since *M. chthonoplastes* has an indispensable requirement for oxygen.

Vertical stratification of functional groups of microorganisms in microbial mats

Four major functional groups of microorganisms have been distinguished so far. These are: the oxygenic cyanobacteria, the anoxygenic phototrophic bacteria, the sulfate-reducing bacteria and the colorless sulfur bacteria. Only the first 2 groups form clearly stratified layers, visible with naked eye. The latter 2 groups are distributed throughout the microbial mat, although the sulfate reducing bacteria may be partitioned into a more oxygen-tolerant population in the top layers and the truly obligate anaerobic species in the permanent anoxic layers.

This vertical stratification of microorganisms may be more complex. In some microbial mats a layer of green sulfur bacteria is found beneath the purple sulfur bacteria (Nicholson *et al.* 1987). The green sulfur bacteria are another group of anoxygenic phototrophic bacteria. Although they share part of the light spectrum with the cyanobacteria, their light requirements are extremely low and when sufficient light impinges on the mat it can be anticipated that these organisms may live phototrophically. Green sulfur bacteria are extremely oxygen sensitive but resist high sulfide concentrations.

The occurrence of a green layer beneath the purple sulfur bacteria does not necessarily indicate the presence of green sulfur bacteria. Sometimes a second layer of cyanobacteria can be found below the layer of purple sulfur bacteria. Measurements of oxygen and photosynthesis have shown that these cyanobacteria perform predominantly an oxygenic mode of photosynthesis (Krumbein *et al.* 1977). The

activity of the purple sulfur bacteria takes care of the elimination of sulfide that otherwise would prevent the occurrence of oxygenic photosynthesis. The oxygen profile as measured by micro-electrodes shows then 2 peaks, separated by an anoxic layer. Although the species composition of the deep layer of cyanobacteria differs from the surface mat, it seems likely that it represents the original surface mat that has been overgrown by a new one and that successively shifts in species composition have occurred. In other occasions 'inverted mats' are encountered where the purple sulfur bacteria form the top layer and the cyanobacteria occur beneath them (Van Gemerden *et al.* 1989, Van Gemerden *et al.* 1989). Such mats form on sediments that receive a high load of exogenous produced organic matter, for instance algae or sea grasses deposited on the beach. Its decomposition result in the production of large amounts of sulfide, preventing growth of cyanobacteria. Mats of purple sulfur bacteria may develop that scavenge the sulfide, allowing cyanobacteria to grow below them.

Layer of ferric iron

The separation of the oxygenic and anoxygenic phototrophic bacterial communities is clearly the result of the opposite gradients of oxygen and sulfide and of course of light. Although the separation appears perfect to the naked eye, there is overlap between the two when observed at the micro-meter scale, giving rise to competitive interactions and the exposure to sulfide and oxygen in the aerobic and anaerobic communities, respectively. In some microbial mats an additional layer can be distinguished between the cyanobacteria and the purple sulfur bacteria. This layer has a rusty color and presumably is composed of iron hydroxides (Stal 1994). This would represent an ideal barrier between the aerobic cyanobacteria and the anaerobic purple sulfur bacteria (Figure 1). Any sulfide diffusing upwards will react with ferric or ferrous iron before it reaches the cyanobacterial mat. Vice versa, any oxygen diffusing downwards will react with ferrous iron or FeS and be unable to interfere with the purple sulfur bacteria. It is presumed that this layer will tend to reduce at night and oxidise during daytime. This hypothesis so far has not been proven experimentally. Apart from being a pure chemical barrier, this rusty layer may also represent a community of anoxygenic phototrophic (purple) bacteria that uses ferrous iron as electron donor, oxidising it to ferric iron (Widdel *et al.* 1993). Such bacteria have been isolated from a variety of environments but it is not known whether they are important in microbial mats. It has also been proposed that cyanobacteria may be capable of iron-dependent anoxygenic photosynthesis but experimental proof for this hypothesis is lacking (Cohen 1984). The involvement of chemosynthetic bacteria in the oxidation of iron in microbial mats is less likely. Some colorless sulfur bacteria are capable of oxidising ferrous iron aerobically, but this process occurs only at extremely low pH (~2), which does not occur in coastal microbial mats. Species such as *Gallionella ferruginea* or *Sphaerotilus natans* which oxidise ferrous iron at neutral pH are unlikely to be able to compete with the chemical oxidation of iron in microbial mats (Emerson and Revsbech 1994).

