RESEARCH ARTICLE

Variability in benthic diazotrophy and cyanobacterial diversity in a tropical intertidal lagoon

Karolina Bauer[1], Beatriz Díez[1], Charles Lugomela[2], Susanna Seppälä[1], Agneta Julia Borg[1] & Birgitta Bergman[1]

[1]Department of Botany, Stockholm University, Stockholm, Sweden; and [2]Department of Fisheries Science and Aquaculture, University of Dar es Salaam, Dar es Salaam, Tanzania

Correspondence: Karolina Bauer, Department of Botany, Stockholm University, S-106 91 Stockholm, Sweden. Tel.: +0046 08 161326; fax: +0046 08 165525; e-mail: bauer@botan.su.se

Present address: Susanna Seppälä, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden.

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Abstract

Benthic nitrogen fixation has been estimated to contribute 15 Tg N year^{-1} to the marine nitrogen budget. With benthic marine nitrogen fixation being largely overlooked in more recent surveys, a refocus on benthic diazotrophy was considered important. Variations in nitrogenase activity (acetylene reduction-gas chromatography) in a tropical lagoon in the western Indian Ocean (Zanzibar, Tanzania) were monitored over a 3-year period (2003–2005) and related to cyanobacterial and diazotrophic microbial diversity using a polyphasic approach. Different nitrogenase activity patterns were discerned, with the predominant pattern being high daytime activities combined with low nighttime activities. Analyses of the morphological and 16S rRNA gene diversity among cyanobacteria revealed filamentous nonheterocystous (Oscillatoriales) and unicellular (Chroococcales) representatives to be predominant. Analyses of the *nifH* gene diversity showed that the major phylotypes belonged to noncyanobacterial prokaryotes. However, as shown by cyanobacterial selective *nifH*-denaturing gradient gel electrophoresis analysis, cyanobacterial *nifH* gene sequences were present at all sites. Several *nifH* and 16S rRNA gene phylotypes were related to uncultured cyanobacteria or bacteria of geographically distant habitats, stressing the widespread occurrence of still poorly characterized microorganisms in tropical benthic marine communities.

Introduction

Despite the recognition of the globally wide-spread marine planktonic nitrogen-fixing cyanobacteria and their important contributions to the marine nitrogen budget (Capone *et al.*, 1997; Zehr *et al.*, 2001; Falcon *et al.*, 2004), considerably less attention has been given to benthic microbial communities of tropical coastal areas. These communities, mainly dominated by cyanobacteria, are conspicuous and are often recognized as stratified 'microbial mats' (Stal, 2000). However, the term 'microbial mat' may also refer to thinner and less stratified benthic microbial communities covering more exposed areas such as sand flats and rocky shores (Charpy-Roubaud & Larkum, 2005; Díez *et al.*, 2007).

In the 1980s, the general perception was that benthic bacterial communities were contributing most of the 'new' nitrogen in the marine environment via biological nitrogen fixation, and estimates suggested 15 Tg N to be fixed per year as compared with 4.8 Tg N fixed by planktonic diazotrophs (Capone & Carpenter, 1982). Since then, estimates on planktonically derived nitrogen fixation have been upshifted 20–40-fold (Karl *et al.*, 2002), while contributions via benthic nitrogen fixation have not been re-evaluated, nor the actors responsible. However, the introduction of molecular approaches has disclosed previously unidentified diazotrophs (using the *nifH* gene, encoding the small subunit of the nitrogenase enzyme complex, as a marker) in a wide variety of marine coastal environments, such as hypersaline lagoons, rocky shores and as symbionts of corals (Pinckney & Paerl, 1997; Lesser *et al.*, 2004; Omoregie *et al.*, 2004a; Ley *et al.*, 2006; Yannarell *et al.*, 2006; Díez *et al.*, 2007). If integrated with previous data, records of nitrogen fixation from these recent studies are likely to enhance the estimated benthic nitrogen fixation.

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Most microbial mats harbor a rich and intermixed diversity of photoautotrophic cyanobacteria and heterotrophic bacteria, and with representatives in both groups potentially possessing *nif* genes (Zehr *et al.*, 1995; Omoregie *et al.*, 2004b; Steppe & Paerl, 2005; Musat *et al.*, 2006; Díez *et al.*, 2007). Recent data show that cyanobacteria, per biomass, may dominate such nitrogen-fixing communities, while the composition of the communities may shift on a seasonal basis (Yannarell *et al.*, 2006). Indeed, heterotrophic bacteria may be the more active diazotrophs (Zehr *et al.*, 1995). Attempts were recently made to identify diazotrophs in microbial mats by examining the expression of the *nifH* gene (Omoregie *et al.*, 2004a; Yannarell *et al.*, 2006), but mainly without effort to link phylotypes to morphotypes. As several clusters of *nifH* phylotypes retrieved from natural habitats show no or low relationship with sequences from cultured microorganisms (Omoregie *et al.*, 2004b; Musat *et al.*, 2006), it follows that important groups of diazotrophs are yet to be identified. Exploring complex microbial consortia using a polyphasic approach, i.e. combining molecular and morphological data (Vandamme *et al.*, 1996; Abed *et al.*, 2002; Komárek & Anagnostidis, 2005), may help overcome such problems. This is particularly valid when attempting to identify the morphologically highly diverse cyanobacterial community. However, both approaches are imbued with limitations. There is still no consensus method for efficiently extracting DNA from natural microbial consortia, the very fundament of any genetic study, and PCR-based DNA amplification bias has to be considered (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998). In addition, morphological characters may vary as a response to changes in the environment (Komárek & Anagnostidis, 1998; Silva & Pienaar, 2000; Komárek & Anagnostidis, 2005). The identification of such variations in morphotypes requires comprehensive experience. Further, it has been claimed that molecular approaches may reveal a higher diversity than morphological studies alone (Wilmotte & Golubic, 1991; Nübel *et al.*, 1997).

As the knowledge about diazotrophs and their diversity in globally wide-spread tropical coastal habitats is limited, a comprehensive study, integrating molecular and morphological characterization with estimates of nitrogen-fixation activities, seemed urgent. The site selected is located in the western Indian Ocean, being a particularly underrepresented environment in coastal microbial community studies (Bergman, 2001). Recently, however, a rich cyanobacterial diversity was unveiled in coastal zones of Tanzania (Lugomela *et al.*, 2001a) and in eastern South Africa (Silva & Pienaar, 2000). Although comprehensive, these studies were exclusively based on morphological characters. Here, a 3-year study was undertaken in an intertidal sand flat of a tropical lagoon, potentially representing a common coastal habitat in tropical areas. With the focus on cyanobacteria, a detailed characterization of the microbial community was performed by combining genetic and morphological characteristics and estimates of the corresponding nitrogen fixation activities.

Materials and methods

Sample collection

Sampling of natural populations of cyanobacteria and field experiments were carried out during three periods in 2003 through 2005 in Paje, Zanzibar (Tanzania). The Paje lagoon is located on the east coast of Zanzibar Island in the western Indian Ocean (06°16′S, 30°32′E). A coral reef (*c.* 2 km out) runs parallel to the shoreline, creating an intertidal lagoon between the reef and the shoreline. The bottom consists of white fine and medium-grained coral sand. Water temperatures in this area normally range from 30 to 37 °C and the salinity from 14 to 32 psu (Ndaro & Olafsson, 1999). The tidal range is about 4 m.

Two sites in the intertidal lagoon, with pronounced cyanobacterial coverage, were chosen for nitrogenase activity measurements in 2003, 2004 and 2005 and for diversity studies in 2003 and 2005. In 2005, two additional sites were included. The sites will be referred to as A and B (sites examined in 2003, 2004 and 2005) and C and D (additional sites in 2005).

