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Composition and heterogeneity of the microbial community in a coastal microbial mat as revealed by the analysis of pigments and phospholipid-derived fatty acids

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ABSTRACT

The beaches of the North Sea barrier island Schiermonnikoog (The Netherlands) are covered by microbial mats. Five types of microbial mats were distinguished based on a variety of characteristics, located along a transect perpendicular to the coast. Biomass abundance and composition of the microbial community were analyzed in these mats using pigments and phospholipid-derived fatty acids (PLFA) as biomarkers. Biomass per gram sediment increased more than six-fold from the mats at the low water mark to mats found at the edge of the dunes. Microscopic analysis revealed that the increase in biomass was accompanied by a change in species composition. Pigment- and PLFA composition reflected the changes in species composition. The PLFA data could be used to estimate the relative group abundance using the matrix factorization program CHEMTAX., whereas the pigment data were found not to be suitable for this purpose.

1. Introduction

Microbial mats are vertically stratified communities of microorganisms that are found in a variety of different environments, for example intertidal and supratidal coastal sediments, marine salterns, hot springs, hypersaline ponds and deserts. Steep gradients of physical and chemical parameters, such as irradiance, oxygen and sulfide, provide niches for a variety of microorganisms, including aerobic and anaerobic photoautotrophs, chemoautotrophs and heterotrophs (Revsbech et al., 1983; Stal, 2000).

In mats on intertidal and supratidal sandy beaches, cyanobacteria are usually the most conspicuous microorganisms. Together with diatoms, which are also commonly present, these oxygenic phototrophs enrich the sediment with organic carbon through the fixation of CO_2 (Martinez-Alonso et al., 2004; Stal, 2000). Many cyanobacteria are capable of nitrogen fixation and can thus also supply nitrogen to the mats (Pinckney et al., 1995; Stal, 2003). The organic matter serves as substrate for a variety of heterotrophic bacteria, becoming available through excretion of metabolites or by death and lysis of the microorganisms (Martinez-Alonso et al., 2004; Stal, 2000; Wieland et al., 2003). Sulfate-reducing bacteria play an important role in the decomposition of organic matter. They produce sulfide that precipitates as iron-sulfide or pyrite which colors the deeper layers of the mat black (Stal, 2000). Sulfide serves as an electron donor for anoxygenic photosynthesis by purple sulfur bacteria (under anaerobic conditions) or for colorless chemoautotrophic sulfur oxidizing bacteria (both aerobically and anaerobically) (Caumette et al., 1994; Wieland et al., 2003).

Microbial mats on intertidal and supratidal beaches are found on several of the barrier islands in the Waddensea along the north-west coastline of the Netherlands, Germany and Denmark (Stal, 1999; Wieringa et al., 2000; Noffke, 2003; Stal, 2003). Here, we report on the composition of microbial mats on the island Schiermonnikoog (The Netherlands). The north-western part of the sandy beaches on this island is covered by extensive microbial mats. The beach is located behind a sand bar that protects it from wave impact. We observed spatial differences in the development of the mats as well as in their composition with regard to microorganisms. Along a transect perpendicular to the coast a variety of different types of mats were distinguished. Communities that were loosely bound to the sediment and which contained low biomass were present at the low water mark, while mature, well-structured, dense mats formed close to the dunes.

The objective of our study was twofold. Firstly, we wanted to study the heterogeneity of the mat in terms of microorganism composition and abundance of biomass. Secondly, we wanted to investigate the usefulness of PLFA as biomarkers in this system, with the intention of using stable-isotope labeling of PLFA to determine the activity of the mat organisms in an upcoming study.