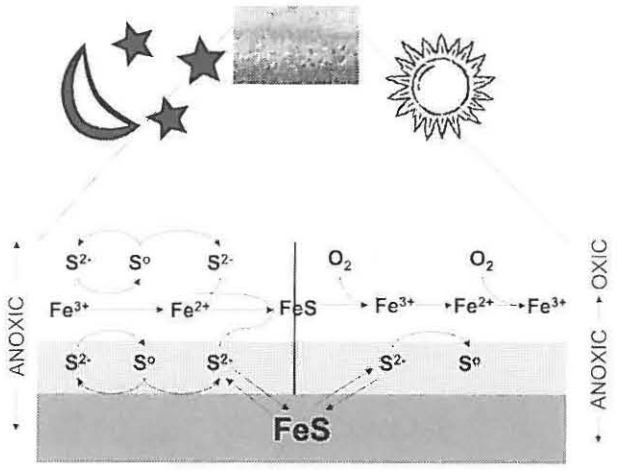

Figure 1: A conceptual model of a multi-layered microbial mat with a layer of iron as the buffer between the aerobic and anaerobic communities of phototrophic microorganisms. At night, sulfate-reducing bacteria (SRB) produce sulfide throughout the mat and the top layer of cyanobacteria and the layer of purple sulfur bacteria below, reduce zero valence ('elemental') sulfur to sulfide. This reduces ferric iron (Fe^{3+}) while the sulfide is oxidised back to sulfur. Sulfide subsequently reacts with ferrous iron (Fe^{2+}) to produce the black insoluble FeS. During the day, when the cyanobacteria evolve O_2 in the course of photosynthesis, FeS is oxidised. This prevents O_2 from diffusing into the layer of the anaerobic, anoxygenic bacteria. The sulfide produced by the SRB reacts with the Fe^{3+} and prevents it from reaching the cyanobacteria.

Mat-forming cyanobacteria are protected by an iron coat

The rusty layer that sometimes separates the green and purple communities may prevent sulfide diffusing from below into the cyanobacterial mat but it does not help against sulfide which is produced inside the cyanobacterial mat by sulfate-reducing bacteria or by the cyanobacteria themselves. The mat-forming cyanobacterium *M. chthonoplastes* has been shown to accumulate iron in its polysaccharide sheath (Stal 1994). This layer of iron may serve a similar function as the rusty layer of the mat (Figure 2). When during fermentation in the dark *M. chthonoplastes* reduces sulfur to sulfide, the latter reacts with ferric iron, oxidising the sulfide back to zero valence 'elemental' sulfur, and producing ferrous iron. The net result of this fermentation is the reduction of iron. Another process coupled to fermentation by which iron is reduced is the oxidation of formic acid to CO_2. During the subsequent light period the ferrous iron is oxidised back to ferric iron by the oxygen produced during photosynthesis. This will keep the oxygen partial pressure low in the cell and in its immediate vicinity which is beneficial for the organism because it reduces losses of photosynthate by photorespiration.

Nitrogen fixation: without it coastal microbial mats would not develop

Nitrogen comprises 7–10% of cell dry weight matter and represents therefore the second most important element. In the

