Diazotrophy and diversity of benthic cyanobacteria in tropical coastal zones

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Till mamma och pappa

Abstract

Discoveries in recent years have disclosed the importance of marine cyanobacteria in the context of primary production and global nitrogen cycling. It is hypothesized here that microbial mats in tropical coastal habitats harbour a rich diversity of previously uncharacterized cyanobacteria and that benthic marine nitrogen fixation in coastal zones is substantial.

A polyphasic approach was used to investigate cyanobacterial diversity in three tropical benthic marine habitats of different characters; an intertidal sand flat and a mangrove forest floor in the Indian Ocean, and a beach rock in the Pacific Ocean. In addition, nitrogenase activity was measured over diel cycles at all sites. The results revealed high cyanobacterial diversity, both morphologically and genetically. Substantial nitrogenase activity was observed, with highest rates at daytime where heterocystous species were present. However, the three habitats were dominated by non-heterocystous and unicellular genera such as *Microcoleus, Lyngbya, Cyanothece* and a large group of thin filamentous species, identified as members of the Pseudanabaenaceae family. In these consortia nocturnal nitrogenase activities were highest and *nifH* sequencing also revealed presence of non-cyanobacterial potential diazotrophs. A conclusive phylogenetic analysis of partial *nifH* sequences from the three sites and sequences from geographically distant microbial mats revealed new clusters of benthic potentially nitrogen-fixing cyanobacteria. Further, the non-heterocystous cyanobacterium *Lyngbya majuscula* was subjected to a physiological characterization to gain insights into regulatory aspects of its nitrogen fixation. The data demonstrated that nitrogenase activity is restricted to darkness, which called upon a re-evaluation of its diazotrophic behaviour.

Keywords: Marine cyanobacteria, benthic nitrogen fixation, diversity, diazotrophy, *Lyngbya majuscula,* Zanzibar, western Indian Ocean

List of Publications

This thesis is based in the following publications/manuscripts and additional unpublished data. Publications will be referred to in the text by roman numerals as follows:

I Lundgren, P., **Bauer, K.,** Lugomela, C., Söderbäck, E., & Bergman, B. (2003): Re-evaluation of the nitrogen fixation behaviour in the marine non-heterocystous cyanobacterium *Lyngbya majuscula*. *J. Phycol.* 39, 310-314.

II Díez, B., **Bauer, K.** & Bergman, B. (2007): Epilithic cyanobacterial communities of a marine tropical beach rock (Heron Island, Great Barrier Reef): diversity and diazotrophy. *Manuscript accepted with revisions in Appl. Environ. Microbiol.*

III **Bauer, K.,** Díez, B., Lugomela, C., Seppälä, S., Borg, A.J. & Bergman, B. (2007): Variability in diazotrophy and cyanobacterial diversity in a tropical intertidal lagoon. *Manuscript submitted to Appl. Environ. Microbiol.*

IV **Bauer, K.**, Bergman, B. & Díez, B. (2007): New nitrogen-fixing tropical marine cyanobacteria: a case-study of microbial mats in a mangrove habitat. *Manuscript*

Paper I is reproduced with the permission of the Journal of Phycology. My contributions to the papers were as follows: (I) Taking part in planning, sampling, analysing and writing the manuscript, performing the majority of the acetyelene reduction assays, performing the western blots and immunogold assays. (II) Taking part in planning, sampling, analysing and writing the manuscript, performing the acetyelene reduction assays and morphological identification (III). Taking part in planning, analysing and writing of the manuscript, performing the majority of the analyses. (IV). Taking part in planning, sampling, analysing and writing the manuscript, performing the major part of the analyses except for the image analysis.
I am corresponding author for paper I, III and IV.

Related paper not included in the thesis:
Falcon, L.I., Lindvall, S., **Bauer, K.**, Bergman, B & Carpenter E.J. (2004): Ultrastructure of unicellular N_2 fixing cyanobacteria from the tropical North Atlantic and subtropical North Pacific Oceans. *J. Phycol.* 40, 1074-1078.

Contents

Abbreviations

abs.	absolute
ARA	acetylene reduction assay
ATP	adenosine triphosphate
Au	gold
C	carbon
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
eDNA	environmental DNA
EPS	extracellular polymeric substances
EtOH	ethanol
FSW	filtered seawater
gDNA	genomic DNA
IAA	isoamyl alcohol
LM	light microscopy
Ma	million years
N	nitrogen
NA	nitrogenase activity
OTU	operational taxonomic unit
PCR	polymerase chain reaction
Psu	practical salinity unit
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase – polymerase chain reaction
SEM	scanning electron microscopy
TEM	transmission electron microscopy
WIO	western Indian Ocean

1. Introduction

1.1. Cyanobacteria

1.1.1 Evolutionary aspects and cyanobacteria of today

The Cyanobacteria is one of about 10 major phyla of eubacteria according to 16S rRNA phylogeny (Woese, 1987). They inhabited earth long before most present-day life forms. Geological evidence suggests that their origin might date as far back as 3,500 Ma (Schopf, 2000). During their inconceivably long evolution these organisms have diverged and colonised a wide variety of habitats, but it has been suggested that the first cyanobacteria were unicellular and inhabiting freshwater environments (Sanchez-Baracaldo, 2005). Recent findings suggest that differentiated cyanobacteria arose between 2.450 and 2.100 Ma ago (Tomitani et al., 2006). Some of the earliest evidence of life includes microfossils of stromatolites (Fairchild et al., 1996), analogues to modern day microbial mats, laminated communities often dominated by cyanobacteria (Stal, 2000). Cyanobacteria are also believed to have been the first oxygen evolving photoautotrophs, hence contributing to the shift from an anaerobic to an aerobic atmosphere. There is a strong evolutionary relationship between cyanobacteria and higher plants (Giovannoni et al., 1988; Martin et al., 2002; Douglas and Raven, 2003) as cyanobacteria through endosymbiosis gave rise to photosynthetic organelles (Margulis, 1970).

Over the years cyanobacteria have been extensively studied in terrestrial environments, ranging from arctic crusts to desserts. However, the scope of this thesis is on the most recently noticeable group; marine cyanobacteria. Traditionally, focus of aquatic cyanobacterial research has been on freshwater and brackish systems (Oliver and Ganf, 2000), dealing with issues such as toxin production and eutrophication. In the recent two decades evidence for the importance and ecological significance of marine cyanobacteria has been overwhelming (Charpy and Larkum, 1999; Paerl, 2000; Karl et al., 2002). Especially the nitrogen-fixing *Trichodesmium* has fascinated the scientific community (Capone et al., 1997), not only because of its widespread

distribution but also because of its unusual nitrogen-fixation behaviour for a non-heterocystous cyanobacterium, with nitrogen fixation restricted to the light period (Berman-Frank et al., 2001; El-Shehawy et al., 2003). Further, the discovery of the tremendous capacity of marine planktonic picocyanobacteria in primary production have in many ways changed the understanding of nutrient cycling in the world's oceans (Chisholm et al., 1988; Partensky et al., 1999). In addition, relatively recently it has been shown that also some unicellular planktonic cyanobacteria may contribute substantially to marine nitrogen fixation (Zehr et al., 2001; Falcon et al., 2004b). Benthic cyanobacteria, being important primary producers, and in some cases also nitrogen-fixers in microbial mats have also received attention (Stal, 2000). However, most recent studies focus on mats in extreme habitats (Ward et al., 1998; Nübel et al., 1999; Abed et al., 2002; Taton et al., 2003; Jungblut et al., 2005; Ley et al., 2006), while the diversity is likely to be higher in environments with more favourable conditions, such as tropical coastal areas, which still remains largely uncharted in terms of cyanobacterial diversity (Golubic, 1999).