Collected cores were dried (2003), or immediately frozen in DNA buffer (50 mM Tris-HCl, 40 mM EDTA) (2005) for subsequent DNA extraction. Parallel samples were preserved in 2.5% glutaraldehyde in sterile-filtered seawater and used for morphological analyses.

DNA extraction and PCR

DNA was extracted using a modified phenol : chloroform : IAA protocol. Extracted DNA (2003) was cleaned using a DNA kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma-Aldrich). The 16S rRNA gene was partially sequenced (~675 bp) using cyanobacteria-specific primers (Nübel *et al.*, 1997). The PCR conditions used for the 16S rRNA gene cyanobacterial amplification were as described previously (Nübel *et al.*, 1997). Each reaction contained 10 pmol of each primer, 100 μmol deoxynucleoside triphosphates (dNTP Mix 10 mM, Applied Biosystems), 2 μL 10X PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7, Qiagen), 4 μL 5X Q Buffer (Qiagen) and 0.625 U polymerase (HotStar Taq polymerase, Qiagen), and was made up to 25 μL with DNAse/RNAse free H_2O (ultraPURE, Gibco). Partial *nifH* genes were amplified as above using degenerate primers PolF and PolR as described previously (Poly *et al.*, 2001) and with cyanobacteria-selective primers CNF and CNR (Olson *et al.*, 1998), which amplify a fragment *c.* 359 bp long, with a 40 nucleotide GC clamp at

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the 5′end of the forward primer for denaturing gradient gel electrophoresis (DGGE) analyses (Díez *et al.*, 2007). The PCR conditions for CNF/CNR comprised a denaturation step of 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 47 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step of 7 min; the composition of the PCR reaction was as described above.

DGGE

DGGE was carried out with a Dcode system (BioRad) to reveal the *nifH* cyanobacterial diversity present in the different samples. PCR products were loaded onto a 0.75 mm 6% polyacrylamide gel (37.5 : 1 acrylamide : bisacrylamide) in a linear gradient of denaturing agents (urea and formamide) of 35–80%. The running conditions were 1 × TAE buffer, 60 °C and 75 V for 16 h. The nucleic acids in the gels were then stained in 1 × TAE buffer containing SYBRGold Nucleic Acid Stain (1 : 10 000 dilution; Molecular Probes) and visually recorded using a molecular imager (ChemiDoc XRS system, BioRad). The gel was placed on a UV table with a blue light filter and predominant bands were cut out, submerged in 20 μL DNAse/RNAse free H_2O (ultraPURE, Gibco) and stored at 4 °C overnight. An aliquot of the eluted DNA was subjected to an additional PCR amplification using the same primers and sequenced in both directions on an ABI 3130XL system (DNAtechnology, Aarhus, Denmark). BLAST searches were performed at www.ncbi.nlm.nih. gov/blast (Altschul *et al.*, 1997).

Cloning and restriction fragment length polymorphism (RFLP)

Fresh PCR products were cloned using a commercial cloning kit (TOPO TA, Invitrogen) according to the manufacturer's instructions. *Escherichia coli* grown overnight in Luria–Bertani were used directly as a template and the 16S rRNA gene or *nifH* gene was reamplified using the same primers as above. A total of six clone libraries with 16S rRNA genes were constructed: four from sites A and B (years 2003 and 2005), and two from sites C and D (year 2005). Four libraries from site A–D (2005) were constructed with *nifH* genes and 132 clones were screened using RFLP. The *nifH* gene was digested using restriction enzymes MnlI and HaeIII. Restriction patterns were analyzed using FINGERPRINTINGII software (BioRad). Clones from which the *nifH* genes could not be amplified or that gave products that were unrestricted after incubation (a total of 17 clones) were excluded from further analysis. A total of 29 *nifH* clones representing the different RFLP patterns obtained and 221 16S rRNA gene clones were sequenced using the M13reverse primer on an ABI 3130XL system (DNAtechnology, Aarhus, Denmark).

Sequence analysis

Sequences were aligned in Bioedit using CLUSTALW and manually corrected. A distance matrix was computed in PHYLIP (Felsenstein, 1993) using the Kimura two-parameter model (Kimura, 1980). Single operational taxonomic units (OTUs) of 16S rRNA gene sequences were determined using the farthest-neighbor criterion in DOTUR (Schloss & Handelsman, 2005), and 16S rRNA gene sequences with a maximum farthest-neighbor distance of 0.03 were treated as representing one OTU (38). All sequences were subjected to BLAST searches and the closest relatives from GeneBank were included for phylogenetic analysis; only sequences from published studies or culture collections were included. The 16S rRNA gene and *nifH* clone and DGGE sequences and the reference taxa were used for phylogenetic inference from distance approximations by the neighbor-joining method and Kimura two-parameter (Kimura, 1980) and Jukes Cantor correction (Jukes & Cantor, 1969), respectively, in PAUP (version 4.0b10, Sinauer Associates Inc.). Branch support was measured by bootstrap analysis with parsimony criterion settings and a heuristic search strategy with 1000 bootstrap replicates for the 16S rRNA gene data set (Fig. 1) and the *nifH* data set (Fig. 2). Bayesian analyses were carried out using MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), and MrAic (Nylander, 2004) was used to compare nucleotide substitution models to be implemented in MrBayes. The best-fitting model was a general time-reversible model with a γ-distributed rate and a proportion of invariant sites (GTR+γ+I). Posterior probabilities above 0.95 are shown in the tree. The 16S rRNA gene sequences of *Gloeobacter violaceus* and *Bacillus subtilis*, and the *nifH* gene sequence of *Methanocaldococcus jannaschii* were used as outgroups.

Morphological diversity

For scanning electron microscopy, cores were fixed in 2.5% glutaraldehyde and sterile-filtered seawater, washed in a 1 : 1 mixture of sterile-filtered seawater and ddH_2O 2 × 10 min, dehydrated 10 min each in 25%, 50%, 70%, 90%, 95% and 100% ethanol and placed in 100% acetone, which was exchanged every 10 min for 30 min. Samples were dried in a critical point drier, mounted on stubs and sputter-coated with Au in an E5100 High-Resolution Sputter Coater (Polaron, England). Samples were examined and photographed using a Cambridge Stereoscan 260 electron microscope equipped with a digital camera. Both freshly collected and fixed cyanobacteria were examined using light microscopy (Olympus BH-2 or Zeiss Axiovert) equipped with interference contrast. Morphological characters were studied in detail in samples from sites A-D (2005). The morphological identification was according to (Desikachary,