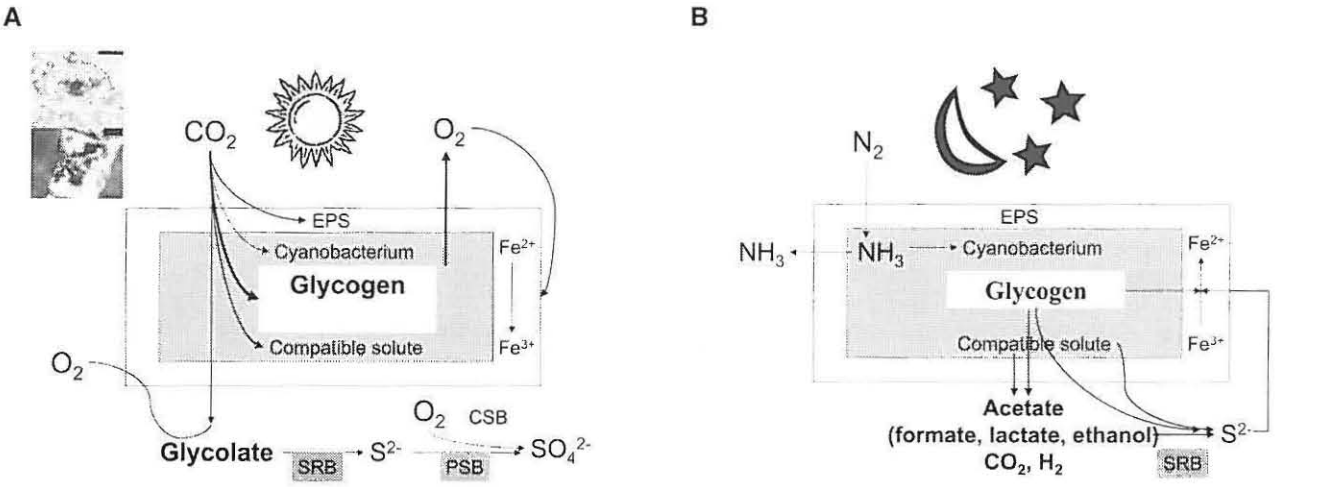

Figure 2: A conceptual model showing the possible role of iron accumulating in the sheath of a mat-forming cyanobacterium. The inserted photo shows a transmission electron microscope image of the cyanobacterium Microcoleus chthonoplastes with iron accumulated at the sheath visible as electron-dense particles. **A.** During the day, the cyanobacterium fixes CO_2 as result of photosynthesis. The photosynthate is used for the largest part to fill up the glycogen storage pool. It is further used for the synthesis of structural cell material and compatible solute (osmotica) and is excreted as extracellular polysaccharides (EPS). The organism evolves O_2. This may result in photorespiration and a loss of photosynthate as glycolate. In order to prevent O_2 from accumulation, ferrous iron in the sheath is oxidised. **B.** During the night, glycogen and the compatible solute is fermented and concomitantly sulfur is reduced to sulfide. The fermentation products are end-oxidised by sulfate-reducing bacteria (SRB) and sulfide is formed. The sulfide reacts with ferric iron, preventing the accumulation of sulfide to toxic levels while regenerating ferrous iron.