1.1.2 Diversity and cyanobacterial taxonomy

The use of culture-independent techniques has revolutionized the field of microbial diversity (Hugenholtz et al., 1998; Muyzer, 1999). Estimates suggest that standard culture techniques fail to isolate more than 99% of all bacteria encountered in nature (Amann et al., 1996; Handelsman, 2004). Diversity of cyanobacteria have been investigated in numerous natural habitats, in recent years mainly by using molecular techniques, but the issue of identifying individual taxa remains problematic (Komárek and Anagnostidis, 1998; Golubic, 1999; Komárek and Anagnostidis, 2005). Mainly two different approaches have been applied to cyanobacterial taxonomy; the traditional botanical approach and the considerably more recent bacteriological approach. Due to their photosynthetic properties and pigment composition cyanobacteria were at first classified together with eukaryotic algae and termed 'blue-green algae'. The botanical approach to cyanobacteria taxonomy dates back to the 19th century and is based on phenotypic descriptions, including cell and sheath morphology, colony formation, pigmentation, mode of reproduction and to some extent physiology and biochemistry. Further, each species has to be described in Latin and its reference is a herbarium specimen. Thuret (1875), Gomont (1892) and Bornet and Flahaut (1886-1888) were the first to use this approach for taxonomy of the families Oscillatoriaceae, Nostocaceae and Stigonemataceae. Totally over 2000 species of cyanobacteria have been validly published under the botanical code of nomenclature. The most comprehensive and updated taxonomic revision, based on the botanical approach, was published by Komárek and Anagnostidis (1998; 2005).

However, the discovery of the prokaryotic nature of cyanobacteria and limitations of the botanical approach, especially for cyanobacteria with simple morphologies, opened up for the bacteriological approach. This approach to cyanobacterial taxonomy was first proposed by Stainer and others (1978). The bases are physiological and genotypic characters such as pigment composition, fatty acid analysis, heterotrophic growth, nitrogenase activity, DNA base composition and genome length. It requires a live specimen to be cultured in one of the official cyanobacterial collections of the world. The taxonomy according to this approach was published by Rippka and others (1979) and is summarized in Table 1.

Table 1. Cyanobacterial taxonomy after Rippka et al. (1979).

Section I	Unicellular cyanobacteria that reproduce by binary fission or by budding.
Section II	Unicellular cyanobacteria that reproduce by multiple fission.
Section III	Filamentous non-heterocystous cyanobacteria that divide in only one plane.
Section IV	Filamentous heterocystous cyanobacteria that divide in only one plane.
Section V	Filamentous heterocystous cyanobacteria that divide in more than one plane.

Initially, molecular tools for taxonomy included the use of chemotaxonomic markers (e.g. lipid composition, polyamines, carotenoids and biochemical features). However, methods based on nucleic acids and proteins are now much more well-established and commonly used. The 16S rRNA gene has proven to be a useful marker for investigating phylogenetic relationships and is the most commonly used marker for distinguishing identities between prokaryotic organisms at the genus level. For filamentous cyanobacteria the more variable *hetR* gene were shown to distinguish organisms even at the strain level (Janson et al., 1999a; Janson et al., 1999b). Recently, the internal transcribed spacer (ITS) between the 16S and 23S rRNA genes has also been used as a complement to 16S rRNA analysis for diversity studies in environmental samples (Taton et al., 2003). The ITS is a variable sequence and has been successfully used to distinguish between cultured strains (Scheldeman et al., 1999; Iteman et al., 2000; Boyer et al., 2002). However, the study by Taton et al. (2003), in which the cyanobacterial diversity in a microbial mat was investigated, showed that no meaningful alignment could be made based on different ITS types. Also the nitrogenase encoding gene *nifH* has been used extensively as a genetic maker among nitrogen-fixing phylotypes. The rather short length of the normally amplified *nifH* fragments

(Zehr and McReynolds, 1989; Olson et al., 1998; Poly et al., 2001) is afflicted with limitations for resolving phylogenetic relationships. However, a comparison of bacterial 16S rRNA and *nifH* based phylogenetic reconstructions demonstrated high consistency (Zehr et al., 2003). Up till now, few cyanobacteria have been characterized and validly published under the bacteriological code of nomenclature (Castenholz, 2001). The most updated system based on the bacteriological approach is available online through Bergey's manual of bacteriology and a summary is given in Table 2. (Garrity et al., 2001).

Table 2. Classification of cyanobacteria according to Bergey's manual of bacteriology. Adapted from (Garrity et al., 2001).

Class	Subsection	Family	Form genus
Cyanobacteria	I	I	*Chamaesiphon, Chroococcus, Cyanobacterium, Cyanobium, Cyanothece, Dactylococcopsis, Gloeobacter, Gloeocapsa, Gloeothece, Microcystis, Prochlorococcus, Prochloron, Synechococcus, Synechocystis*
	II	I	*Cyanocystis, Dermocarpella, Stanieria, Xenococcus*
		II	*Chroococcidiopsis, Myxosarcina, Pleurocapsa*
	III	I	*Arthrospira, Borzia, Crinalium, Geitlerinema, Halospirulina, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Oscillatoria, Planktothrix, Prochlorothrix, Pseudanabaena, Spirulina, Starria, Symploca, Trichodesmium, Tychonema*
	IV	I	*Anabaena, Anabaenopsis, Aphanizomenon, Cyanospira, Cylindrospermopsis, Cylindrospermum, Nodularia, Nostoc, Scytonema*
		II	*Calothrix, Rivularia, Tolypothrix*
	V	I	*Chlorogloeopsis, Fisherella, Geitleria. Lyengariella, Nostocopsis*

An approach using polyphasic taxonomy, the term first introduced by Colwell (1970), was attempted to reach a consensus in bacterial systematics by integrating genotypic, phenotypic and phylogenetic information (Vandamme et al., 1996). It has proved to be a valuable tool for bacteriologists, and particularly for cyanobacteriologists (Lehtimaki et al., 2000; Abed, 2002; Suda et al., 2002; Abed et al., 2003). Due to the increasing amount of

taxonomically informative data on cyanobacteria generated from modern techniques such as genetic sequencing, ultrastructure and ecophysiological characterization, a revision of cyanobacteria taxonomy, integrating new information is under way (Komárek and Anagnostidis, 1998; Hoffmann et al., 2005; Komárek and Anagnostidis, 2005) and a new system have been proposed (Fig. 1). However, it is evident that certain orders and families remain problematic and require further revision. Moreover, with new information, such as whole-genome sequences accumulating, the taxonomic system will inevitably continue to change and evolve.

Table 3. Proposal of a new cyanobacterial taxonomic system. Only genera supported by molecular and ultrastructural markers are included in the generic category. Taxa in parentheses are not yet validly described. Adapted from Hoffmann et al. (2005). →

