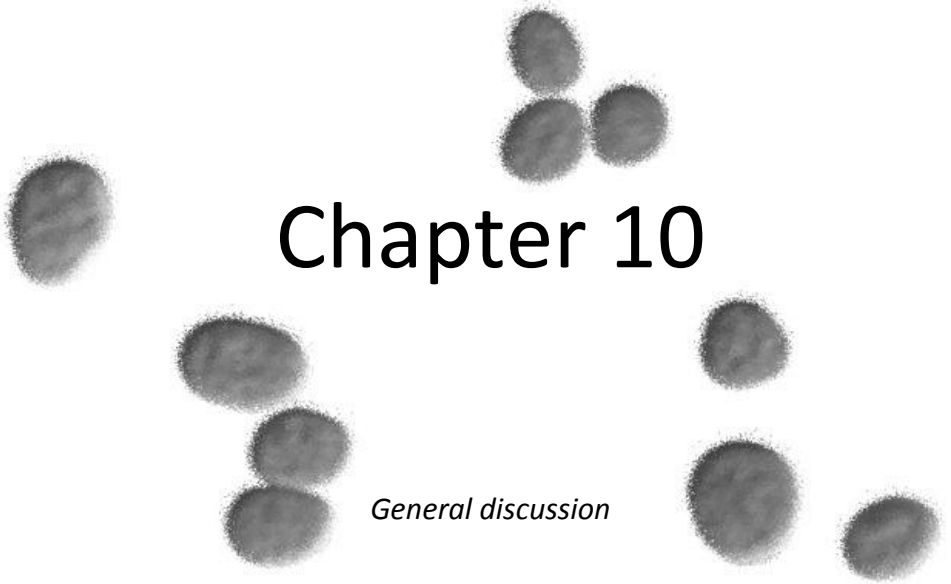

Chapter 10

General discussion

The fixation of dinitrogen (N_2 fixation) is the only process compensating for the loss of bio-available nitrogen compounds. The ability to fix N_2 is widespread among *Bacteria* and *Archaea* but not found in *Eukarya*, except for symbiotic associations with *Bacteria* (Zehr et al., 2003). Free-living N_2 fixing organisms (diazotrophs) exist in terrestrial, freshwater and marine environments. Microbial mats are distributed all over the world and harbor a variety of diazotrophic organisms (Chapter 2). Only their versatility with respect to species composition and metabolic pathways can explain the success of these micro-ecosystems in a variety of different habitats, including those generally considered as 'extreme'. In the marine environment microbial mats are often found in intertidal areas. In these coastal habitats highly fluctuating environmental conditions largely exclude grazers and higher plants and therefore allow for the development of microbial mats. *Cyanobacteria* are the most conspicuous structural part of the majority of microbial mats. Their low nutritional demands and their capability of photosynthesis, N_2 fixation, fermentative pathways and EPS-production (Stal, 2001) enable them to colonize the nutrient poor and particularly nitrogen-depleted habitats. However, a number of studies demonstrated the high diversity of diazotrophs in microbial mats (Omoregie et al., 2004a; Steppe & Paerl, 2005; Chapter 4) along with complex patterns of N_2 fixation which did not always correspond to the major cyanobacterial diazotrophs (e.g., Bauer et al., 2008).
This thesis investigated coastal microbial mats on the Dutch barrier island Schiermonnikoog with regard to their diazotrophic community and N_2 fixation. Molecular tools as well as measurements of nitrogenase activity (NA) were used to elucidate key players as well as spatial and temporal changes in microbial mat diazotrophy.

Dynamic N_2 fixation patterns and complex microbial communities

Although microbial mats have been studied extensively for more than 30 years, the physiology of microbial mats as well as the metabolic activities and their controlling factors is still poorly understood. Measurements of NA have suffered from a lack of detail and resolution due to the unavailability of adequate methods. The new on-line incubation of natural samples or cultures of diazotrophic *Cyanobacteria* has shown its superiority compared to the traditional batch incubation in closed vials (Staal et al., 2001) and allowed for the measurement of light response curves. The deduction of curve parameters for the characterization of NA enabled us to follow changes in NA characteristics over a daily cycle (Chapter 3). The rapid change of these curve parameters caused the daily patterns of microbial mat NA and hinted to variations in the activity levels of the diazotrophic organisms due to an adaptation to changing conditions, to shifts in the active community during a 24 h day, or to a combination of both.
NA is tightly controlled by a number of factors, including the availability of fixed nitrogen, oxygen concentrations and light intensities. The inhibiting effect of nitrogen compounds on NA has been shown for cultured organisms as well for microbial mats (e.g., Ohki et al., 1991; Martín-Nieto et al., 1991; Pinckney et al., 1995b; Cheng et al., 1999). The presence of oxygen is also generally considered to constrain NA (for extensive reviews see Fay, 1992 and Gallon, 1992). However, under certain circumstances oxygen is needed for the energy