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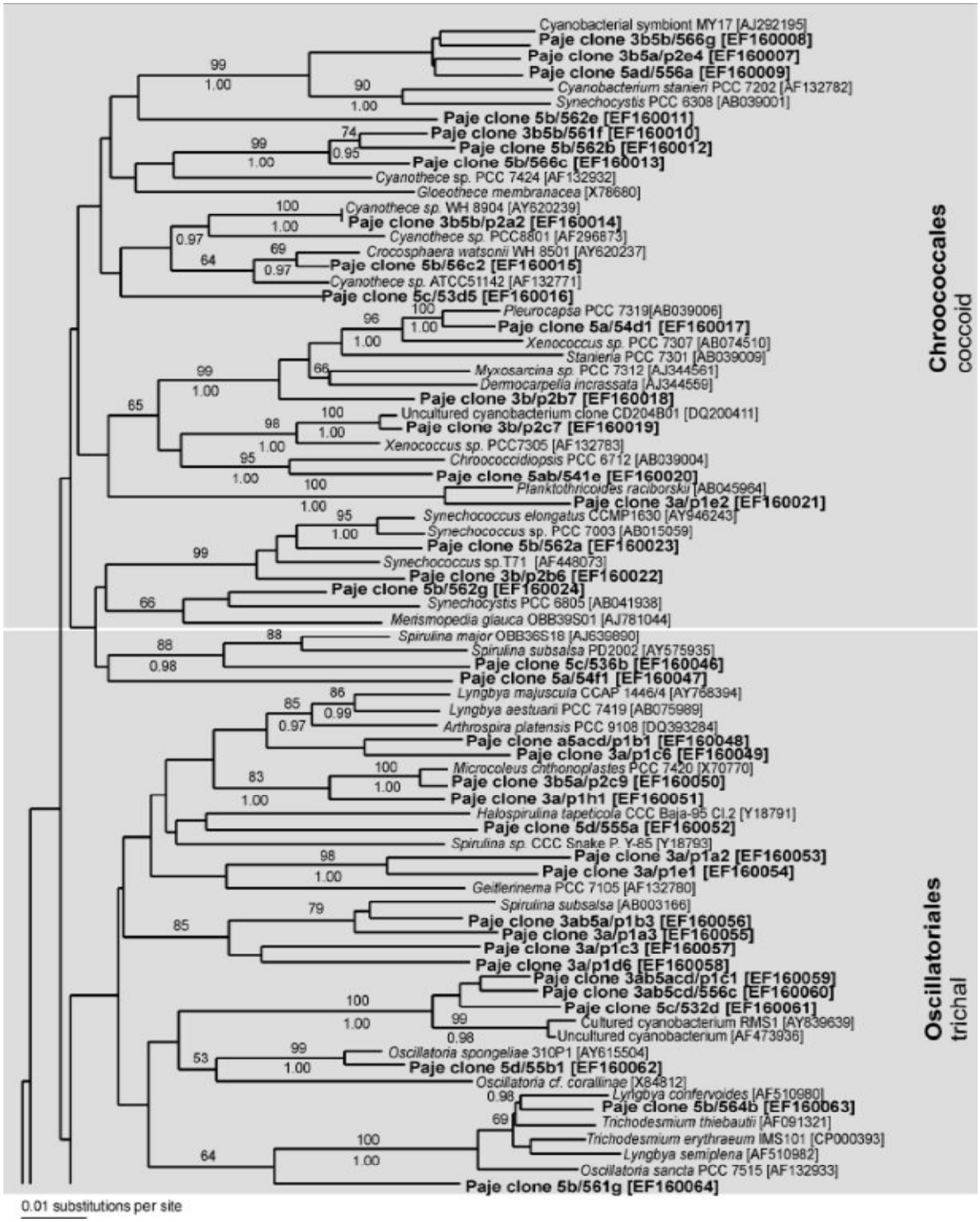


Fig. 1. Neighbor-joining tree from analysis of cyanobacterial 16S rRNA gene sequences from sites A and B (2003) and A–D (2005) collected in the Paje lagoon (western Indian Ocean, Zanzibar), constructed using Kimura 2-parameter model. Sequences from the present study are given in bold. OTU names are coded as follows: 3x5x/y, where 3 and 5 refers to samples collected in year 2003 and 2005, respectively, 'x' refers to the site or sites were the OTUs were found and 'y' refers to the sequence clone which was used in the analysis. Bootstrap support > 50 and Bayesian posterior probabilities values > 0.95 are shown.

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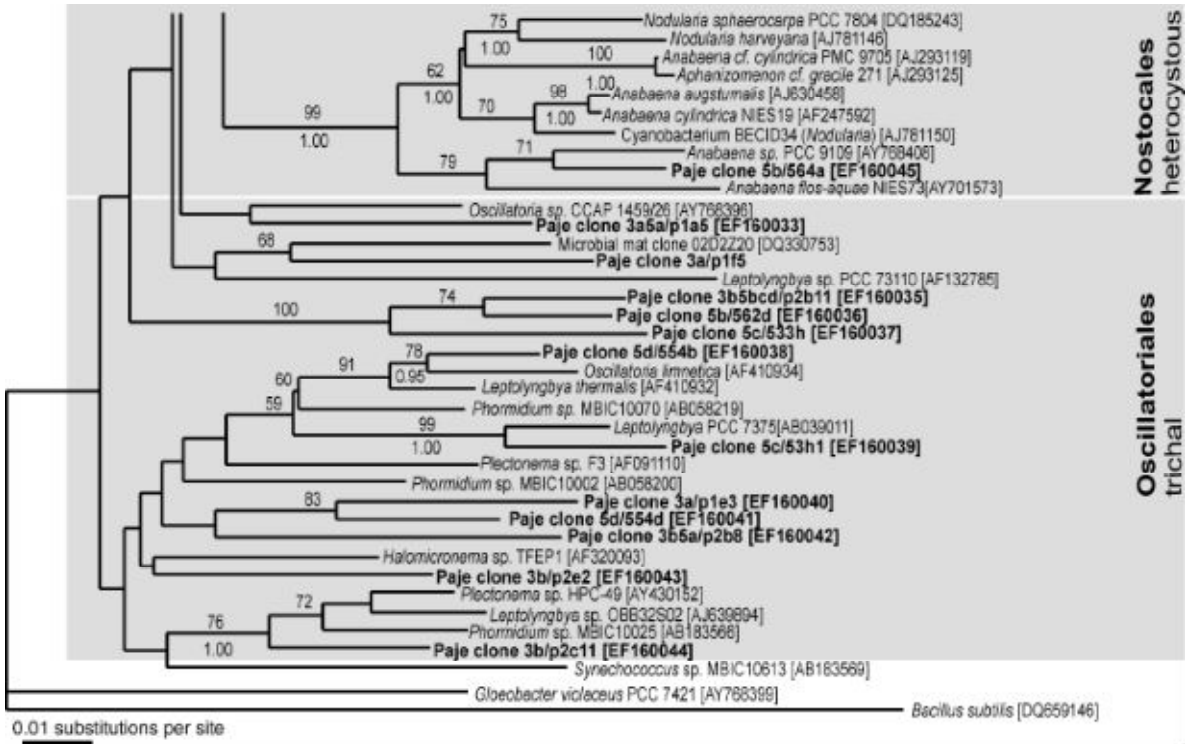


Fig. 1. Continued

1959; Komárek & Anagnostidis, 1998; Silva & Pienaar, 2000; Lugomela *et al.*, 2001a; Komárek & Anagnostidis, 2005).

Nitrogenase activity

Acetylene reduction assays (ARAs) were carried out on lagoon bottom cores, each with a diameter of 10 mm and a thickness of 2–3 mm, collected using a cork borer from sites A and B (2003 and 2004) or A–D (2005). Each core was incubated for 2 h in 17-mL glass incubation vials containing 1 mL sterile-filtered seawater and 10% acetylene gas (generated from calcium carbide) (Stal, 1988). After incubation, 5 mL of the gas phase was retracted and injected into 5-mL vacutainers (Becton and Dickinson) and stored until the ethylene content could be analyzed using a gas chromatograph (Shimadzu GC-8A) equipped with a Porapac N column.

Statistical analysis

Statistical analysis of nitrogenase activity consisted of a one-way ANOVA and a multiple comparison test (Tukey HSD) using STATISTICA (Statsoft Inc.).