	Order	Family	Genus (selected)
G*	Gloeobacterales	Gloeobacteraceae	*Gloeobacter*
Synechococcophycidae	[Synechococcales] **coccoid**	Synechoccaceae	*Aphanothece* (small-celled types*), Cyanobium, Prochlorococcus, Synechococcus*
		Merismopediaceae	*Aphanocapsa, Synechocystis* (small-celled)
		Chamaesiphonaceae **heteropolar**	*(Chamaesiphon* subg. *Euchamaesiphon)*
		Acaryochloridaceae	*Acaryochloris*
	[Pseudanabaenales] **trichal**	Pseudanabaenaceae	*Geitlerinema, Halomicronema, Limnothrix, Leptolyngbya, Prochlorothrix, Pseudanabaena*
		Schizorichaceae	*Shizothrix*
Oscillatoriophycidae	Chroococcales **Coccoid/trichal**	(Cyanobacteriaceae) **coccoid**	*Aphanothece* (large-cell type)*, Cyanobacterium, Cyanothece, "Euhalothece", Myxobaktron*
		Microcystaceae **coccoid**	*Microcystis*
		Gomphosphaeriaceae **coccoid**	*Snowella, Woronichinia*
		Prochloraceae **coccoid**	*Prochloron*
		Chroococcaceae **coccoid**	*Chroococcus*
		Entophysalidaceae **polarized**	*Enthophysalis, Cyanoarbor*
		Stichosiphonaceae **polarized**	*Chamaecalyx*
		Dermocarpellaceae **polarized**	*Cyanocystis, Dermocarpella, Stanieria*
		Xenococcaceae **polarized**	*Chroococcidiopsis, Myxosarcina, Xenococcus*
		Hydrococcaceae **polarized**	*Hyella, Pleurocapsa*
		(Spirulinaceae) **trichal**	*(Halospirulina, Spirulina)*
	Oscillatoriales **trichal**	Borziaceae **necrides -**	*Borzia, Komvophoron*
		Phormidaceae **necrides +**	*Arthrospira, Microcoleus, Phormidium, Plantothrix, Symploca, Trichodesmium, Tychonema*
		Ammatoideaceae	*Ammatoidea*
		Oscillatoriaceae **necrides +**	*Blennothrix, Hormoscilla, Lyngbya, Oscillatoria*
		Gomontiellaceae **trichal, necrides +**	*Crinalium, Starria*
Nostochphycidae	Nostocales **heterocystous**	Scytenemataceae **isopolar, false branching**	*Scytonema*
		Symphynemataceae **true branching**	*Symphyonema,* "Y-Stigonematales"
		Borzinemataceae	*Borzinema*
		Rivulariaceae **heteropolar, hairs**	*Calothrix, Gloeotrichia, Rivularia*
		Microchaetaceae **heteropolar**	*Microchaete, Spirirestis, Tolypothrix*
		Nostocaceae **isopolar, without branching**	*Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermopsis, Cylindrospermum, Nodularia, Nostoc, Trichormus*
		Chlorogloeopsidaceae **simple true branching**	*Chlorogloeopsis*
		Haplosiphonaceae **true branching**	*Fisherella, Mastigocladus,* "T-Stigonematales"
		Loriellaceae	*Loriella*
		Stigonemataceae **true branching, multiseriate**	*Stigonema*

* Gloeobacterophycideae

1.1.3 Metagenomic analyses – current status

The most recent advancements in microbial diversity include the community genomic or 'metagenomic' approach. High-throughput DNA sequencing has resulted in an enormous increase in genomic information related to microorganisms during the last decade. Today, close to 20 cyanobacterial genomes (gDNA) have been sequenced (Liolios et al., 2006), and several more are to be expected in the years to come. This development will likely revolutionize our knowledge and understanding of physiological properties of individual marine microbes and microbial consortia. Ongoing application of metagenomic methods to ocean water samples (Venter et al., 2004) is creating an unimaginable dataset on genetic diversity and metabolic pathways in marine microbes. Metagenomic methods provide tools to evaluate evolution, ecosystem functioning and predictions of responses to environmental perturbations in the world's oceans. These methods also allow comprehensive evaluation of physiological properties of individual organisms. Venter and coworkers (2004), when introducing marine microbial whole genome eDNA analyses, found not less than 1.800 tentative new species and astoundingly 1.2 million new genes in the Sargasso Sea (Atlantic Ocean), even though only the smallest cell size fraction <0.8 μm was analyzed, and that the Sargasso Sea is already one of the most studied seas in the world in terms of environmental genomics (Beja, 2002a; 2002b; 2002c). It is crucial that we begin to attribute biogeochemical processes, such as cycling of C and N to individual components of the microbial populations. In this context, cyanobacteria have a specific role as they all are photoautotrophic and a large proportion also diazotrophic. Genomic methodologies will allow such high resolution characterizations. Although the whole genome eDNA analyses generates huge amount of valuable genetic data for the various microbial consortia, there is no direct mechanism available to connect this data to uncultured morphotypes and thereby to the individual microbial actor(s), and therefor cyanobacterial and other bacteria phenotypic identification may still rely on visual observations at some level of resolution (LM, SEM or TEM).

1.1.3 Ecophysiology and mat-building

Photosynthetic properties and the ability to fix atmospheric nitrogen leave some cyanobacteria with little dependence on their surrounding environment in regards to supply of nutrients. Approximately 90% of total nitrogen in seawater is in the form of N_2 (Kennish, 2001) and thereby only available for diazotrophs. For nitrogen-fixing cyanobacteria phosphorus is therefore generally the limiting nutrient, but also deficiencies in iron are known to restrict growth (Karl et al., 2002). In cyanobacterial photosynthesis (with the exception of prochlorophytes) light is harvested by phycobilisomes and chlorophyll a and through the electron transport chain ATP is formed. Aerobic

respiration is an additional source of energy, taking place in the absence of light. These processes take place in thylakoid and cytoplasmic membranes, respectively. If anoxic conditions prevail, e.g. in microbial mats, fermentation function as an alternative energy source.

Cyanobacteria dominated microbial mats have steep gradients in light penetration and the euphotic zone is often not more then 1 mm (Lassen et al., 1994). The complex structure and activities in mats cause also gradients in oxygen levels, nutrient concentrations, pH and redox-potential. Common for many mat-forming cyanobacteria are high production of extracellular polymeric substances (EPS), contributing to the cohesiveness of the mat matrix. Mats are mainly formed by filamentous cyanobacteria, trapping sediment particles. EPS stabilizes the sediments and enables the establishment of the mat. Also some unicellular species form mats, usually less coherent in structure. For a microbial mat to develop certain conditions have to be fulfilled (Stal, 2000):

> "(i) the environmental conditions must allow growth of the mat-building organism
>
> (ii) the growth rate of the mat-building organisms must be faster than consumption by grazing organisms
>
> (iii) sedimentation rates should not be exceedingly high to allow stabilized colonization of the surface by the mat-building organism
>
> (iv) destruction forces such as burrowing organisms and mechanical and chemical erosion must be absent or at least not prevent accretion of organisms."

When these criteria are met, cyanobacterial mats commonly develop in a wide variety of habitats with an extreme rage of physical conditions, such as hot springs, arctic lakes and hypersaline lagoons. Microbial mats also thrive in environments with large daily fluctuations in light, temperature and salinity, such as inter-tidal habitats (Stal, 2000).

1.2 Nitrogen Fixation

1.2.1 The process

Nitrogen is one of the most essential nutrients for all living beings and dinitrogen gas (N_2) is the major component of the Earth's atmosphere. However, the inert gas is not biologically available to most organisms. Nitrogen

has to exist in the form of NH_3 or NO_X to be utilized by higher plants and animals. The only organisms with access to the immensely large reserve of dinitorgen in the atmosphere are the diazotrophs, e.g. selected groups among archea and eubacteria with the capability to reduce dinitorgen to ammonia. This process is known as biological nitrogen fixation and is catalyzed by enzyme complex termed nitrogenases (Stacey et al., 1992).

Among cyanobacteria, many species combine the ability to perform oxygenic photosynthesis and fix atmospheric dinitrogen (N_2). The latter process is performed according to the following equation:

$$N_2 + 8\ e^- + 8\ H^+ \longrightarrow 2NH_3 + H_2$$

It is an energy demanding process that requires 16 ATP to reduce one molecule of dinitorgen (Gallon and Chaplin, 1987). The reaction is catalysed by nitrogenase, composed of two subunits: dinitrogenase reductase and dinitrogenase. The nitrogenase most commonly found in cyanobacteria is often referred to as conventional or classic nitrogenase and has two components. The smaller component, dinitrogenase reductase (Fe protein), is encoded by the *nifH* gene and the larger dinitrogenase (MoFe protein) is encoded by *nifD* and *nifK*. Dinitrogenase reductase serves as an electron donor for the catalytic dinitorgenase and has two identical subunits bridged by a single [4Fe-4S] cluster. Dinitrogenase is a tetramer and has two P clusters [8Fe-7S] and two FeMo cofactors which are proposed binding sites for dinitrogen (Smith et al., 1985; Igarashi and Seefeldt, 2003). There are also alternative nitrogenaseses, which do not contain molybdenum. In the first alternative nitorgenase the Fe protein is coded by *vnfH*, in which v stands from vanadium that replaces molybdenum. The second alternative nitrogenase contains neither molybdenum nor vanadium and the corresponding gene is *anfH* (Dixon and Kahn, 2004). A fourth and fundamentally different type of superoxide dependent nitrogenase was recently described in *Streptomycetes thermoautotrophicus* (Ribbe et al., 1997). Including the structural genes, about 20 nif genes are expressed and involved in biosynthesis and regulation of proteins involved in the nitrogen fixation process (Johnston et al., 2005).