supply of nitrogenase via respiration (e.g., Jensen et al., 1983b), e.g., in *Cyanobacteria* which fix N_2 in the dark (Villbrandt et al., 1990; Currin & Paerl, 1998; Staal et al., 2003a). Light is the major source of energy in phototrophs and also fuels NA in these organisms and in the diazotrophic communities dominated by phototrophs (e.g., Bebout et al., 1993; Grabowski et al., 2008). Daily variations in oxygen and light availability, as they occur in microbial mats, can therefore likely alter NA rates and result in a dynamic daily NA pattern. The impact of environmental factors can also lead to a shift in the active diazotrophic community by affecting the activity of the various diazotrophs to a different extent (e.g., Yoch & Gotto, 1982; Gallon et al., 1993; Moisander et al., 2002).

The observation that daily integrated NA under natural light was almost independent from the daily integrated incident light could be indicative for chemotrophic bacteria as the only diazotrophic agents in these microbial mats. However, the light response curves as well as the light action spectra of nitrogenase activity provide a strong support for the dominant role of *Cyanobacteria*. If chemotrophic diazotrophs would be the only active diazotrophs in the mats, no stimulation of nitrogenase activity with increasing light intensities could be observed. Nevertheless, NA patterns are the result of joint activities of a variety of different diazotrophs and therefore only of limited value for identifying key players of N_2 fixation in complex microbial systems.

Exploring the functional diversity to yield further insight in metabolic processes taking place in an ecosystem is challenging. Although the construction of clone libraries is biased (von Witzingerode et al., 1997), it is used to generate lists of 'operational taxonomic units' (OTUs) that identify the major microbial constituents of an ecosystem. In various microbial mats *Cyanobacteria*, as well as *Proteobacteria* are dominant microorganisms but *Chloroflexi*-like bacteria are also common members of the microbial community, especially in hypersaline mats (e.g., Sørensen et al., 2005; Ley et al., 2006). However, not all of the mat-organisms are diazotrophs. Therefore, analyses of *nifH*, the functional gene for one of the enzymes of nitrogenase, were used to identify diazotrophs in microbial mats. In agreement with the previous 16S rRNA analyses, *Cyanobacteria* and *Proteobacteria* dominated (e.g., Yannarell et al., 2006) but the relative contributions to the *nifH* clone libraries differed. For instance, *nifH* sequences belonging to heterotrophic diazotrophs prevailed in *Cyanobacteria*-dominated mats (Zehr et al., 1995; Omoregie et al., 2004a, b; Bauer et al., 2008).

Analyses of the microbial communities of the Schiermonnikoog-mats with the two molecular markers (16S rRNA gene and *nifH*) revealed that *Cyanobacteria* were indeed the dominant component, also with regard to the diazotrophic fraction of the community (Chapter 4). Other important members of the diazotrophic community of both stations were *Gamma-* and *Deltaproteobacteria* although the contribution of the sub-divisions of the *Proteobacteria* to the *nifH* clone libraries was strikingly different between the different types of microbial mats. This difference might be caused by dissimilar niches being present in the microbial mats, with respect to, e.g., oxygen profiles and light attenuation.

Spatial and temporal variability in diazotrophy

The different patterns of NA in each of the mat types can most likely be attributed to the dissimilar diazotrophic communities at the three stations (Chapter 9). As for the daily pattern of NA, the diazotrophic communities of Station II and III were more similar to each other than to Station I. This was confirmed by the comparison of common OTUs. The reason for this is not exactly know. Pore water salinity and desiccation time were proposed to be the main environmental variables determining community composition of the mats on Schiermonnikoog (Dijkman et al., 2010) but environmental conditions other than salinity and desiccation time might have influenced the diazotrophic community. The biggest difference in diazotrophic community composition between Station I as opposed to Station II and III, i.e. the almost complete lack of cyanobacterial *nifH* at Station II and III, still lacks an explanation. We can only hypothesize that the filamentous non-heterocystous *Cyanobacteria* observed at these two stations microscopically may have been non-diazotrophic. Therefore, the dominant cyanobacterium, although being a known diazotroph, may not be the main contributor to *nifH* clone libraries, as shown for other microbial mats (e.g., Omoregie et al., 2004a, b). However, a discrepancy between clone libraries based on *nifH* or its transcripts has been demonstrated within this thesis (Chapter 4) and in other ecosystems (e.g., Hewson et al., 2007; Man-Aharonovich et al., 2007). Hence, organisms not detected in *nifH* clone libraries might still contribute to *nifH* expression.