Emphasis was put on the oxygenic phototrophic organisms that are the key organisms and that dominate the biomass of the mats. Various methods are available to study the species composition of microorganisms each supplying information on a different level of detail. Microscopy can be used to identify and enumerate species. However, quantifying species in terms of abundance and biomass by microscopy is tedious, demands great skill and involves the measurement of

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cellular dimensions to be able to convert cell numbers to biomass. Biomarkers usually can not give information on the species level but are more easily obtained for a larger number of samples and are more easily converted to biomass. We used pigments and phospholipid-derived fatty acids (PLFA) as biomarkers to study the heterogeneity of the microbial mats on Schiermonnikoog.

Phospholipids are the main cellular membrane lipids to which a large diversity of fatty acids can be esterified. We used phospholipid-derived fatty acids rather than total fatty acids since the former represent living biomass. Upon cell death, the majority of phospholipids is rapidly degraded to neutral lipids (Pinkart et al., 2002; Drenovsky et al., 2004). Neutral lipids are much more persistent and can remain present in the sediment for extended periods of time. In addition, neutral lipids include storage lipids, the cellular content of which can be highly variable. Moreover, the fatty acid composition of these storage lipids can be very different from the composition of phospholipid-derived fatty acids (Piorreck et al., 1983). Thus, PLFA represent a less variable fraction than total fatty acids. In contrast to pigments, which are limited to phototrophic organisms, PLFA are present in all eukaryotes and bacteria, allowing the study of a wider range of organisms.

After microscopic observations of the species relevant to the mats, we determined the biomarker composition of species relevant to the microbial mats both from culture experiments and literature data, and used this information to interpret the variations in pigment and PLFA composition and abundance in the microbial mats of Schiermonnikoog. The matrix factorization program CHEMTAX was used to estimate group abundance from the PLFA composition.

2. Material and methods

2.1. Research area

The beaches at the north-west side of the barrier island Schiermonnikoog (The Netherlands) are covered by microbial mats (Fig. 1). At the low water mark mats are submerged twice a day during high tide. Higher on the beach, the mats are irregularly submerged and receive freshwater through rainfall and well water from the nearby dunes. At the low water mark the presence of microorganisms could only be verified through microscopy, although sometimes scant green patches were visible. Higher on the beach, mats develop that possess the typical sedimentary structure and contain high phototrophic biomass. These mats can sometimes be lifted in one piece from the sediment and consists almost completely of organic matter. Some mature mats had an orange appearance, which is usually attributed to decaying organic material.

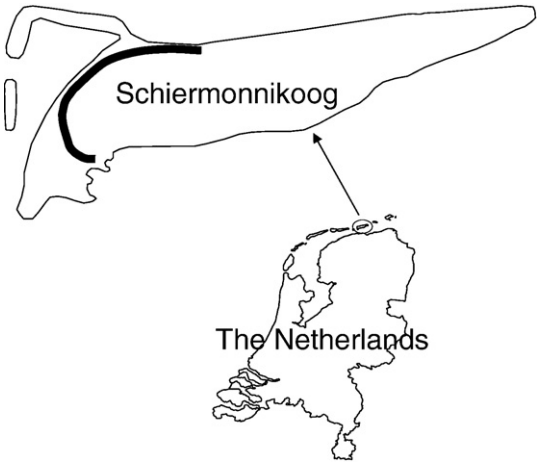

Fig. 1. Map of the North Sea barrier island Schiermonnikoog (The Netherlands). Microbial mats were found on the beaches on the north-west side of the island, indicated by a black bar. All sampling sites were within 500 m of 53°29′18″N, 6°08′27″E.

Five types of microbial mats were defined, based on the visual appearance of the mat. The different mat types, sampled at five different sites, are described in Table 1. The five sites were selected along a transect perpendicular to the coast. The beach was approximately 300 m wide from the waterline the edge of the dunes, and the sampling sites were spaced more or less equally over this distance. The mats were sampled in June 2002.

To avoid semantic juggling, the term 'mats' is used throughout this manuscript for all types observed, with the understanding the at the low water mark the 'mats' should rather be characterized as 'biofilms', as no real mat structure is present.