1.2.2 Ecophysiological strategies

Nitrogenase is irreversibly inactivated when exposed to oxygen. Not only does O_2 affect the protein structure but it can also inhibit synthesis of nitrogenase in many diazotrophs (Wolk, 1996; Bergman et al., 1997). For oxygen evolving photosynthetic cyanobacteria it is essential to protect the nitrogenase from inactivation and for this purpose various strategies have evolved. The most apparent strategy is the differentiation of heterocysts,

cells specialized for nitrogen fixation, which lack the capability to perform photosynthesis. They have thicker cell walls to reduce O_2 diffusion, no PSII activity and show higher respiration rate than vegetative cells. About 5-10% of the cells differentiate into heterocysts, evenly spaced along the filament (Adams and Duggan, 1999). For the non-heterocystous nitrogen-fixing cyanobacteria the strategies are more variable and complex. Unicellular cyanobacteria use, instead of spatial separation with heterocysts, temporal separation with nitrogen fixation taking place during the night and oxygenic photosynthesis during the day (Gallon et al., 1988; Reddy et al., 1993). Ultrastructure analysis of nitrogen-fixing unicellular cyanobacteria from the tropical North Atlantic and the North Pacific suggest that carbohydrates are stored in polysaccharide granules during photosynthesis as a strategy to later on supply cells with energy during nocturnal nitrogen fixation (Falcon et al., 2004a). Additionally, a large group of cyanobacteria are capable of nitrogen fixation only under micro-oxic or anoxic conditions (Bergman et al., 1997). One example is the filamentous *Plectonema boryanum*, which fixes nitrogen only under micro-oxic conditions. It is however unable to fix nitrogen when grown completely anaerobically, which stresses the need for respiration as a source of ATP for the highly energy demanding nitrogen-fixing reaction (Rai et al., 1992). The likewise non-heterocystous genus *Trichodesmium* has yet another strategy for nitrogen fixation, combining spatial and temporal separation of nitrogen fixation and photosynthesis. Immunological studies showed that nitrogenase is restricted to a novel type of specialized cell (Bergman and Carpenter, 1991), later termed diazocyte (Fredriksson and Bergman, 1997). Diazocytes constitute approx. 15% of total cells, organized into subsets of cells (Janson et al., 1994; Lin et al., 1998; El-Shehawy et al., 2003). More recently, a temporal separation in *Trichodesmium* was disclosed, with photosynthetic rates decreasing at midday when nitrogenase activity peaked (Berman-Frank et al., 2001).

1.2.3 The actors – *nifH* diversity

Only species within archea and eubacteria are known to fix atmospheric nitrogen. Nitrogen-fixers are commonly referred to as diazotrophs and are widespread in diverse habitats both free-living and in various symbiotic associations. Sequencing and phylogenetic analyses of *nifH* genes have become a common approach to study diazotrophs and have confirmed their presence in habitats ranging from termite guts and marine invertebrates to mangroves and coral reefs (Zehr et al., 2003). Not all species express their *nifH* genes, and certainly not at all times, and to target the most active diazotrophs in a community it is advisable to extract and amplify RNA. Generally *nifH* sequences divide into four major phylogenetic clusters. *nifH* and some *vnfH* sequences (coding for the conventional molybdenum nitrogenase and alternative vanadium nitrogenases) in cyanobacteria and proteobacteria

form cluster I. Genes encoding nitrogenase in some archaea and the second alternative nitrogenase form cluster II. Cluster III contains *nifH* genes from gram positive bacteria, such as *Clostridium,* and alternative nitrogenase from the archaeon *Methanosarcina*. Cluster IV contains sequences coding for nitrogenase homologues from archaea and chlorophylide (Chien and Zinder, 1996; Zehr et al., 2003).
Diversity studies using *nifH* genes as markers have shown large variations between different environments such as open oceans, salt marshes, freshwater and hypersaline lakes, estuaries and terrestrial systems, suggesting that nitrogen-fixing actors differs between ecosystems (Zehr et al., 2003). Recently, attempts have been made to assess *nifH* diversity using microarrays (Steward et al., 2004; Moisander et al., 2006) with promising results. This approach will be useful for monitoring community changes and *nifH* expression, but to thoroughly explore diversity in remote locations where new phylotypes are frequently encountered traditional cloning techniques, or indeed metagenomic analyses, combined with isolation of potential diazotrophs is essential.

1.3 Marine cyanobacteria and nitrogen fixation

1.3.1 Overview

Nitrogen fixation by benthic cyanobacteria was explored already in the 60s by Stewart, who performed ^{15}N studies on *Calothrix* scopulorum in benthic areas in Scotland (Stewart et al., 1967). From being regarded as less prevalent and diverse than cyanobacteria in terrestrial and limnic systems, marine cyanobacteria are now recognized as important primary producers and nitrogen-fixers in oceans world-wide (Capone et al., 1997; Partensky et al., 1999; Paerl, 2000; Karl et al., 2002). On account of its oligotrophy, the open ocean is well suited for diazotrophic and photosynthetic cyanobacteria. However, heterocystous species, with a few exceptions (Carpenter and Janson, 2001), seems to be ‘mysteriously’ excluded from such waters. It has recently been suggested that temperature may exclude heterocystous cyanobacteria from tropical oceans (Staal et al., 2003). The theory is that heterocystous cyanobacteria are out-competed by the diazocytic *Trichodesmium*, as differentiation of heterocysts do not provide any advantage in tropical waters. Nevertheless, the great advantage of diazocytes over heterocysts is still unknown and the low abundance of heterocystous species in cold waters remains enigmatic.

Although mostly dominated by non-heterocystous filamentous and unicellular species, marine microbial benthic communities in tropical and temperate coastal habitats harbor heterocystous cyanobacteria such as *Calothrix*, *Anabaena, Nodularia* and *Rivularia* (Stal, 1995; Stal et al., 1996; Lugomela et al., 2001a). Another heterocystous genus, *Richelia* lives symbiotically within marine diatoms (Jansson et al. 1999a). Other marine symbioses have been observed between nitrogen fixing unicellular cyanobacteria and various marine eukaryotes such as dinoflagellates, sponges, cnidarians, ascidians, echiuroid worms and corals (Carpenter and Foster, 2002; Janson, 2002; Karl et al., 2002; Raven, 2002a, 2002b; Lesser et al., 2004; Foster et al., 2006a; Foster and Zehr, 2006b). There are also intertidal lichens with cyanobacterial symbionts (Janson et al., 1993; Carpenter and Foster, 2002).

Massive accumulation of nitrogen-fixing cyanobacteria at surfaces ('blooms') are in certain waters afflicted with environmental problems such as toxin production. Summer bloom of the toxin producing *Nodularia spumigena* is an escalating and persistent issue in the Baltic Sea (Stal et al., 2003) and toxic blooms of *Lyngbya majuscula* are an increasing problem in coastal zones of Queensland, Australia (Albert et al., 2005; Watkinson et al., 2005).

Studies of marine cyanobacteria have progressed rapidly in the recent decades due to technical advancements such as remote sensing and an increase in oceanic research cruises, which provides access to large, remote areas of the open oceans. The vast majority of these expeditions are however restricted to oceans surrounding developed countries (e.g. the Tropical Atlantic Ocean and parts of the Pacific Ocean). Other marine territories, such as the Indian Ocean, still remain largely understudied. Recent focus on marine research in western Indian Ocean (WIO), exposed major knowledge gaps concerning e.g. identification and activities of primary producers and it was pointed out that this area requires immediate attention (Björk et al., 1996). Cyanobacterial research in the region has recently gained momentum but additional studies are called for (Bergman, 2001) and the microbiota of benthic communities are the least studied. Nitrogen fixation by *Trichodesmium* in coastal zones of Tanzania was reported already 1981 (Bryceson and Fay, 1981) and recent studies investigated morphological diversity, nitrogen fixation and productivity of both planktonic and benthic cyanobacteria around Zanzibar (Lugomela et al., 2001a; Lugomela et al., 2001b; Lugomela et al., 2005). With a large part of the population in the region strongly depending on natural resources in coastal zones for their livelihood, sustainable productivity in the marine environment is of crucial importance. For an in-depth understanding of nitrogen cycling dynamics, the most limiting nutrient for marine productivity, in the WIO region extensive surveys and quantifications of nitrogen fixation is necessary.