Besides the differences caused by the position of the microbial mats along the intertidal gradient, the change in beach morphology during the three years of our investigation might have had an impact on the microbial mats. This has already been indicated for the bacterial and diazotrophic community in 2006 and 2007 (Chapter 4). Comparing the diazotrophic communities of all stations over the period of three years showed that the most dramatic shift between subsequent years happened from 2006 to 2007. The shift in community composition from one year to the next might have been caused by morphological changes of the beach area, as visible in the increase of vegetation from 2006 to 2008, as well as by the difference in climatic conditions between these years, especially with respect to temperature, light and water availability. These year-to-year differences, although not investigated in further detail, are likely causes for shifts in the diazotrophic community and, hence, the daily patterns and integrated rates of NA. In order to test this hypothesis, we compared the characteristics of NA by calculating the daily cycle of NA based on the light response curves generated in 2006 and 2007 and the light intensities measured in 2008. Similar NA characteristics in the subsequent years should have resulted in similar daily NA patterns and rates. The striking differences of the daily patterns of NA in the three years are in line with the shift in diazotrophic community composition. Different diazotrophs adapt differently to changing environmental conditions within a 24h cycle and different diazotrophic communities are therefore expected to exhibit different daily patterns of NA. Moreover, lower daily integrated chlorophyll *a*-normalized NA rates calculated for Station I in 2007 and Station II in 2008 as compared to the other years at these stations were also attributed to the shift in diazotrophic community composition. In both cases, a decrease in the contribution of

Oscillatoriales to the diazotrophic community might have caused the lower NA rates. An increase of *nifH* sequences related to *Oscillatoriales* at Station I in 2008 coincided with an increase in daily integrated chlorophyll *a*-normalized NA rates and substantiates this hypothesis.
Studies to follow community composition and metabolic activity of microbial mats over the course of several years are rare. Investigations of microbial mats situated in the intertidal of Bird Shoal within the Rachel Carson National Estuarine Research Reserve (RCNERR) during the last decade revealed a seasonal change in NA pattern and rate but a rather stable diazotrophic community over seasons and years and less dramatic changes in daily NA patterns (Zehr et al., 1995; Paerl et al., 1996; Steppe & Paerl, 2002; 2005).

Active diazotrophs

Cyanobacteria and *Proteobacteria* were the main contributors to the *nifH* pool in the mats but the existence of a functional gene does not necessarily demonstrate expression and therefore potential activity. Therefore, we also investigated the *nifH* transcripts and observed a discrepancy between contribution to *nifH* DNA and cDNA clone libraries (Chapter 4). This discrepancy can be explained by assuming that not every diazotroph expresses *nifH* under the changing conditions during a 24h cycle. Some might not be fixing N_2 at all. This was confirmed by the analysis of OTUs shared between the present and active diazotrophic community which indicated that the active diazotrophic taxa were genetically redundant and in general well represented by the DNA genotypes although unique active diazotrophic taxa were found in the cDNA libraries. This indicates that these organisms were not only present and presumably active; they also showed a higher expression of *nifH* compared to other (active) diazotrophs and, hence, might be major contributors to N_2 fixation in this mat. There were OTUs in the *nifH* cDNA library which were absent in the *nifH* DNA library because they were probably not sufficiently abundant to be retrieved by PCR of the *nifH* DNA. However, because *nifH* is expressed it presumably is an active diazotroph. We also found OTUs overrepresented in the DNA *nifH* library. These organisms contributed to the pool of potential diazotrophs but seemed not to contribute to the fixation of N_2 in this mat.
Although filamentous non-heterocystous *Cyanobacteria* of the *Lyngbya*-type were identified as the structurally dominant components of the Schiermonnikoog microbial mats, *nifH* expression analyses showed that the expression pattern did not correspond to the recorded NA pattern (Chapters 5 and 6). This may not be surprising since gene expression does not necessarily translate directly into an active enzyme (e.g., Ludden & Roberts, 1989; Ohki et al., 1991; Du & Gallon, 1993; Zehr et al., 1993). Moreover, cell-specific *nifH* expression by this cyanobacterium was low compared to the other diazotrophs. *NifH* expression of less than 1 transcript copy^{-1} could have two reasons. On the one hand, all cells could be transcribing but only with a very low rate. On the other hand, transcription per cell could be high but only taking place in very few cells. The conditions at this station might not have supported diazotrophic growth of this cyanobacterium and *Lyngbya* sp. might be outcompeted by heterocystous *Cyanobacteria*