2.2. Sampling

At each site, samples were taken for pigment and phospholipid-derived fatty acid composition, carbon and nitrogen analysis and microscopic observations. Sampling was done using polyethylene cores with a diameter of 24 mm. Six cores were taken from each mat. The top 2 mm of each core was sliced off, packed in aluminum foil and immediately frozen in liquid nitrogen. The top 2 mm contained the complete firm mat structure at the sites with well developed mats. Closer to the low water mark biomass was more dispersed in the sediment. We have chosen to sample the top 2 mm only nonetheless to be able to compare the different sites. The samples were transported in liquid nitrogen to the laboratory and subsequently stored at −80 °C until analysis. The samples of three cores were lyophilized, pooled and homogenized to obtain enough biomass for the analyses. Hence, for each site duplicate samples were analyzed.

2.3. Cultures

PLFA and pigment composition were determined for one diatom species and eight species of cyanobacteria. Species were chosen that are relevant to the microbial mats. Some of the cyanobacteria tested were originally isolated from the microbial mats of Schiermonnikoog. Cultures were grown in Erlenmeyer flasks containing the appropriate medium (see Table 2) at an irradiance of 40 μmol $m^{-2}s^{-1}$ at 16 °C and a light–dark cycle of 14:10 h. The cultures in the logarithmic growth phase were harvested by filtration on glass microfiber filters (GF/F, Whatman). Samples were frozen immediately in liquid nitrogen and stored at −80 °C until analysis.

2.4. Pigments

Pigments were extracted in 90% acetone and analyzed by HPLC using a reversed-phase analytical column (Novapak C_{18}, 4 μm, 15 cm; Waters Millenium HPLC system) (Rijstenbil, 2003). Most pigments were quantified at 450 nm using the appropriate standards, except for echinenone, myxoxanthophyll and scytonema for which standards were not available. These pigments were tentatively identified from

Table 1
Description of the sites sampled for this study.

Site	Description	Dominant species/key species
1	No visible coloring or faint green coloring, loose sand	Pennate diatoms (*Navicula* sp., *Diploneis* sp., *Amphora* sp., *Cylindrotheca closterium*), *Lyngbya* sp.
2	Green/brown coloring visible in the sand, no coherent mat structure	*Microcoleus chthonoplastes*, *Lyngbya* sp.
3	Non-coherent green mat visible at or just below the surface	*Microcoleus chthonoplastes*, *Lyngbya* sp.
4	Coherent mat at or just below the surface; often on dark sediment	*Nostoc* sp, *Calothrix* sp., *Anabaena* sp., *Microcoleus chthonoplastes*, *Lyngbya* sp.
5	Coherent mat, partly senescent with orange coloring on top of mat; intense black sediment	*Nostoc* sp, *Calothrix* sp., *Anabaena* sp., *Microcoleus chthonoplastes*, *Lyngbya* sp.

Table 2
Pigment- and PLFA composition of oxygenic phototrophic organisms relevant to the microbial mats.