1.3.2 Marine benthic nitrogen fixation

Up until the 1990s benthic nitrogen-fixing was considered the main source of 'new' nitrogen in the world's oceans (Capone, 1988). Estimations suggested that 15.4 Tg N was fixed annually by benthic nitrogen fixers (Capone and Carpenter, 1982), as seen in Table 4. The estimates are based on relatively few studies and would benefit from a re-evaluation that would include data collected during the last 20 years.

Table 4. Estimates of benthic marine nitrogen fixation approximated to habitat area (Capone and Carpenter, 1982).

Environment	Area	N_2 Fixation	
	(km^2 x 10^6)	(g/m_2 year)	(Tg/year)
Depth			
> 3000 m	272	0	0
2000 to 3000 m	31	0.0007	0.022
1000 to 2000 m	16	0.001	0.016
200 to 1000 m	16	0.01	0.16
0 to 200 m	27	0.1 ± 0.04	2.7
Bare estuary	1.08	0.4 ± 0.07	0.43
Sea grass	0.28	5.5	1.5
Coral reefs	0.11	25 ± 8.4	2.8
Salt marsh	0.26	24 ± 10.5	6.3
Mangroves	0.13	11	1.5
Total	363		15.4

Cyanobacterial mats have been in focus in benthic nitrogen fixation studies (e.g. Stal et al., 1984; Stal and Krumbein, 1985a, 1985b, 1985c; Stal, 1988; Villbrandt et al., 1991; Bebout et al., 1993; Paerl et al., 1996; Pickney and Paerl, 1997; Steppe et al., 2001; Omoregie et al., 2004a, b; Charpy-Roubaud and Larkum, 2005), but cyanobacteria are not the only potential nitrogen-fixers in these systems. Recent studies have shown that also heterotrophic bacteria, such as *Clostridium, Desulfovibrio, Klebsiella* and *Azotobacter*, are potentially important nitrogen-fixers in microbial mats (Zehr et al., 1995; Steppe and Paerl, 2002; Yannarell et al., 2006). The main challenge of benthic nitrogen fixation research today is to expand the focus beyond a few geographical 'hot spots' in Mexico and U.S. (see references above), and to search for geographical distribution patterns of important benthic diazotrophs. For large scale global quantification, numerous additional studies are needed and for physiological characterization of significant actors, isolation and culturing efforts are crucial.

2. Aim

The overall aim of the thesis was to search for and refocus on marine benthic nitrogen fixation, and to raise the awareness of current challenges and solutions related to cyanobacterial taxonomy. This was accomplished through;
I) exploring the occurrence of marine benthic cyanobacteria, with focus on diazotrophs in the western Indian Ocean coastal zones, using a polyphasic approach and;
II) deepening our knowledge in regards to nitrogen-fixation strategies and capacities of benthic cyanobacteria.
II) challenging the proposed hypothesis for nitrogen-fixing behaviour of *Lyngbya majuscula,* a globally wide-spread non-heterocystous cyanobacterium.

3. Comments on the methodology

3.1 Estimating diversity of cyanobacteria in nature

When investigating biodiversity certain definitions need to be clarified. Firstly, diversity refers here to taxonomic richness, i.e. the number of species identified in a specified habitat. Secondly, a species, or preferably operational taxonomic unit (OTU), is defined as sharing less the 97% similarity of 16S rRNA sequences with other OTUs.

In order to estimate diversity of marine cyanobacteria at the sites selected it was necessary to optimize the accuracy of the taxonomic identification. For this reason, morphological studies using LM and SEM and two different molecular methods, denaturing gradient gel electrophoresis (DGGE) and cloning of the 16S rRNA and/or *nifH* partial gene sequences were explored. This approach raised some methodological issues when preliminary examining cyanobacterial diversity in the Paje lagoon, Zanzibar. For instance, differences in DNA extraction efficiency between protocols and samples, the selection and specificity of oligonucleotide primers for PCR, and shortcomings in existing molecular data from environmental samples were occasionally apparent. These questions arose from discrepancies found first between the morphological identification and the data obtained from sequencing of 16S rDNA fragments from the same samples collected and separated using DGGE. An attempt to evaluate different DNA extraction methods resulted in the conclusion that commercially available kits tested were not suitable for extracting DNA from the microbial mats in question. More specifically, a standard phenol:chlorophorm:isoamylalcohol protocol was more efficient than a commercially available DNA extraction kit (GeneElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, USA). Additional kits were evaluated in terms of recovery and purity of DNA but were all less efficient then various versions of phenol:chlorophorm:isoamylalcohol extraction. One of the most successful extraction methods were the combination of mechanical lysing using a bead-beater (FastPrep, matrix E) in combination with a xanthogenate buffer with addition of SDS (Jungblut and Neilan, 2006), and a subsequent purification with phenol:chlorophorm:isoamylalcohol.

The choice of oligonucleotide primers for amplification has crucial impact on the out-come of molecular diversity studies. Primers general for bacteria might overlook cyanobacteria in terms of retrieving 16S rRNA gene sequences from complex mixed environmental samples. In the present study a

combination of cyanobacterial specific/selective and/or general bacterial primers were used (Tab. 5). General bacteria primer-pairs 27F/1392R and 341FGC/907R for amplification of 16S rRNA did not reflect cyanobacterial diversity satisfactory in samples collected in Paje, Zanzibar, nor did the general *nifH* primers polR/polF. The *nifH* primers nifH1/nifH2 did not amplify cyanobacteria or bacteria successfully from several samples and were excluded from further analysis. However, discrepancies and variation between replicate samples may also to some extent be caused by high heterogeneity in nature, problems with homogenizing samples or interfering substances.

The choice of culture independent technique for separating sequences from environmental samples is also of great importance. Denaturing gradient gel electrophoresis has proven useful (Muyzer, 1999). Its fingerprinting properties make it particularly attractive for comparative studies of complex communities. However, if the number of species in a sample is high the excision of bands can be challenging and molecular cloning will preferably be the method of choice for identifying sequences. Moreover, species of low abundance might be impossible to detect using DGGE. A recent study by Taton et al. (2003) suggests that clone libraries, which yielded a higher number of different sequences, provide a more complete picture of diversity then DGGE, by which the most abundant organisms in that study could not be detected.

Table 5. Oligonucleotide primers tested. Primers in bold were used in papers I-IV.