cell it is mainly present in its reduced form. Ironically, in its most ubiquitous form, atmospheric dinitrogen (N_2) it is not accessible to most organisms and the bound forms such as nitrate, ammonia or organic nitrogen are usually in low supply, particularly in the marine environment. A limited group of, exclusively, prokaryotic organisms possesses the ability to reduce the extreme stable triple bond between the two molecules of N in N_2. These organisms all contain the enzyme complex nitrogenase which catalyses the reduction of N_2 to NH_3 at the expense of a large amount of energy and low-potential electrons (ferredoxin) (Peters *et al.* 1995). Among cyanobacteria many species are known to be capable of fixing nitrogen. As oxygenic photo-autotrophic organisms they are particularly well equipped to meet the energy and electron demands of nitrogen fixation. On the other hand, nitrogenase is extremely sensitive to oxygen and diazotrophic cyanobacteria therefore developed strategies to circumvent this problem (Gallon 1992). Clearly, the best adaptation has been evolved by the heterocystous cyanobacteria. These filamentous cyanobacteria differentiate special cells, the heterocysts, which have lost the ability of oxygenic photosynthesis and CO_2 fixation and which have become the sites of nitrogen fixation. Nitrogen fixation in these organisms is strongly light dependent although some activity can be sustained in the dark, driven by aerobic respiration. Heterocystous cyanobacteria are remarkably rare in coastal microbial mats (Stal *et al.* 1994). More frequently, non-heterocystous filamentous cyanobacteria have been shown to fix nitrogen in these environments. These organisms can be distinguished with respect to their strategy to fix nitrogen in two major groups (Stal 1995, Bergman *et al.* 1997). One group is capable of fixing nitrogen only under anaerobic conditions and its strategy can be described as 'avoidance of oxygen'. There are a number of ways by which this can be achieved. First of all, nitrogen fixation can be confined to the dark period, when the mat has become anoxic. The disadvantage of this strategy is that only a limited nitrogenase activity can be sustained under such conditions because of the low energy yield of fermentation. Experiments and calculations have unequivocally demonstrated that some nitrogen fixation can be supported by fermentation by mat-forming cyanobacteria such as *Oscillatoria limosa* (Stal and Moezelaar 1997). Another way is to realise a spatial separation of oxygenic photosynthesis and nitrogen fixation in the mat. At the bottom of the cyanobacterial mat oxygenic photosynthesis may not be possible because only far red light (>700nm) predominates here, which is specifically harvested by the anoxygenic photosystem I (Stal *et al.* 1985). Moreover, sulfide may also reach higher concentrations in this part of the mat and this is a potent inhibitor of oxygenic photosynthesis. It has indeed been shown that the specific (chlorophyll-normalised) nitrogenase activity increased with depth in the mat. Sulfide has been shown to induce nitrogenase in mat-forming cyanobacteria in the light (Villbrandt and Stal 1996). The problem with this strategy is that the nitrogen-fixing cyanobacteria do not fix CO_2 (except when sulfide-dependent anoxygenic photosynthesis occurs) and the cyanobacteria in the surface lay-

ers are still devoid of nitrogen. Transport of fixed nitrogen outside the cells seems inefficient and would rather favor non-diazotrophic organisms in the mat. It seems more likely that in this case the cyanobacteria move up and down between the two sites. Motility of mat-forming cyanobacteria has been shown in several occasions but these were all controlled by light (Garcia-Pichel *et al.* 1994, Kruschel and Castenholz 1998). Motility controlled by the nitrogen status of cyanobacteria still awaits experimental proof.

The second group of non-heterocystous diazotrophic cyanobacteria is capable of fixing nitrogen under fully aerobic conditions (Bergman *et al.* 1997). It is not precisely known by which mechanism these organisms protect nitrogenase from oxygen inactivation. Although they are capable of diazotrophic growth in culture under fully aerobic conditions and under continuous illumination, they fix nitrogen exclusively during the dark when grown under an alternating light-dark cycle. The same day-night pattern of nitrogen fixation can be found in most microbial mats. Presumably, oxygen concentrations reach too high levels in the mat during daytime. Sometimes, two peaks of nitrogenase activity can be observed during a day-night cycle in a mat of aerobic nitrogen-fixing cyanobacteria, one each at sunrise and sunset and low or zero activity at night and during the day (Villbrandt *et al.* 1990).

In the few examples of microbial mats formed by heterocystous cyanobacteria, nitrogen fixation occurs during daytime (Stal 1995). It is not precisely known why these organisms are excluded in many microbial mats. It has been suggested that heterocystous cyanobacteria are more sensitive to sulfide than non-heterocystous species (Howsley and Pearson 1979). However, it is H_2S which freely diffuses into the cell and exerts is toxicity and because of the alkaline conditions in coastal microbial mats the concentrations of this gas remains extremely low. Another suggestion is that heterocystous cyanobacteria do not tolerate anoxic conditions in the dark. Indeed, mats of heterocystous and non-heterocystous cyanobacteria occurring close to each other on an inter-tidal flat in Baja California, Mexico, differed markedly in their oxygen dynamics (Stal 1995). The mat of the heterocystous cyanobacterium *Calothrix* sp. did not turn anoxic during the night and lacked the black layer of FeS and purple sulfur bacteria. This also hinted to the absence of sulfide in this mat. During daytime oxygen concentrations in the *Calothrix* mat did not reach excessive high levels. This, and the availability of oxygen during the dark could have been critical for the heterocystous cyanobacterium to proliferate.