Results

The sites

The four sites examined, denoted A–D, were located along a one-km transect, parallel to the shoreline. The sites were completely exposed for *c.* 4 h, two times per diurnal cycle at low spring tide. The sites varied in size, ranging from $>> 10\,m^2$ (sites C and D) to $\sim 5\,m^2$ (site A), down to isolated patches of cyanobacterial tufts within an area *c.* $2\,m^2$ (site B). Sites A, C and D shared a similar macroscopic structure, with the exception of a patchy macroalgal (*Cladophora* sp.) coverage in site C. The cyanobacteria formed a coherent *c.* 2 mm thick green layer (mat/biofilm) with a thinner pink layer beneath, suggesting the presence of purple sulfur bacteria. However, no indication of sulfide production was detected. No additional stratification was visible using stereomicroscopy. Site B showed a non-mat-like appearance, consisting of a patchy green layer of cyanobacterial tufts.

Cyanobacterial diversity (morphological and partial 16S rRNA gene sequence analyses)

The gross morphologies of the encountered cyanobacteria, from observations using light- or scanning electron microscopy, are shown in Table 1. The distinctly pigmented cyanobacteria present in the mats varied considerably in their morphology, in terms of pigmentation, size and shape, and representatives from unicellular, filamentous and heterocystous genera were apparent. However, sites A, C and D were clearly dominated by filamentous nonheterocystous genera such as *Lyngbya*, *Microcoleus*, *Spirulina* and

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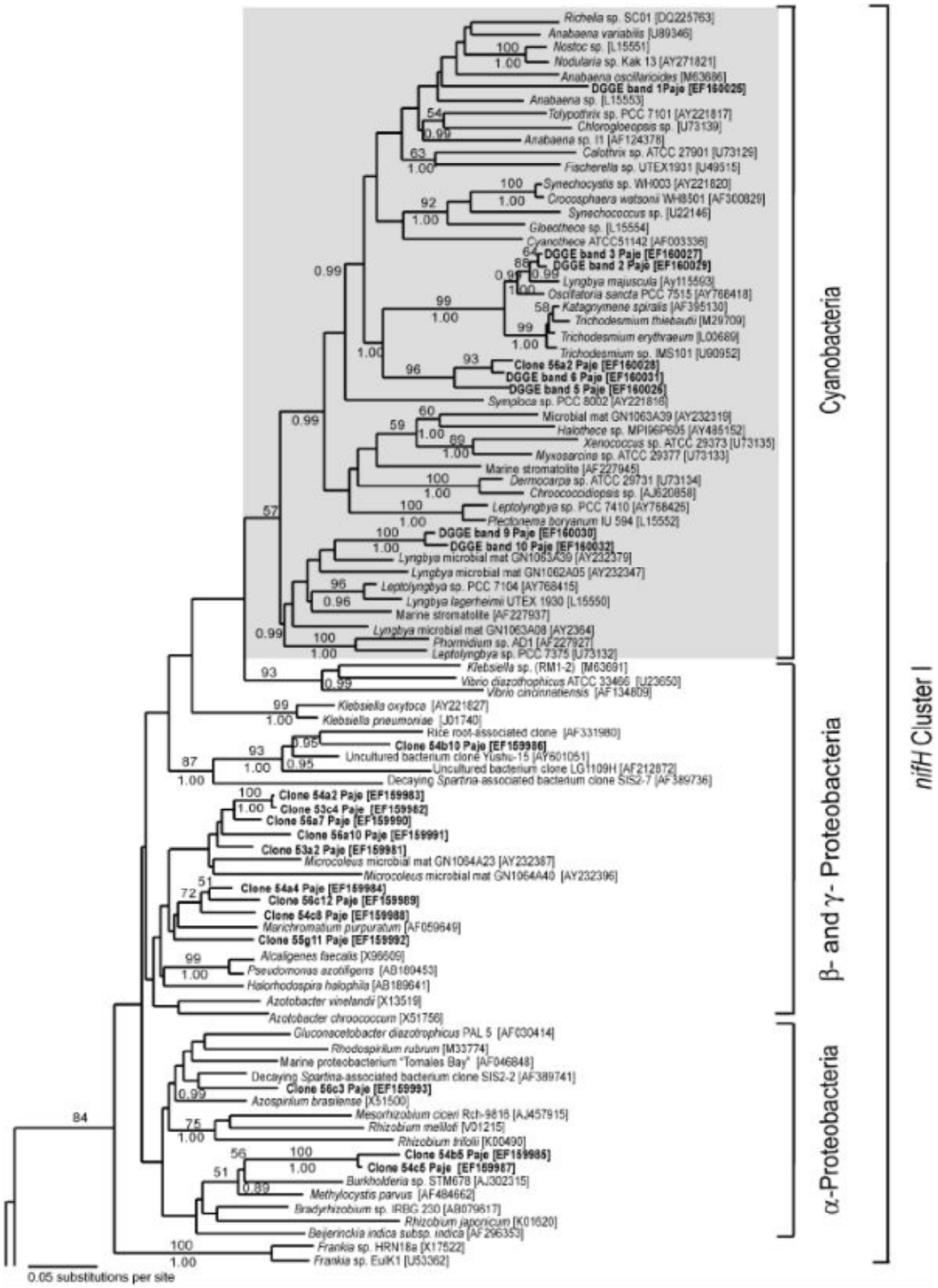


Fig. 2. Neighbor-joining tree from analysis of bacterial *nifH* gene sequences from sites A–D (2005) in Paje lagoon (western Indian Ocean, Zanzibar). These were retrieved using Jukes-Cantor model and neighbor-joining distance method. Sequences from the present Paje study are given in bold. Shaded box in gray color represent cyanobacterial sequences from *nifH* cluster I. Bootstrap values > 50 and Bayesian posterior probabilities > 0.95 are shown.

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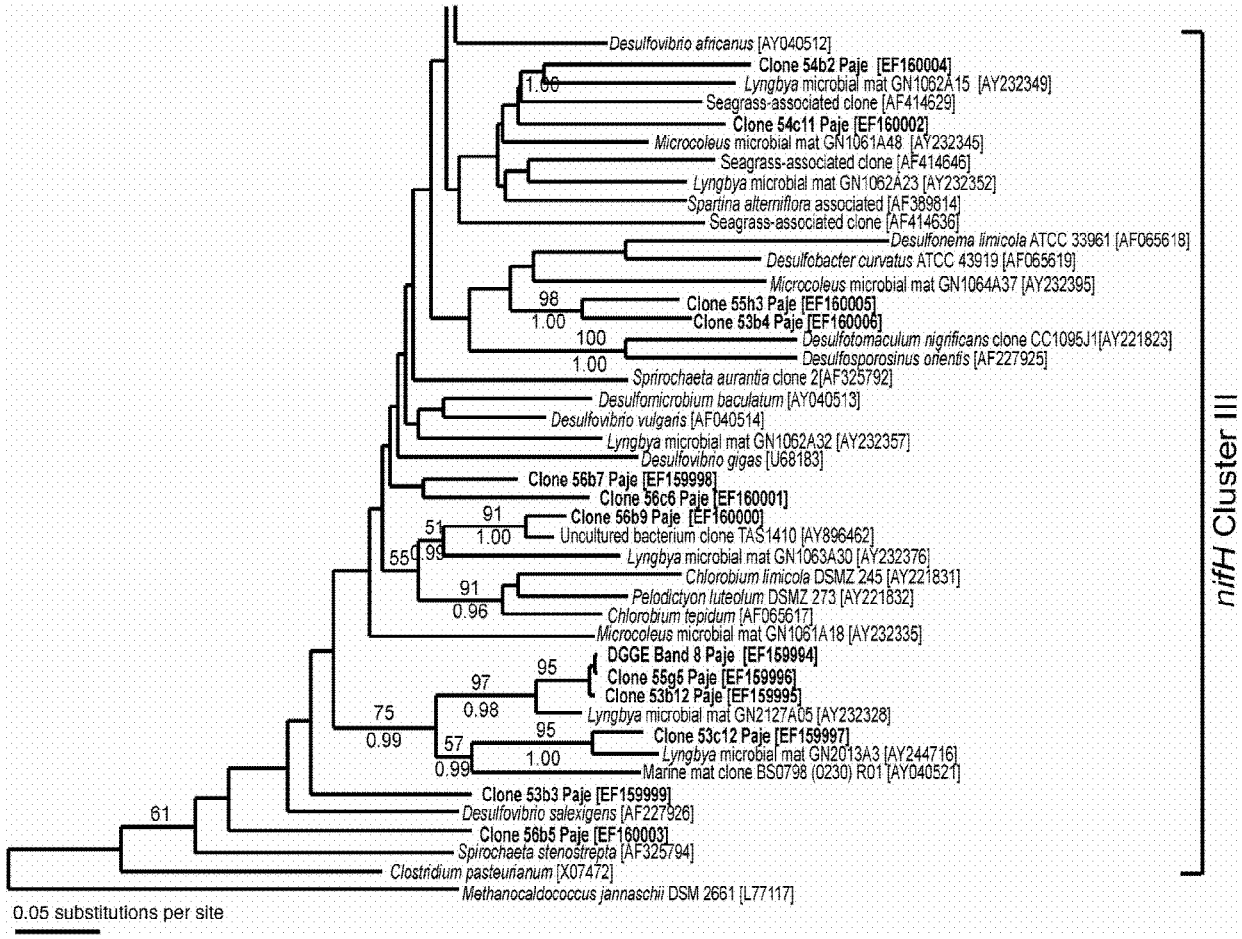
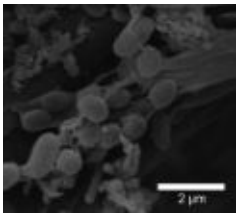
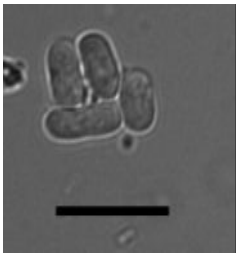
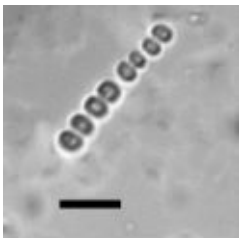
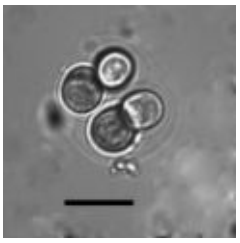