Gene	Primer	Sequence (5' to 3')	Reference
16S rRNA	**CYA106F***	CGGACGGGTGAGTAACGCGTGA	(Nübel et al., 1997)
16S rRNA	**CYA359F***	CGGACGGGTGAGTAACGCGTGA	(Nübel et al., 1997)
16S rRNA	**CYA781R(a)**	GACTACTGGGGTATCTAATCCCATT	(Nübel et al., 1997)
16S rRNA	**CYA781R(b)**	GACTACAGGGGTATCTAATCCCTTT	(Nübel et al., 1997)
16S rRNA	**27F**	AGAGTTTGATCMTGGCTCAG	(Lane, 1991)
16S rRNA	**1392R**	ACGGGCGGTGTGTRC	(Lane, 1991)
16S rRNA	341F*	CCTACGGGAGGCAGCAG	(Muyzer et al., 1998)
16S rRNA	907R	CCGTCAATTCCTTTRAGTTT	(Muyzer et al., 1998)
nifH	**PN1**	CGTCACGGTCAAAGAATCAT	Paper I
nifH	**PN2**	ACACCACCAGCATGAGCATA	Paper I
nifH	**PolF**	TGCGAYCCSAARGCBGACTC	(Poly et al., 2001)
nifH	**PolR**	ATSGCCATCATYTCRCCGGA	(Poly et al., 2001)
nifH	**CNF***	CGTAGGTTGGCACCCTAAGGCTGA	(Olson et al., 1998)
nifH	**CNR**	GCATACATCGCCATCATTTCACC	(Olson et al., 1998)
nifH	nifH1F	TGYGAYCCNAARGCNGA	(Zehr and McReynolds , 1989)
nifH	nifH2R	ANDGCCATCATYTCNCC	(Zehr and McReynolds , 1989)

* With an additional GC-clamp - CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G

By sequence analysis a number of hypotheses, such as phylogenetic relationships and diversity richness, can be investigated. A range of statistical methods and diversity indices from ecology have been adopted by environmental microbiologists (Hughes and Bohannan, 2004) and several software tools have been developed to facilitate the statistical analysis of sequence data (Schloss and Handelsman, 2005, 2006). In paper III DOTUR, a software tool for assigning operational taxonomic units and estimating species richness, was used. Such tools are extremely helpful when handling large datasets comprising sequences from hundreds of genetic clones and reduces the amount of data that has to be included in further phylogenetic analysis.
When reconstructing phylogeny a large set of methods has to be evaluated. They are generally divided in character-based or distance methods. Chartacter-based methods include maximum parsimony and maximum likelihood. Traditionally maximum parsimony has been most commonly used among systematists to infer phylogeny. Parsimony is a principle assuming that the simplest solution is the best, i.e. the most parsimonious tree is the one assuming the fewest evolutionary changes. Maximum likelihood is a character-based method that estimates the likelihood of an evolutionary hypothesis given the data and a specific model and is proportional to the probability of observing the data given the hypothesis. The method searches for the phylogenetic tree with the maximum likelihood (Swofford et al., 1996). Another way to infer phylogeny is by using distance methods, which are based on a matrix of par-wise distance values between sequences in an alignment. Probably, the most frequently used distance-method is neighbor-joining (Saitou and Nei, 1987). The most recently emerging method for estimation of phylogeny is the application of Bayesian interference (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), based on posterior probabilities of trees. Similar to the maximum-likelihood method certain nucleotide substitution models are applied to the data. Software are also available for evaluating which substitution model that is best suited for the given data (Posada and Crandall, 1998; Nylander, 2004). Moreover, numerous software packages for phylogenetic inferences are available. In this study PAUP, PHYLIP and MrBayes were used for the majority of the analyses.

3.2 Measuring nitrogen fixation

Acetylene reduction assay (ARA) is by far the most commonly used technique to assay for nitrogen fixation (i.e. nitrogenase activity). Due to its simplicity, affordability and accuracy this indirect way of measuring nitrogen fixation is generally preferred over the more tedious ^{15}N tracer technique. Stewart and coworkers (Stewart et al., 1967, 1968) as well as Hardy and coworkers (Hardy et al., 1968), developed ARA in the late 60s. The capacity

of nitrogenase to reduce C_2H_2 to C_2H_4 instead of N_2 to NH_3, is the basis for the method. In theory, total amounts of fixed nitrogen could be derived from ARA results using a factor of 3 or 4, depending on if 6 or 8 electrons are involved in acetylene or dinitrogen reduction. It has been shown that a conversion factor 4, rather then 3, most accurately correspond to the true value (Stal, 1988). It is however advisable to determine the exact conversion factor using ^{15}N measurements whenever possible, since this factor can vary considerably. This is true particularly for natural samples (Stal, 1988; Montoya et al., 1996).

In all four studies presented in this thesis, ARA was the method of choice. Some exploratory ^{15}N incubations were carried out along side the acetylene reduction assays in the Paje lagoon, but organic material, other then cyanobacteria, in the benthic samples were likely affecting the results since values were remarkably lower then the corresponding activity rates from ARA.
As an addition to nitrogenase activity the presence and immuno-localization of the Fe protein has been used to successfully couple activity to specific organisms both cellularly and subsellularly (Bergman et al., 1986; Bergman and Carpenter, 1991; Fredriksson and Bergman, 1997; Lin et al., 1998; Berman-Frank et al., 2001). This was the method of choice to study diel variations in distribution of nitrogenase in the non-heterocystous *Lyngbya majuscula* in paper I.

4. Results and Discussion

4.1 Benthic cyanobacterial diversity

Benthic cyanobacteria are successful inhabitants of a variety of coastal habitats from subtidal sea grass beds and coral reefs to intertidal sand flats, hypersaline lagoons and beach rocks. Their success in these, in many ways harsh environments, is largely due to their versatility in both form and function. With physiological processes such as photosynthesis, diazotrophy, production of the protective pigment scytonemin and osmolytes cyanobacteria are readily prepared for harsh environmental stresses such as nutrient limitation, hyper-salinity and high UV-radiation. Cyanobacteria inhabiting coastal, often tidal habitats as those studied here are subject to a range of these stresses. In addition, cyanobacteria are unusually variable in size and morphology for being prokaryotes (Fig. 1, Tab. 2), ranging from less then 1 µm up to 80 µm in size and with simple, unicellular as well and filamentous, differentiated morphotypes. Both unicellular and non-heterocystous filamentous types range from being some of the smallest to the some of the largest species. As a consequence, the larger cyanobacteria present in the mats investigated by many-fold out-size most bacteria present (< 3 µm), although the latter may be more numerous. Hence, the cyanobacterial population of a mat/biofilm typically represents the total larger biomass and possibly thereby represents a more important contributor of 'new' nutrients to the system.

By investigating cyanobacterial diversity in three different tropical marine localities (Paje lagoon and Chwaka Bay, Zanzibar and Heron Island, Great Barrier Reef) a large variety in morpho- and genotypes was disclosed. The three habitats; an intertidal lagoon sand flat (Paje), mangrove muddy sediments (Chwaka) and a beach rock (Heron Island) together represent some of the most common tropical coastal environments around the world. Furthermore, the distant geographical locations, the western Indian Ocean (Zanzibar) and the western Pacific Ocean (Great Barrier Reef), allow some comparisons to be made in regards to prevalence and distribution of species. The

western Indian Ocean, being one of the more understudied marine regions (Bergman, 2001), indeed harboured many not previously characterized cyanobacteria (Papers III and IV). Also in Heron Island several unique sequences with low similarities to known phylotypes were identified (Paper II).