	Diatom	Cyanobacteria							
	C.c	Syn.[a]	Lyn.[b]	Mic.[b]	Ana.[c]	Aph.[c]	Nos.[c]	Cal.[c]	Nod.[c]
A. Pigments									
Pigments (ng (μg Chl *a*)$^{-1}$)									
β,β-carotene		67.2	94.7	121.0	65.8	40.8	60.0	67.6	37.6
Zeaxanthin		221.6	52.7	19.3					
Canthaxanthin					14.3	18.4	17.0	51.3	100.8
Echinenone			26.7	6.5	32.8	24.5	33.9	43.8	56.2
Myxoxanthophyll			9.9	17.1	37.1	6.1	33.9	56.6	25.2
B. PLFA									
PLFA (weight % of total PLFA)									
Saturated fatty acids									
14:0	8.3	16.1	19.2	0.2		0.5			
15:0	0.6								
16:0	17.9	17.8	19.0	36.6	28.1	31.1	29.2	44.7	37.3
18:0	0.3	1.8	0.8	0.8	0.6	0.9			
Mono-unsaturated fatty acids									
14:1		10.6							
16:1ω9c		4.7	2.3	2.4					8.8
16:1ω7c	19.2	45.0	1.4	13.2	14.5	3.0	13.7	3.8	6.8
18:1ω9c	1.9	0.8	1.7	1.8	2.5	1.7	2.2	0.9	0.8
18:1ω7c	0.1	2.2	0.9	1.6	3.0	0.5	1.7	0.9	2.1
Poly-unsaturated fatty acids									
16:2ω7	0.3								
16:2ω4	4.1				4.8		5.4		
16:3ω4	1.7								
16:3ω3			3.6			10.9			
16:4ω3								6.6	
16:4ω1	5.5								
18:2ω6c	3.3		7.6	3.6	5.6	6.3	5.3	4.7	3.4
18:3ω6	3.7							1.1	
18:3ω3	0.2		43.6	42.2	40.0	43.1	40.6	12.0	16.4
18:4ω3	3.4							23.5	24.5
20:4ω6	5.3								
20:5ω3	22.0								
22:6ω3	2.2								
Medium[d]	F/2[e]	BG11	ASN	ASN	BG11	BG11	BG11	BG11	BG11

C.c.: *Cylindrotheca closterium* CCY9601, *Syn.*: *Synechococcus* sp. CCY 0011[f], *Lyn.*: *Lyngbya* sp. CCY 0005[f], *Mic.*: *Microcoleus* sp. CCY0002[f], *Ana.*: *Anabaena* sp. ATCC 27899, *Aph.*: *Aphanizomenon* sp. CCY 9905, *Nos.*: *Nostoc* sp. PCC7120, *Cal.*: *Calothrix* sp. ATCC 27905, *Nod.*: *Nodularia* sp. CCY 0014[f].

[a] Unicellular.
[b] Non-heterocystous.
[c] Heterocystous.
[d] BG11: freshwater medium (Stanier et al., 1971), F/2: artificial seawater enriched with F/2 nutrients (Guillard and Ryther, 1962), ASN: marine medium (Rippka, 1988).
[e] Sand added as substrate.
[f] Isolated from the microbial mat on Schiermonnikoog.

their absorption spectra and quantified using β,β-carotene as standard. Calculated values for these three pigments will only approximate the true values, as response factors differ among pigments. Myxoxanthophyll is the sum of several peaks with the characteristic absorption spectrum of myxoxanthophyll (see e.g Karsten and Garcia-Pichel, 1996). Echinenone partly co-eluted with a Chl *a* epimer that mainly occurred in the field samples. Therefore, echinenone was quantified at its absorption maximum of 461 nm where absorption of the Chl *a* epimer is negligible.

2.5. Phospholipid-derived fatty acids

Total lipids were extracted in a mixture of chloroform, methanol and water (1:2:0.8, v/v) according to Boschker (2004), using an adaptation of the method of Bligh and Dyer (Bligh and Dyer, 1959). Phase separation was induced by the addition of chloroform and water to a final composition of chloroform–methanol–water of 1:1:0.9 (v/v). The chloroform layer containing the total lipid fraction was collected. This total lipid extract was fractionated into neutral-, glyco-, and phospholipids on heat activated silicic acid columns by sequential elution with chloroform, acetone and methanol. The methanol contained the most polar fraction which consists mainly of phospholipids. Derivatives of this fraction were produced by mild alkaline methanolysis yielding fatty acid methyl esters (FAMEs). The FAMEs 12:0 and 19:0 were used as internal standards. FAME concentrations were determined by gas chromatography-flame ionization detection (GC-FID, Interscience HRGC MEGA 2 series) using a polar analytical column (BPX-70, Scientific Glass Engineering, 50 m length, 0.32 mm diameter, 0.25 μm film). The GC was equipped with a split/split-less injector that was used in the split-less mode. The injector temperature was 240 °C, the column pressure was 14 psi and the following temperature program was applied: initial 60 °C for 2 min, then to 110 °C with 25 °C/min, to 230 °C with 3 °C/min, to 250 °C with 25 °C/min and hold 15 min. The split-less period was 1.5 min and the total run time about 55 min. Blanks and a standard mixture were measured regularly in between samples to check for system stability and possible contamination. FAME identification was based on comparison of retention times with known reference standards. The identity of FAMEs not present in the reference standards was determined from mass spectrometry (Finnigan Voyager Mass Spectrometer) on pycolinyl esters prepared from samples as described in Dubois et al. (2006). This allows identification of the molecular mass and the number and position of double bonds in FAMEs. Peak identities and chemical purity were also checked by measuring the samples on an apolar column