Fig. 2. Continued

Oscillatoria as well as by genera within Pseudanabaenaceae. Unicellular taxa were also represented, while heterocystous taxa were rarely encountered. Results from the phylogenetic analysis of partial 16S rRNA gene sequences from six individual clone libraries (A and B, 2003, and A-D, 2005) are shown in Fig. 1. From a total of 221 clones, 50 OTUs were identified based on 0.03 farthest-neighbor distance of the 16S rRNA partial gene sequences (*c.* 600 bp). A summary of the 16S rRNA gene sequence analyses is presented in Table 2. The richness estimates showed no major differences between sites or years, but generally sites A and B were slightly more diverse than C and D. Only five of the OTUs were encountered frequently in the total lagoon community as a whole. Three of these OTUs formed clusters with the filamentous *Lyngbya* (3a5acd/p1b1, 44 clones), *Spirulina* (3ab5a/p1b3, 20 clones) and the unicellular *Cyanothece* (5b/566c, 23 clones). Two OTUs clustered with unidentified cyanobacteria belonging to Oscillatoriales (3ab5cd/556c and 3ab5acd/p1c1, 23 and 17 clones, respectively). The most evident difference in cyanobacterial composition between sites was the presence of a heterocystous cyanobacterium at site B. The phylogenetic analyses suggested *Anabaena* sp. to be the closest relative (Fig. 1), while morphological characters, such as the presence of sheath and truncated cells, suggested affiliation with the genus *Nodularia* (Table 1). Several unicellular morpho- and phylotypes were also identified in the mat at site B, e.g. species of the potentially nitrogen-fixing genera *Cyanothece* and *Gloeocapsa* sp., as well as *Chroococcus* sp. and *Chroococcidiopsis* sp. Filamentous nonheterocystous species also appeared frequently. Nonheterocystous cyanobacteria were predominantly identified at site A, with the main morphotypes being *Microcoleus chthonoplastes*, *Lyngbya* spp. and *Spirulina* spp. Likewise, sites C and D harbored only nonheterocystous (unicellular and filamentous) cyanobacteria. 16S rRNA gene sequence analysis suggested a dominance of several members of Oscillatorales and Chroococcales (e.g. *Cyanothece* sp.) at sites A, C and D. Unicellular phylotypes were particularly well represented at site B, but also occurred in A and C. Species within the genus *Spirulina* were common and present at all four sites. A distinctly smaller spiral-shaped morphotype was identified as *Spirulina*

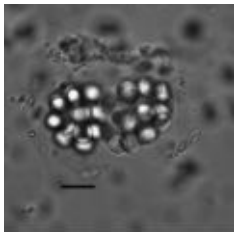
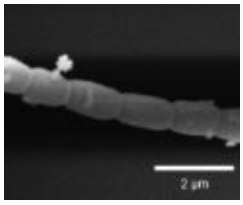
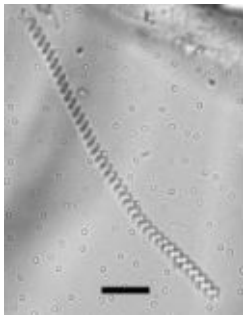
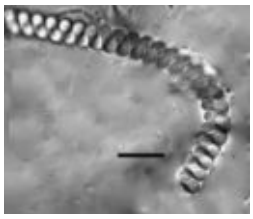
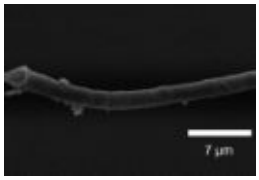
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Table 1. Cyanobacterial morphotypes encountered at sites A–D in the Paje lagoon (western Indian Ocean, Zanzibar) using light and scanning electron microscopy

Order Family *Genus species*	Size	Morphological description	Location	Figure
Chroococcales				
Synechococcaceae				
Aphanothece sp.	Cells 0.8 μm wide and 1.5 μm long	Cells oval with rounded ends, approximately twice as long as wide	D	
Cyanothece sp.	Cells 2.7–3.2 μm wide and 5.5–6.9 μm long	Cells oval with rounded cells	D	
Johannesbaptistia sp.	Cells 3–3.4 μm wide and 2.5–2.8 μm long	Cells arranged in solitary, unbranched pseudo-filaments	B	
Microcystaceae				
Gloeocapsa sp.	Cells without sheath 5.5–7 μm in diameter	Cells spherical, surrounded by a thick colorless sheath	B	
Chroococcaceae				
Chroococcus sp.	Cells 23.6–28.5 μm wide and 17.1–17.3 μm long. Sheath 2.2–2.5 μm thick	Individual cells spherical, subspherical to hemispherical after division. Distinct and lamellated sheath	B	

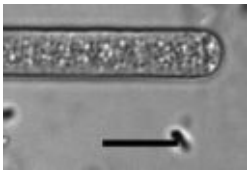
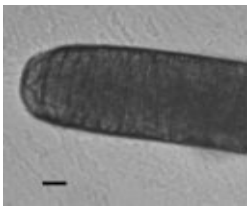
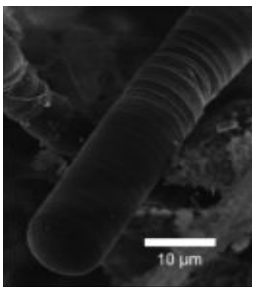
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Table 1. Continued.