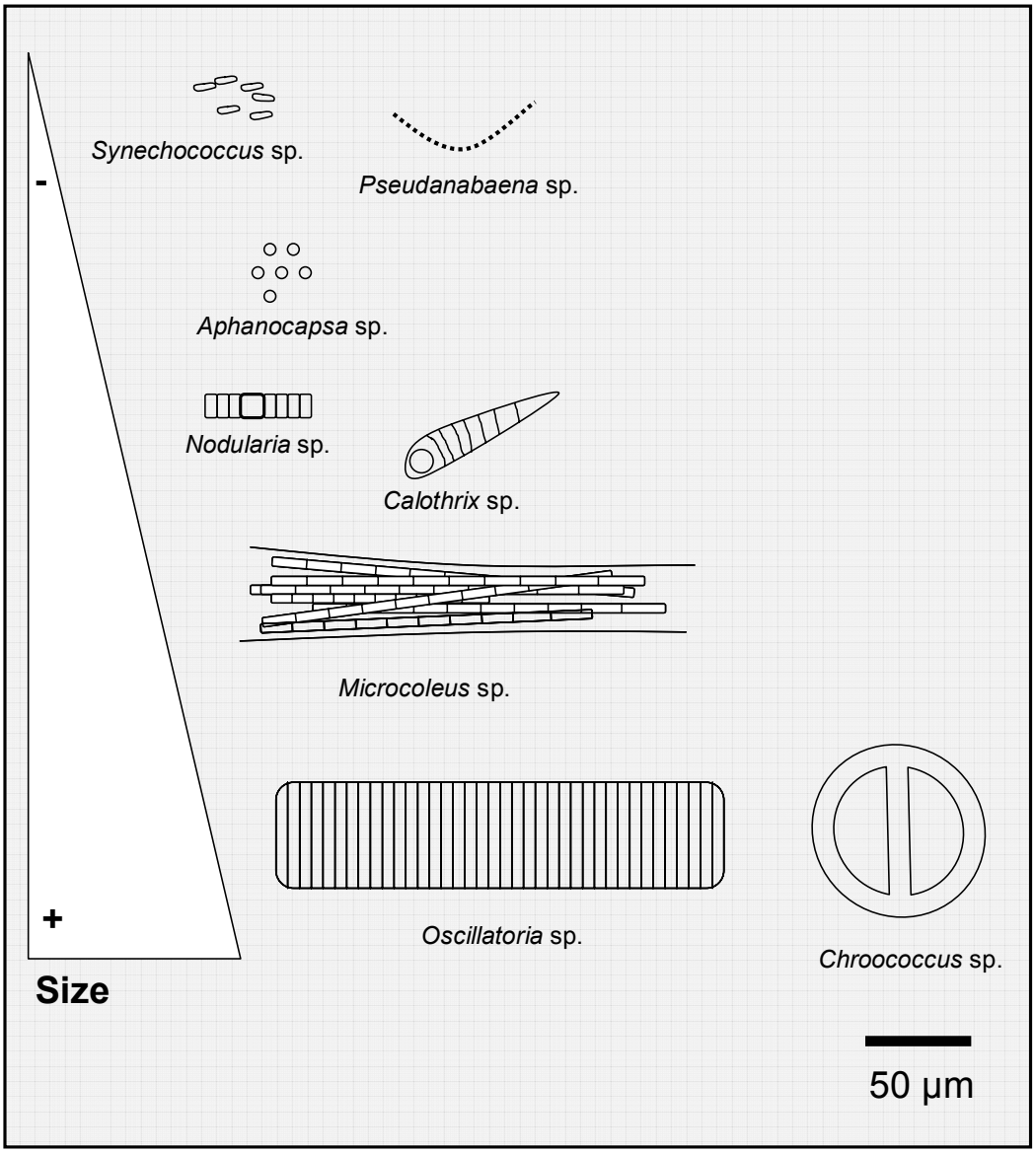

Figure 1. Schematic illustration of size scales among various cyanobacterial morphotypes.

Not unexpectedly, descriptions of morphologies of the encountered cyanobacteria, based on light- and/or scanning electron microscopy analyses, consistently resulted in fewer taxa being identified than those detected using the corresponding 16S rRNA screening (Paper II, III, IV). In addition, the data clearly demonstrate the presence of several heterocystous cyanobacteria, in spite of their typical absence from open water marine systems, using both

visual and genetic analyses. In Heron Island, *Calothrix* spp. and *Kyrtuthrix* sp. dominated part of the beach rock and in Zanzibar both the pheno- and genotype of *Nodularia* sp. was identified in the Paje lagoon. Likewise, an *Anabaena* sp. genotype was retrieved from the mat in Chwaka Bay. In general, the non-heterocystous species outnumbered the heterocystous by far in all three habitats, and the low numerical abundance of heterocystous cyanobacteria (on genera and biomass basis) in the marine environment was confirmed. Temperature has been suggested as a factor affecting distribution of heterocystous species (Staal et al., 2003), but other factors may contribute. Nevertheless, when heterocystous cyanobacteria do inhabit marine benthic habitats, as was observed here (Paper II-IV), their nitrogen fixation strategy (with day time maxima) and contribution to the nitrogen budget is likely to be of complementary importance to that of non-heterocystous genera and their contribution to 'new' nitrogen to the ecosystem, thereby likely significant.

The filamentous non-heterocystous genera *Lyngbya*, *Microcoleus*, *Spirulina* and *Oscillatoria* dominated most sites investigated. *Lyngbya* and *Microcoleus* are known and common mat-forming species (Stal, 1991; Omoregie et al., 2004a). Characteristic for both genera are the well developed sheaths of extracellular polysaccharides, protecting against desiccation and providing adhesive forces in the mat. As shown in paper IV, the sheath of *Microcoleus chthonoplastes* may also serve as a growth substrate for smaller cyanobacteria. Species of *Spirulina* and *Oscillatoria,* lacking a protective sheath, may instead rely on their motility to escape high light intensities, via migration downward in the mat.

Another common and thereby important group of filamentous non-heterocystous cyanobacteria detected at all sites were members of the Pseudanabaenaceae family (Paper II-IV). The filaments are thin and morphological characters for identification are few. Many of the genotypes sequenced showed remarkably low similarity to cultured strains and possibly due to their small size, discrete nature or challenging growth requirements, they have apparently largely been overlooked in most identification and culturing attempts. A reason may be that their potential co-dependence on other organisms in the mat is so vital that axenic growth is prevented.

Unicellular taxa were also present at all sites, with the fewest detected in the mats covering the mangrove sediments in Chwaka Bay. In the Paje lagoon several unicellular morpho- and phylotypes were identified, e.g. the potential nitrogen-fixing genera *Cyanothece* and *Gloeocapsa* sp., as well as *Chroococcus* sp. and *Chroococcidiopsis* sp., with the latter being also one of the major unicellular phylotypes detected in the Heron Island beach rock. Another unicellular genus, *Enthophysalis,* with characteristic morphology but no genotype yet deposited in GeneBank, was also encountered in the beach rock.

The large variety in habitats and geographic location examined allowed comparisons of genotypes retrieved from all sites (paper IV). Results suggested that certain phylotypes may be common to tropical microbial mats regardless of habitat type.

4.2 Diazotrophy

Nitrogen fixation is a another common feature in benthic coastal habitats as shown in the localities investigated here (Paper I-IV). Both loosely attached rumpled filaments of *Lyngbya majuscula,* with a seaweed-like appearance, and microbial mats with more or less coherent structures demonstrated nitrogenase activity. The dominance of non-heterocystous cyanobacteria in the vast majority of the sites examined suggested that nitrogenases activity would reach maximum levels during the dark period when oxygen levels in the cells are at a minimum. This was confirmed by diel measurements which demonstrated clear diurnal patterns with the highest activities at night time (Tab. 6). Only one of the sites had high abundance of a heterocystous cyanobacterium (site B, Paper III). As expected, the nitrogenases activity was highest during day-time at this site, when energy supply for the costly nitrogen fixation is high via photosynthesis. The rates of fixation in the non-heterocystous mats were remarkably similar, with the exception of those in the microbial mat in the mangrove forest of Chwaka Bay (paper IV). The activity recorded was considerably lower than those observed in Paje and Heron Island (paper II and III), but almost in the same range as observed in a microbial mat dominated by *Lyngbya* sp. in Guerrero Negro (Pacific Ocean), reporting 9 μmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$ as the highest rate (Omoregie et al., 2004b). Similar to what was observed in Chwaka, activity in that system was also confined to night-time. The Paje lagoon and Heron Island beach rock are both considerably more exposed localities then the sheltered tidal creeks of Chwaka Bay. With constant erosion from waves, persistence and re-establishment of the mats are necessities for survival and the nutrient demand is high. Coral rocks and sand have low capacity to bind nutrient rich pore water, unlike muddy sediments which may have substantial nutrient content. A reasonable explanation for the lower activity rates in Chwaka is higher availability of dissolved organic nitrogen emanating from the surrounding dense stands of mangrove trees and their litter. On the other hand, it may be expected that nitrogen fixation occurring among mangroves contributes positively to the overall nitrogen budget of these lush stands.

Table 6. Summary of ARA activities from Zanzibar and Heron Island. Maximum rates for each site are given.