(HP-5MS, Agilent, see Boschker (2004) for further details) on which retention times and elution orders are different.

The fatty acid notation consists of the number of carbon atoms followed by a colon and the number of double bonds present in the molecule. The position of the first double bond relative to the aliphatic end of the molecule follows the symbol omega, ω (e.g. 16:1ω7). If more double bonds are present, these are separated by methylene groups. Prefixes 'i' (iso) and 'ai' (anteiso) represent the location of a methyl branch one or two carbons respectively from the aliphatic end (e.g. i15:0). If relevant, 'c' indicates the cis-orientation of the double bonds.

2.6. C/N analysis

Organic carbon and total nitrogen were analyzed using a Carlo Erba elemental analyzer following an *in situ* acidification procedure as described in detail in Nieuwenhuize et al. (1994).

3. Results

3.1. Microscopic observations of the microbial mat

Microscopy was limited to qualitative observations and identification of the main types of oxygenic phototrophic organisms in the mat. Cyanobacteria and diatoms were the most abundant oxygenic photosynthetic taxa. The key species at each site are listed in Table 1. Cyanobacteria of the filamentous genera *Spirulina*, *Phormidium* and *Nodularia*, cyanobacteria of the unicellular genera *Synechocystis*, *Merismopedia* and *Gloeocapsa* and the filamentous green alga *Enteromorpha* sp. were observed in low numbers at all sites. The unicellular cyanobacterium *Synechococcus* was observed in high numbers at all sites. Site 1 was dominated by pennate diatoms like *Navicula* spp., *Diploneis* spp., *Amphora* spp. and *Cylindrotheca closterium*. The filamentous, non-heterocystous cyanobacterium *Lyngbya* sp. was present in low numbers. The non-heterocystous cyanobacterium *Microcoleus chthonoplastes*, characterized by bundles of trichomes in a common sheath, dominated the sites 2 and 3. At these sites *Lyngbya* sp. was also common in addition to the diatom genera *Navicula* and *Diploneis*. At the sites 4 and 5 *M. chthonoplastes* and *Lyngbya* sp. were present, but clearly less abundant than at the sites 2 and 3. In these mats heterocystous cyanobacteria belonging to the genera *Nostoc*, *Calothrix* and *Anabaena* were common whereas they were rare at the other sites. Remarkable was the yellow–brown color of the sheaths of *Lyngbya* sp. and *Calothrix* sp. at site 4. We found many akinetes in the mats at site 5. Akinetes are differentiated cells of certain genera of heterocystous cyanobacteria and serve the survival under conditions that prevent growth and activity of the organism (Barsanti and Gualtieri, 2006). Akinetes were also found in the mats at site 4 but in much lower numbers. Diatoms of the genus *Navicula* were frequently observed at the sites 4 and 5, but *Diploneis* spp. were rare in these mats.