or other diazotrophs without being excluded from the mats. Alternatively the majority of the *Lyngbya* sp. found in this mat might have been of a non-diazotrophic type. This confirms previous observations that structurally dominating diazotrophs might be active but not necessarily the key players in N_2 fixation.

Another structurally important member of many microbial mats, including those at Station III on Schiermonnikoog, is *Microcoleus chthonoplastes* (e.g., Zehr et al., 1995; Paerl et al., 1996; Omoregie et al., 2004 a, b). The repeated failure to detect NA in pure cultures of *M. chthonoplastes* led to the general assumption that this organism lacked the genes for dinitrogen fixation and, hence, lacked the ability to fix N_2 (de Wit et al., 2005; Steppe & Paerl, 2002; Zehr et al., 1995). Nonetheless, Bolhuis et al. (2010) discovered that the genome of *M. chthonoplastes* PCC7420 possesses a complete *nif*-gene cluster and reported that several other strains of this species contained *nifH*, *nifD* and *nifK* (Chapter 7). We therefore expected to find some *nifH* expression in the microbial mats at Station III but repeated analyses showed only low cell specific expression rates (Bolhuis et al., 2010, Chapter 7). However, when taking the measured *nifH* copy number into account, *M. chthonoplastes* appeared to be the most important contributor to overall *nifH* expression and, hence, it might not only be the structurally dominant organisms but also one of the key diazotrophs in this mat type. At another station *nifH* expression by *Oscillatoria*-related diazotrophs was very high. *Oscillatoria* is often observed as the main pioneer organism in different microbial mats (e.g., Stal et al., 1985; Fourçans et al., 2004; Jungblut et al., 2005). The daily NA cycle of these mats (Stal & Krumbein, 1987; Villbrandt et al., 1990) corresponds to the one observed here and might indicate that *Cyanobacteria* are the major diazotrophs at this station.

However, growing evidence from studies analyzing the *nifH* pool in microbial mats suggests organisms other than *Cyanobacteria* as important diazotrophs (e.g., Omoregie et al., 2004a, b). From these mats, proteobacterial *nifH* sequences were retrieved most frequently (e.g., Zehr et al., 1995). The only non-cyanobacterial group of *nifH* sequences that was prominent in the Schiermonnikoog transcript libraries from 2006 was of gammaproteobacterial origin. For these anoxygenic phototrophs, one would expect optimal growth conditions in anoxic but still illuminated parts in the mat. NA at daytime seems plausible under such conditions and would agree with the *nifH* expression pattern. However, copy numbers of this *nifH* sequence were low and therefore the organisms did not seem to be important in whole mat *nifH* expression.

It is unknown whether similar expression levels result in similar activities or not. Owing to the fact that different organisms may be characterized by different post-transcriptional and post-translational regulatory mechanisms, their contribution to whole community NA may still be different from what we might expect based on gene expression levels. Nevertheless, oxygenic phototrophs seem to be the major contributors to NA in the Schiermonnikoog microbial mats.