Locality	Nitrogenase activity (µmol C_2H_4 m^{-2} h^{-1})	Time of day	Reference
Changuu, Zanzibar/*Lyngbya majuscula*	3.5*	01:30	Paper I
Heron Island, Great Barrier Reef/microbial mat	35.9	03:00	Paper II
Paje, Zanzibar/heterocystous mat B	119.2	15:00	Paper III
Paje, Zanzibar/non-heterocystous mat A	57.2	02:00	Paper III
Paje, Zanzibar/non-heterocystous mat C	27.8	00:40	Paper III
Paje, Zanzibar/non-heterocystous mat D	22.9	00:40	Paper III
Chwaka, Zanzibar/non-heterocystous mat CwC	3.9	23:00	Paper IV

* nmol C_2H_4 chl a^{-1} h^{-1}

In addition to meassuring nitrogenase activity in randomly selected sites of the microbial mats, samples were collected along a randomly chosen transect in the Paje lagoon, reaching from the shoreline to 224 meters out into the intertidal sand flat towards the fringing reef. These often 'bare' sediments showed microbial activity in the form of nitrogenase activity. Activity was mainly detected during the night with the highest rate being 19.3 µmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$ at the site located furthest from the shoreline (Fig. 2). Similar to what was observed in the microbial mats this might suggest the presence of nitrogen-fixers with temporal separation as the nitrogenase protection mechanism, e.g. non-heterocystous cyanobacteria or heterotrophic bacteria.

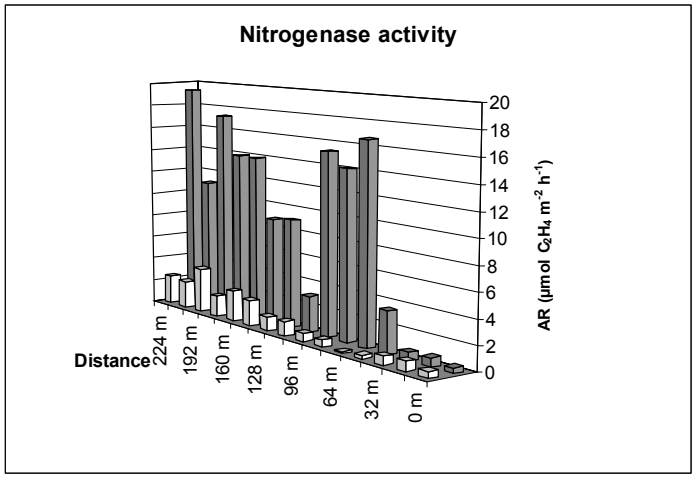

Figure 2. Nitrogenase activity detected along a transect in the Paje lagoon, Zanzibar. Light and grey bars represent samples incubated at day and night, respectively.

The first molecular screening of potential diazotrophs in the Paje lagoon suggested a complete dominance of heterotrophic bacteria (Paper III), but when cyanobacterial primers were used several additional potential nitrogen-

fixers were identified. Two of the sequences retrieved clustered together with the *nifH* sequence from *Lyngbya majuscula* (Paper I), possibly sharing a similar strategy for nitrogen fixation, i.e. with nitrogenase re-synthesized and activated at the onset of the dark period.

We attempted to quantify nitrogen fixation using the ^{15}N isotope tracer technique and incubating mat cores from the four sites investigated in the Paje lagoon. The results obtained where contradictive to what was observed by ARA. The heterocystous *Nodularia* sp. site (B) showed lowest levels of fixed N, in contrast to the highest nitrogenase rates (Table. 7). Conversion factors between C_2H_4 and N_2 in different natural ecosystems might vary considerably from the theoretical 3:1 or 4:1 (Montoya et al., 1996), but the factors detected here were dramatically higher. This could perhaps be explained by large amount of organic material other than nitrogen fixers in the cores or an unusually fast export of fixed nitrogen to the surrounding environment.

Site	N fixed ARA	N fixed ^{15}N
A	**240.5**	**15.2**
B	**329.1**	**2.4**
C	**291.1**	**20.3**
D	**227.8**	**19.0**

Table 7. Nitrogen fixed in sites A-D in Paje lagoon, Zanzibar. Values are given in μg $N \cdot m^{-2} \cdot day^{-1}$. Nitorgenase activity rates were converted using a conversion factor of 4:1 and assuming a 12:12 hour light:dark cycle and fixation only in light (B) or dark (A, C and D).

However, even the lower levels recorded suggested that fixed nitrogen could have profound impact on a nutrient poor ecosystem, such as the Paje lagoon. The lagoon have been extensively used for commercial seaweed farming of the red algae *Eucheuma* spp. since the early 90s, with initially high growth rates that rapidly dropped after several years of intense algal cultivation. A prolonged monitoring of cyanobacterial diversity and nitrogen fixation in pristine tropical coastal zones versus areas used for seaweed farming, or exploited for tourism, is essential for a deep understanding of how the marine environment will change as a response to the potential economical progress and anthropogenic activities in developing countries in tropical zones.

4.3 Concluding remarks

The world's coastal length has been estimated to approx. 1,600 000 km (Burke, 2001), of which large parts still remain poorly characterized in terms of biological activities and cyanobacterial diversities. Coastal regions often harbour highly productive ecosystems such as mangrove stands and fringing coral reefs and to understand how these systems depend on and interact with nitrogen-fixers, such as cyanobacteria, are of vital importance for a large part of the world's population which are depending on marine resources for their livelihood. Understanding how these systems vary in function and biodiver-

sity is fundamental if we are to maintain marine productivity as a whole. Cyanobacteria, functioning as primary producers supporting higher trophy levels with indispensable sources of 'new' carbon and nitrogen are key-organisms in this process.

Large scale distribution patterns and quantification of benthic diazotrophy is called upon in order to enable efficient management of tropical coastal zones. The overall findings of the work presented in this thesis provide the first comprehensive and comparative basis for future studies on variations and similarities in benthic cyanobacterial diversity and diazotrophy patterns in distinctly different and geographically separated coastal habitats.

5. Conclusions and future prospects

The major and general conclusions from this work are:

- Benthic nitrogen fixation in tropical coastal zones is conspicuous and the rule rather then the exception in all habitats investigated.
- *Lyngbya majuscula* deploys a strategy to protect the oxygen sensitive nitrogenase complex typical for the vast majority of non-heterocystous cyanobacteria; i.e. a temporal separation of photosynthesis and nitrogen fixation, the latter nocturnal.
- Microbial mats in variable tropical coastal habitats harbour a large diversity of cyanobacterial pheno- and phylotypes with complementing structural and ecophysiological strategies.
- Diazotrophic communities in microbial mats consist of both cyanobacteria and heterotrophic bacteria, and dominance will vary depending on prevailing physical conditions and habitat.
- Complementary diurnal nitrogen fixation patterns assure a constant supply of nitrogen to these often nutrient deprived systems.
- New pan-tropical or even cosmopolitan, potential nitrogen-fixing, non-heterocystous filamentous cyanobacteria were frequently encountered in all the geographically distant microbial mats investigated.

The morphological and genetic surveys of cyanobacteria undertaken in these waters so far (Lugomela et al., 2001a; Lugomela et al., 2001b; Lugomela et al., 2002a) paper I, III and IV) have generated data that convincingly demonstrate the common occurrence of a range of cyanobacterial taxa, as well as their great physiological importance as primary producers and nitrogen-fixers (Bergman, 1996, 2001; Lugomela et al., 2001a; Lugomela et al., 2002a; Lugomela and Bergman, 2002b; El-Shehawy et al., 2003; Lugomela et al., 2005).
However, many challenges remain in the field of exploring diazotrophy and diversity among benthic cyanobacteria world-wide. One approach that without doubt efficiently will generate information on cyanobacteria/bacteria in these valuable and vulnerable coastal systems is the introduction of the potent eDNA metagenomic approach. Such analyses are, in collaboration with researchers at the J. Craig Venter Institute (USA), currently in progress in our continued research on planktonic and benthic cyanobacteria in the tropi-

cal western Indian Ocean. This metagenomic survey will in combination with morphological and transcriptional analyses enormously extend our insights into community composition and the biology of these perpetually interesting organisms.

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7. References

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