Order Family *Genus species*	Size	Morphological description	Location	Figure
Xenococcaceae				
Chroococcidiopsis sp.	Cells 5.7–6.2 μm in diameter	Cells more or less spherical, in groups surrounded by sheath	B	
Oscillatoriales				
Pseudanabaenaceae				
Pseudanabaena sp.	Trichomes *c.* 1 μm wide, cells 1.3–1.6 μm long	Trichomes distinctly constricted at cross-walls	B, C	
Spirulina labyrinthiformis	Trichomes *c.* 1–1.2 μm wide. Coils 2–3 μm wide and 1.8–2 μm high	Trichomes screw-like regularly coiled	A, B, C	
Spirulina sp.	Trichomes *c.* 2–2.5 μm wide, coils 5.2–5.9 μm wide and 3–3.8 μm high	Trichomes tightly, regularly coiled	A, C, D	
Leptolyngbya sp.	Trichomes *c.* 2 μm wide	Trichomes surrounded by sheath	A, B, C	

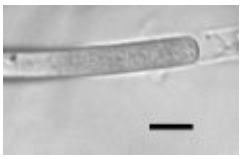
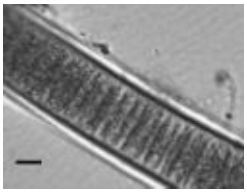
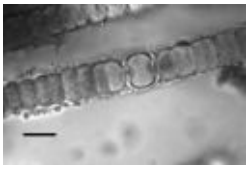
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Table 1. Continued.

Order Family *Genus species*	Size	Morphological description	Location	Figure
Phormidiaceae				
Phormidium sp.	Trichomes c. 3.7 μm. Cells 0.8–1.0 μm long	Trichomes surrounded by sheath, end cells rounded	A, C, D	
Microcoleus chthonoplastes	Trichomes 5.6–6.6 μm wide, Cells 5.7–8.4 μm long. End cells up to 16 μm long	Several trichomes together in one filament, surrounded by sheath. End cells attenuated or rounded	A,D	
Oscillatoriaceae				
Oscillatoria sp.1	Trichomes 6.5–6.9 μm wide, cells 1.8–2.7 μm long	Cells granulated, end cells rounded	A, C, D	
Oscillatoria sp.2	Trichomes c. 37 μm wide, cells 5.2–5.4 μm long	End cells slightly attenuated, cells not constricted at cross-walls	B	
Lyngbya aestuarii	Trichomes 14.2–15.8 μm wide and cells 1.6–2.8 μm long, sheath 1.5–2.2 μm thick	End cells rounded. Filaments blue–green	B, D, C	

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Table 1. Continued.

Order Family *Genus species*	Size	Morphological description	Location	Figure
Lyngbya sp. 1	Trichomes 6–8 μm, thin sheath c. 1 μm.	End cells rounded. Filaments blue–green	A, C, D	
Lyngbya sp. 2	Trichomes 30–32 μm wide, cells 2–2.5 μm long, sheath approx. 3.8 μm thick	End cells rounded filaments yellow–green	B	
Nostocales Nostocaceae *Nodularia* sp.	Cells 9.5–11 μm wide and 4.5–5.5 μm long, heterocysts 11 μm wide and 7.8–9.2 μm long, sheath c. 1.5 μm	Trichomes surrounded by diffluent sheath, constricted at cross-walls, heterocysts intercalary, end cells rounded	B	

Scale bars in light micrographs are 10 μm.

labyrinthiformis (coils 2–3 μm wide), while the larger morphotype (coils 5.2–5.9 μm wide) did not fit the description of any revised taxa, but may represent a slightly larger strain of *Spirulina subsalsa* or a marine strain of *Spirulina robusta*. The presence of at least two *Spirulina* phylotypes (or possibly *Halospirulina*) was also confirmed by partial 16S rRNA gene sequence analysis (Fig. 1). Phylotypes related to *Oscillatoria* recovered from corals with black band disease (Sussman *et al.*, 2006) were also well represented at sites A and C. A total of seven OTUs clustered genetically with sequences of the genera *Leptolyngbya* and *Phormidium*, and were also identified morphologically. The partial 16S rRNA gene sequences from this study are deposited in GeneBank with accession numbers EF160007–160024 and EF160033–160064.

NifH diversity

DNA extracted from samples collected at sites A-D in 2005 was used as template to amplify and analyze *nifH* diversity. RFLP analysis of *nifH* gene fragments amplified using the general PolR/F primers (Poly *et al.*, 2001) resulted in *c.* 25 different restriction patterns. With the exception of one, all OTUs clustered with bacteria other than cyanobacteria. Although the cyanobacterial sequence obtained originated from site B, it did not seem to represent a heterocystous cyanobacterium. Using the cyanobacterial-selective primers CNF/R (Olson *et al.*, 1998), however, additional cyanobacterial *nifH* gene sequences were retrieved when analyzed by DGGE. One genotype isolated from site B belonged to a heterocystous phenotype, possibly Nostocales, while the other sequences were derived from nonheterocystous cyanobacteria. Two of the latter sequences were closely related to *Lyngbya majuscula,* while two other sequences clustered with sequences isolated from microbial mats in waters outside Mexico and Bahamas (Steppe *et al.*, 2001; Omoregie *et al.*, 2004b) and appeared to be members of Pseudanabenaceae. The remaining three sequences formed a well-supported cluster and were only distantly related to previously reported *nifH* gene sequences. These two clusters are therefore likely to include novel groups of nitrogen-fixing benthic cyanobacteria.

Bacterial sequences, other than cyanobacteria but also belonging to the *nifH* cluster I, were well represented in the phylogenetic analyses (Fig. 2). The Gammaproteobacteria were particularly diverse and heterogeneous. At least two

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Table 2. 16S rRNA gene clone library analysis obtained from microbial mat samples in the Paje lagoon (western Indian Ocean, Zanzibar, Tanzania)

Library	No. Clones	OTUs	ACE	Chao1	Shannon–Weiner index
A2003	40	14	38.5	32	2.12
B2003	36	14	20.2	15.7	2.35
A2005*	35	10	13.5	12	1.83
B2005	39	15	20.5	18.8	2.47
C2005	33	7	13	9	0.97
D2005†	38	8	10.7	9.5	1.51

OTUs and diversity indices and estimations (Abundance-based Coverage Estimator, Chao1 and Shannon–Weiner) were calculated in DOTUR using a 0.3 farthest neighbor limit. Six individual clone libraries were constructed from samples collected in Paje, Zanzibar in 2003 (site A and B) and 2005 (sites A–D).

*Furthest neighbor distance for OTU was 0.4.

†Furthest neighbor distance for OTU was 0.2.

well-separated groups of sequences, composed exclusively of 'uncultured' bacteria with very low similarity to known Gammaproteobacteria, were present at sites A, B and C, respectively. Several sequences from sites A, B and D were closely related to the Gammaproteobacterium *Marichromatium purpuratum*. Finally, one OTU each from sites C and B clustered with Alphaproteobacteria, and two OTUs each from sites A and B clustered with Betaproteobacteria.

The cyanobacterial-selective primers CNF/R combined with DGGE analyses identified one noncyanobacterial sequence. This sequence was detected at three of the four sites examined (A, C and D; Fig. 3), i.e. in the nonheterocystous cyanobacterial mats, using both sets of primers and methods. It was clearly the most dominant sequence in the corresponding clone libraries, representing 43 out of the 132 clones screened, and the relatively most abundant DGGE band recovered. The DGGE theoretically confirmed the high abundance of these noncyanobacterial potential nitrogen fixers in the Paje lagoon.