Controlling factors of N_2 fixation

The large (metabolic) diversity of mat-organisms enables these micro-scale ecosystems to persist under a wide variety of conditions. These conditions, as much as they differ from habitat to habitat, select for certain habitat-specific communities and also shape their metabolic performance, including N_2 fixation which was an important process in all mats that were investigated for it. Temperature and salinity are proposed to be of major importance for the degree of mat development and diazotrophy.
In 2008 a significant impact of variations in light intensities on NA was found for the station harboring a considerable amount of cyanobacterial diazotrophs (Chapter 9). Curiously, variations in light intensity also influenced NA in Station II although we hardly found any diazotrophic *Cyanobacteria* at this station. However, anoxygenic phototrophic diazotrophs might have been important at this station as indicated by the gammaproteobacterial *nifH* in our clone libraries. On the other hand, diazotrophic *Gammaproteobacteria* were also found at Station III where light appeared to have no effect on NA. The reason for that is not clear. At both Station II and III most of these *Gammaproteobacteria* were phototrophs (purple sulfur bacteria). Nevertheless, we expect that also chemotrophic diazotrophs depend indirectly on light, because the photosynthesis by the *Cyanobacteria* drives the microbial mat.
We also measured nitrogenase activity (NA) in three different microbial mat types over a salinity range from 0 PSU (freshwater) to 165 PSU (fivefold the natural salinity) and analyzed the *nifH* transcripts to investigate the effect salinity changes have on the active diazotrophic community and its performance with respect to N_2 fixation (Chapter 8). Studies of this kind are rare and have been limited to hypersaline microbial mats (Pinckney et al., 1995a; Yannarell et al., 2006). The microbial mat highest up in the littoral zone, was less influenced by seawater than the other two stations. Highest NA at salinities ranging from freshwater to ambient seawater salinity demonstrates the adaptation to conditions likely to occur at this station but also the intolerance of the present diazotrophic community with regard to elevated salinities. The shift within the active diazotrophic community seemed more pronounced for the transition from freshwater to natural salinity than from natural to fivefold the natural salinity. This might illustrate the plasticity of the diazotrophic community within the natural salinity range at this station. The diazotrophic fraction of the community best adapted to each of these salinities is most active and ensures high NA. No such plasticity is expected for conditions unlikely to occur and in agreement with decreasing NA at higher salinities. The diazotrophic community active and presumably responsible for high NA under freshwater and seawater conditions showed a remarkable change within the most common groups, *Cyanobacteria* and *Proteobacteria*. The reason why members of the structurally dominant *Oscillatoriales* seemed to be better adapted to lower salinities whereas members of the *Chroococcales* contributed the major part of cyanobacterial *nifH* transcripts at ambient salinity is unknown. The significantly lower contribution of cyanobacterial nifH transcripts to the clone libraries at higher salinities coincided with lower NA rates. The microbial mat close to the low water mark and therefore frequently covered with seawater harbored the most stable active diazotrophic community under increasing salinities, except for the increase in

contribution of *Deltaproteobacteria*. Along with that we also observed smaller differences in NA at the different salinities. This could be caused by the selection for a halotolerant diazotrophic community resulting from the more frequent changes of salinity experienced at the lower intertidal region during the tidal cycle. NA in general was lower at this station, likely caused by the low contribution of *Cyanobacteria* to the active diazotrophic community at all salinities.

Conclusions and outlook

As in most other microbial mats, *Cyanobacteria* are the main structural elements of the intertidal mats of Schiermonnikoog. Filamentous non-heterocystous as well as unicellular forms prevail and have also found to be the most active ones with regard to *nifH* expression. Although we cannot directly translate expression into activity, these oxygenic phototrophs are assumed to be the main diazotrophs in the investigated mats. The dominant cyanobacterium *Lyngbya* sp., however, exhibited only low *nifH* expression levels compared to the other diazotrophs we investigated. Apart from *Cyanobacteria*, *Proteobacteria* were main constituents of the mats, *Gamma-* and *Deltaproteobacteria* being the dominant representatives. Their ecology with regard to N_2 fixation is not well understood but the anoxygenic phototrophs within the *Gammaproteobacteria* are likely to benefit from the anoxic conditions in the mats just beneath the cyanobacterial layer where they still receive sufficient light for energy supply because their pigments absorb in the NIR part of the light spectrum which is less attenuated with depth. The joint activities of this diverse community of diazotrophs shapes the daily pattern of NA and a shift in the diazotrophic community is likely to be responsible for shifts in Na patterns and rates. These shifts were observed on a spatial as well as temporal level. Spatially, the three mat types also exhibit a dissimilar response to altered salinities, both in the active diazotrophic community and in NA rates, most likely due to adaptations to the degree of salinity changes they usually experience during the tidal cycle.
In order to fully understand the dynamics within this highly diverse ecosystem sampling has to be carried out more frequently, especially to elucidate seasonal changes. Along with the mat sampling, environmental conditions have to be monitored in detail to explain the shifts in community composition and NA. To further investigate the influence of environmental conditions, the most predominant conditions (e.g., oxygen supersaturation or anoxia, water saturation or desiccation) could be mimicked in mesocosm experiments. Diazotrophic community composition and NA characteristics under these conditions can then be compared to major shifts *in situ* and used to elucidate driving factors behind ecosystem changes.