The rarity of heterocystous cyanobacteria is not only the case in marine microbial mats but extends to the whole marine environment. The most important nitrogen-fixing cyanobacterium in the marine plankton is the non-heterocystous *Trichodesmium*, whereas in freshwater and brackish environments only heterocystous species occur (Paerl 1990, Moisander and Paerl 2000). Hence, it could also be that high salinity selects against heterocystous species, although the precise mechanism remains to be elucidated. The mats of *Calothrix* in Baja California occur high in the inter-tidal sediments and are rarely submersed and may be are therefore not strongly influenced by the seawater.

Whatever may be the reason, the fact that nitrogen fixation in most microbial mats is accomplished by non-heterocystous cyanobacteria and that this occurs predominantly under anaerobic conditions implies that this process is far from efficient. It can be anticipated that growth of the cyanobacteria is strongly nitrogen-limited in these mats.

Is nitrogen-limited growth of the cyanobacterial mat preventing the formation of a stromatolite?

When nitrogen is limiting growth of the mat-building cyanobacteria these organisms will divert the fixed CO_2 to non-nitrogenous compounds, mainly carbohydrates. This mode of growth is termed 'unbalanced'. During balanced growth all cell components are synthesised in the right proportions, but when nitrogen is unavailable, proteins, nucleic acids and cell walls can not be produced. However, photosynthetic CO_2 fixation is not impaired and carbohydrates are synthesised. Intracellular, glycogen is stored, but since limited space is available polysaccharides are also excreted into the medium as mucilage and sheath material. Many marine microbial mats are therefore embedded in a thick gelatinous polysaccharide matrix.

Photosynthetic CO_2 fixation results in the formation of carbonate ion (CO_3^{2-}). It has therefore been hypothesised that cyanobacterial photosynthesis would promote calcification (Krumbein and Giele 1979). However, in most marine microbial mats, calcification was not spatially associated with the cyanobacteria (Lyons *et al.* 1984, Chafetz and Buczynski 1992). This is remarkable because with the concentration of calcium ion in seawater it would easily exceed the solubility product of calcium carbonate minerals such as aragonite and calcite. Thus, it was obvious to suppose a mechanism that would prevent calcification. Since it is known that polysaccharides can bind Ca^{2+} and/or Mg^{2+} one possible mechanism that has been proposed is that the extracellular polysaccharides produced by the cyanobacteria in the mat would lower the activity of these ions to such extend that calcium carbonate precipitation does not occur (Borman *et al.* 1982, Westbroek *et al.* 1994). Alternatively, polysaccharides may interact with crystallisation nuclei, preventing their growth, or a combination of both mechanisms.

Hence, a chronic nitrogen depletion occurring in many marine microbial mats, leading to excessive extracellular polysaccharide production, may offer an explanation for the fact that the majority of these systems do not lithify and produce stromatolites.

Concluding remarks

The classic example of a microbial mat is built by cyanobacteria and these oxygenic phototrophic microorganisms fulfill a key role in the systems function and metabolism. Most, if not all, of the primary production of these cyanobacteria is more or less directly transferred to the microbial mat system and not primarily used for the synthesis of structural cell material. Probably the most important mechanism is through the excretion of fermentation products. But also other mechanisms are responsible for the transfer of fixed carbon to the microbial community that is fueled in this way. The solar energy fixed by the cyanobacteria is utilised by the sulfate

reducing bacteria and the sulfide they produce, is utilised by anoxygenic phototrophic bacteria and colorless sulfur bacteria. In this way, the microbial mat functions as an internally closed system, driven by solar energy. The mat behaves like a tissue, displaying its own physiology. The net exchange with the atmosphere and geosphere is limited because the cycles of the elements are essentially closed. In many microbial mats, decomposition is complete but in a few other systems a slow accumulation of organic matter may occur. Rarely, microbial mats calcify and lithification results in the formation of stromatolites.

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