This dominant phylotype clustered together with other 'uncultured' bacteria from microbial mats (Steppe & Paerl, 2002; Omoregie *et al.*, 2004a) and belongs to *nifH* cluster III comprising anaerobic diazotrophs. Belonging to the same cluster III were also several sequences recovered from site A. These formed a separate cluster of 'uncultured' sequences closely related to microbial mats and bacteria recently found to be epiphytically associated with sea grasses at a nearby western Indian Ocean site (coastal area of Kenya) (Uku *et al.*, 2007). Four unique OTUs in *nifH* cluster III from sites A, B and C showed less than 90% similarity to any other sequence in the database (Fig. 2). Finally, two OTUs from sites C and D were closely related to Gammaproteobacteria and one OTU from site B clustered with *Chloroflexi* (anaerobic bacterium) and bacteria denoted 'uncultured' in other microbial mats (Yannarell *et al.*, 2006). Partial *nifH* gene sequences from this study are deposited in GeneBank with accession numbers EF159981–160006 and EF160025–160032.

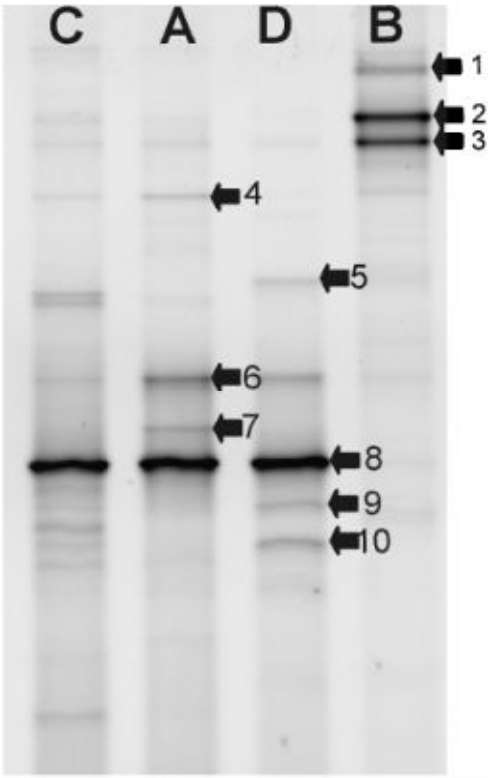


Fig. 3. Selective cyanobacterial *nifH*-DGGE fingerprint image retrieved by amplification using primers CNF (with a 40 oligonucleotide long GC clamp) and CNR from samples A–D (2005) from the tropical Paje lagoon (western Indian Ocean, Zanzibar). The numbers represent all the bands that were identified by sequencing and included in Fig. 2.

Nitrogenase activity

As can be seen in Fig. 4, nitrogenase activities were assayed in 2003 (site A and B) and in 2005 (site A–D). More limited measurements were also performed in 2004 (data not shown). All measurements were performed at the same time of the year (February–March). The highest nitrogenase activities during daytime were observed at site B, harboring

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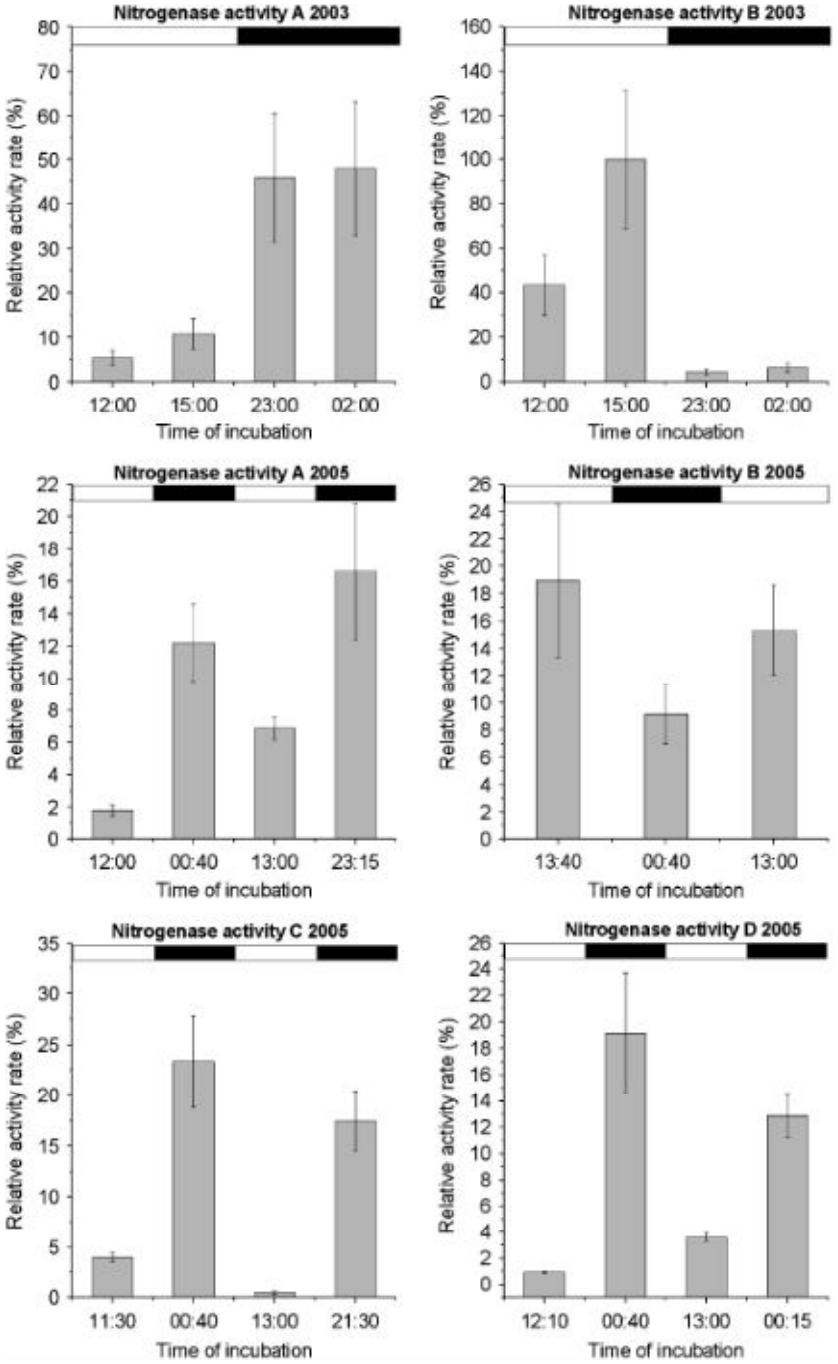


Fig. 4. Nitrogenase activity in sites A and B (2003) and A–D (2005) in the tropical Paje lagoon (western Indian Ocean, Zanzibar). Nitrogenase activity was assayed using the acetylene reducation technique and gas chromatography. Relative values are given as percentage of maximum activity rate (119 nmol C_2H_4 cm^{-2} h^{-1}). Error bars = SE (±).

the heterocystous *Nodularia* sp. The maximum activity observed reached about 120 nmol C_2H_4 cm^{-2} h^{-1} (assayed at 15.00, 2003). Owing to the risk of dilution during transfer of the gas sample to vacutainers, the relative values are presented in Fig. 4 as percentage of the maximum value. In 2003, daytime and nighttime rates were significantly different ($P < 0.05$), while this was not the case in 2005. Nitrogenase activities were also substantial at sites with a nonheterocystous cyanobacterial predominance. However, activity maxima were observed during nighttime, with the highest activities approaching 50 % of the activity at site B (2003), while the highest daytime fixation rates in these mats

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reached about 10 %. In both 2003 and 2005, daytime and nighttime rates in site A differed significantly [2003 $P < 0.001$, 2005 $P < 0.05$ (between 12:00 and 23:15)]. In 2004, nitrogenase activity rates were considerably lower at both sites (data not shown) and showed large variations within each site, indicating a pronounced heterogeneity in terms of metabolic activity and/or species composition. However, the rates from individual single cores showed the same diurnal patterns as in 2003 and 2005, i.e. site B exhibited the highest daytime rate, while the maximum rate at night was detected at site A. In March 2005, the activity was more consistent and similar to observations in 2003 (Fig. 1a and b). Sites C and D shared diel patterns with site A, with the highest nitrogenase activities encountered at night. In site C, all nighttime rates differed significantly ($P < 0.001$) from daytime rates, and at site D one of the nighttime rates was significantly different from the daytime rates ($P < 0.05$).