3.2. Pigment and PLFA content of cultivated strains of cyanobacteria and diatoms

Based on the microscopic observations, the pigment- and PLFA content of several relevant cultivated strains of cyanobacteria and diatoms were analyzed. The pigment composition of the pennate diatom *Cylindrotheca closterium* is similar to that of other diatoms (R. Forster, personal communication; Klein, 1988) and was therefore not analyzed. The pigment composition of the cyanobacteria varied. In addition to chlorophyll *a* (Chl *a*) and β,β-carotene, which are present in all cyanobacteria, a number of different pigments was found. Zeaxanthin was detected in the unicellular *Synechococcus* and the non-heterocystous filamentous *Lyngbya* and *Microcoleus*, but not in the heterocystous species *Anabaena*, *Aphanizomenon*, *Nostoc*, *Calothrix* and *Nodularia* (Table 2A). Canthaxanthin was found in cyanobacteria lacking zeaxanthin. Echinenone and myxoxanthophyll were present in all cyanobacteria except *Synechococcus*. We tentatively identified at least four different myxoxanthophyll-like pigments, the sum of which is presented as 'myxoxanthophyll'.

The pennate diatom *C. closterium* contained the PLFA 14:0, 16:0, 16:1ω7c, 16:2ω4, 16:3ω4, 16:4ω1, 18:4ω3, 20:4ω6, 20:5ω3 and 22:6ω3 (Table 2B). This PLFA composition agreed well with the PLFA composition of centric diatoms (Dijkman and Kromkamp, 2006) and with literature reports on the total fatty acid composition of diatoms (e.g Volkman et al., 1989), for which reason no further diatoms were tested in this study. The PLFA composition of the cyanobacteria varied (Table 2B). *Synechococcus* sp. contained 14:0, 14:1ω10, 16:0 and a large fraction of 16:1ω7c while the poly-unsaturated fatty acids (PUFA) 18:2ω6c, 18:3ω3 and 18:4ω3 were absent. *Microcoleus*, *Anabaena*, *Aphanizomenon* and *Nostoc* contained 16:0, 16:1ω7c, 18:2ω6c and 18:3ω3 as important PLFA. The PLFA composition of *Lyngbya* was similar to these four strains, but it contained 14:0 as an additional major PLFA and possessed less 16:0 and 16:1ω7c. *Calothrix* and *Nodularia* contained 18:4ω3 in addition to 16:0, 16:1ω7c, 18:2ω6c and 18:3ω3. Species possessing 18:4ω3 contained less 18:3ω3 than species lacking it 18:4ω3. Some cyanobacteria contained PUFA with a chain length of 16 carbon atoms: *Anabaena* and *Nostoc* contained 16:2ω4; 16:3ω3 was present in *Lyngbya* and *Aphanizomenon*, while *Calothrix* contained 16:4ω3.

3.3. Biomass of the microbial mat

Total organic carbon, total organic nitrogen, Chl *a*, and total PLFA, expressed per gram of sediment, increased from site 1 to site 5 (Table 3). These parameters were used as proxies for biomass. Chl *a* relatively increased more than total PLFA, leading to a decrease in the ratio PLFA:Chl *a* from site 1 to site 5. The C:N ratio varied between 9.4 and 14 and was significantly higher at the sites 1–3 than at the sites 4 and 5 (ANOVA, $P<0.005$).

3.4. Pigment composition of the microbial mat

In addition to the difference in Chl *a* content of the mats, the relative abundance of the other pigments (expressed per Chl *a*) differed between sites (Fig. 2). Zeaxanthin abundance was low in site 1 compared to the other sites (Fig. 2a). The highest relative abundance of zeaxanthin was found at the sites 2 and 3. Canthaxanthin (Fig. 2a), echinenone (Fig. 2b) and myxoxanthophyll (Fig. 2b) were absent at site 1 and more abundant at the sites 4 and 5 than at the sites 2 and 3. All of these pigments are common in cyanobacteria, albeit in different species (see Table 2A). The cyanobacterial sheath pigment scytonemin was absent at site 1 and had a maximum abundance at site 4 (Fig. 2c). Chlorophyll *b*, indicative of green algae, was detected at the sites 2 to 5 (Fig. 2c). Its abundance was much higher at site 5 than at the other sites. Fucoxanthin and chlorophyll c_{1+2}, both important pigments in diatoms, were relatively highest at site 1 and lowest at site 3 (Fig. 2d).