Discussion

Recent reports on benthic nitrogen fixation mostly deal with microorganisms in 'extreme' habitats, such as hypersaline ponds or lagoons (Pinckney & Paerl, 1997; Paerl *et al.*, 2000; Omoregie *et al.*, 2004a, b; Yannarell *et al.*, 2006). Globally widespread marine environments, such as nutrient-poor tropical lagoons exhibiting 'typical' marine salinity levels, exemplified by the Paje lagoon (western Indian Ocean), examined here have been largely neglected. Even when sheltered by fringing coral reefs, such lagoons offer challenging conditions including high tidal variations, extreme light and temperature conditions (especially at low tide), strong wave actions and unstable bottom surfaces, often composed of small grained, almost white, coral sand. In such highly variable environments (affected also by tourism and/or aquaculture events), cyanobacteria apparently thrive well enough to establish thin mats without much competition from other micro- and macrophytes, often more dependent on a constant supply of nutrients and less disturbed physical conditions.

In spite of the modest macroscopic structure of the microbial biofilms/mats observed in the Paje lagoon, and their occasional but regular coverage by thin layers of fine coral sand, these were composed of a rich cyanobacterial diversity (morphological and genetic). Indeed, the phylogenetic 16S rRNA gene and *nifH* gene analyses verified a high genetic diversity within the cyanobacterial population in the lagoon and the presence of novel genera/species. This potentially also includes previously unidentified nitrogen-fixers with a pan-tropical or even global occurrence (Steppe *et al.*, 2001; Omoregie *et al.*, 2004b; Musat *et al.*, 2006; Díez *et al.*, 2007). The present data also corroborate the morphological studies performed previously on benthic cyanobacterial communities in waters around Zanzibar (Lugomela *et al.*, 2001a). Likewise, a rich morphological variation was identified among the cyanobacteria, ranging from unicellular to filamentous nonheterocystous and heterocystous genera. The genetic diversity, investigated in the area for the first time, revealed the presence of at least 50 different phylotypes compared with the 18 morphotypes observed based on morphological criteria.

16S rRNA gene analyses showed that nonheterocystous cyanobacteria were the most common microorganisms in the euphotic zone of the mats, while *nifH* gene analyses in general proved noncyanobacterial nitrogen-fixers as being the most abundant diazotrophs. This suggests that although cyanobacteria clearly dominate the microbial mats on a biomass basis, diazotrophs are represented both by cyanobacteria and the smaller Proteobacteria.

The photoautotrophic nature of the cyanobacteria is likely to support the heterotrophic bacteria indirectly, via their photosynthesis, with the energy needed for maintaining diazotrophic activities.

The polyphasic approach that was used here (combining molecular and morphological data) considerably improved the attempts to characterize the cyanobacterial diversity in the mats. As is obvious from the data, on neglecting morphological aspects and the challenge of linking phylotypes to morphotypes in complex environmental consortia such as microbial mats, the full microbial diversity may easily be overlooked and underestimated. This is reflected in the large number of cyanobacterial sequences deposited in public databases as 'uncultured' or 'unidentified'. The two cyanobacterial approaches to taxonomy used today (botanical and bacteriological) add to the current confusion. Cyanobacterial taxonomy suffers from unresolved issues and certain cyanobacterial taxa, described using the botanical approach, might be of polyphyletic origin and therefore in need of revision. Especially families with small and simple filamentous morphologies, such as members of the Pseudanabenaceae family identified here, require specific attention. Together, however, previous (Silva & Pienaar, 2000; Lugomela *et al.*, 2001a; Ley *et al.*, 2006; Yannarell *et al.*, 2006; Díez *et al.*, 2007) and the present data suggest these particular morpho- and phylotypes to be widespread as well as vital components in often geographically distant tropical coastal habitats. This group may potentially include diazotrophic genera whose ecological significance therefore needs to be further explored. The polyphasic approach used stresses the occurrence and vital importance of both the more obvious (larger and pigmented) cyanobacteria and the considerably smaller and less conspicuous nonphotosynthetic bacteria.

It is also apparent from the data that the microbial mats at the four sites in the Paje lagoon, dominated by cyanobacteria, fix 'new' nitrogen in distinct diurnal and complementary rhythms. This complementary nitrogen fixation behavior

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between the mats (either activity maxima in the daytime or in the nighttime) may therefore continuously provide the lagoon with new nitrogen.

Heterocystous cyanobacteria have recently been considered to be largely excluded from tropical oceans due to the prevailing high temperatures (Staal *et al.*, 2003), although some have occasionally been encountered in benthic tropical habitats (Silva & Pienaar, 2000; Lugomela *et al.*, 2001a). The present data also show that the heterocystous genus *Nodularia* is a semi-permanent member of the microbial community in the tropical Paje lagoon, consistently being found at a specific site all 3 years. Not surprisingly, the *Nodularia* mat (site B) also showed the highest nitrogenase activities over the years and as expected predominantly in the daytime. Lower, but substantial, nitrogenase activity rates were recorded at night at the sites dominated by the filamentous, nonheterocystous cyanobacteria (site A, C, D). However, as the *nifH* phylogeny demonstrated the presence of diazotrophic heterotrophic bacteria deploying a nighttime nitrogen-fixing strategy (Omoregie *et al.*, 2004a), their importance as contributiors of 'new' nitrogen to the area may likewise be substantial. The diurnal activity rhythms found also suggest the operation of complementary but different strategies to protect the oxygen-sensitive nitrogenase. The variable nitrogenase activities (within and between sites) also stress the instability and unevenness in species distribution, also verified by visual observations. Particularly wavering was the *Nodularia*-dominated mat, far less coherent and more heterogeneous than its nonheterocystous counterparts. These data also illustrate the challenge encountered when trying to estimate the ecological significance of benthic communities. Establishing significant differences over diel cycles is particularly challenging in an intertidal area, with accessibility for short periods twice per diel cycle and for only a few days per moon cycle, which results in a limited resolution. Some of the higher nitrogenase activities recorded (possibly underestimated due to dilution during sample transfer) correlate with the rates presented as typical for microbial mats subjected to a tidal regime in temperate regions (Stal, 1999). Moreover, the large temporal (inter-year, inter-diurnal) and spatial (within and between sites) variations recorded in the Paje lagoon clearly stress the need for long-term, seasonal and diurnal analyses at many sites, preferentially also including isotope tracer techniques, to be able to quantify the input of 'new' nitrogen into the ecosystem.

In summary, a rich benthic cyanobacterial diversity including several novel species was discovered in the intertidal mats in the sand-covered tropical lagoon examined (Zanzibar, Tanzania), as verified using both morphological and genetic analyses. Known and potentially new N_2-fixers, including both cyanobacteria and heterotrophic bacteria, were identified by *nifH* phylogeny and via nitrogen fixation activity measurements. The present data also illustrate the great natural variations in diazotrophic activities and the cyanobacterial diversity over time (short and long term) and space, illustrating the need for long-term field studies if one is to quantify marine nitrogen fixation more reliably in tropical coastal regions.

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