Table 3
Biomass parameters (μg g^{-1} (sediment)), PLFA:Chl *a* ratio and C:N ratio of the 5 mat types.

Mat type	PLFA	Chl *a*	ratio PLFA: Chl *a*	Total organic nitrogen	Total organic carbon	Ratio C:N
1	19.6 (3)	8.9 (4)	2.2	80 (45)	943 (38)	14.0 (2.2)
2	27.8 (13)	14.1 (10)	2.0	110 (15)	1168 (9)	12.6 (2.4)
3	48.5 (6)	38.9 (6)	1.2	174 (17)	1958 (12)	13.3 (1.4)
4	97.4 (15)	78.1 (4)	1.2	350 (13)	3002 (6)	10.1 (1.1)
5	116.1 (2)	131.7 (20)	0.9	468 (6)	3739 (9)	9.4 (0.2)

Standard deviations are given between brackets.

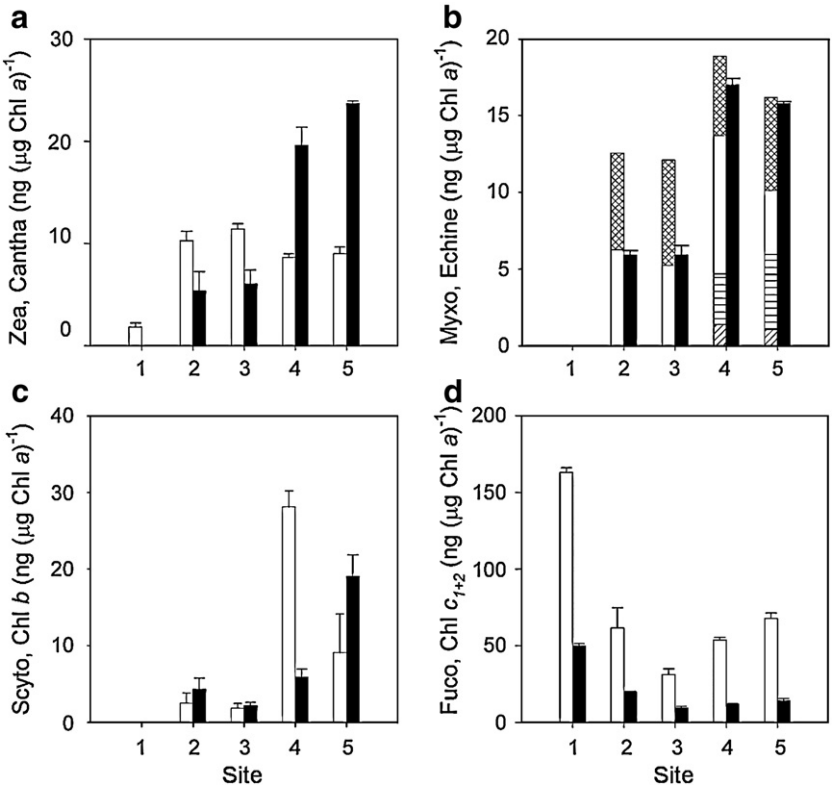

Fig. 2. Relative abundance (normalized to Chl *a*) of several important pigments in the five mat types. a. Zeaxanthin (Zea, white bars) and Canthaxanthin (Cantha, black bars), b. Myxoxanthophyyl (Myxo, white bars, the various myxoxanthophylls in the samples are indicated by the different patterns) and echineneone (Echine, black bars). c. Scytonema (Scyto, white bars) and chlorophyll *b* (Chl *b*, black bars). d. Fucoxanthin (Fuco, white bars) and chlorophyll c_{1+2} (Chl c_{1+2}, black bars). Absolute values for Chl *a* are listed in Table 3.

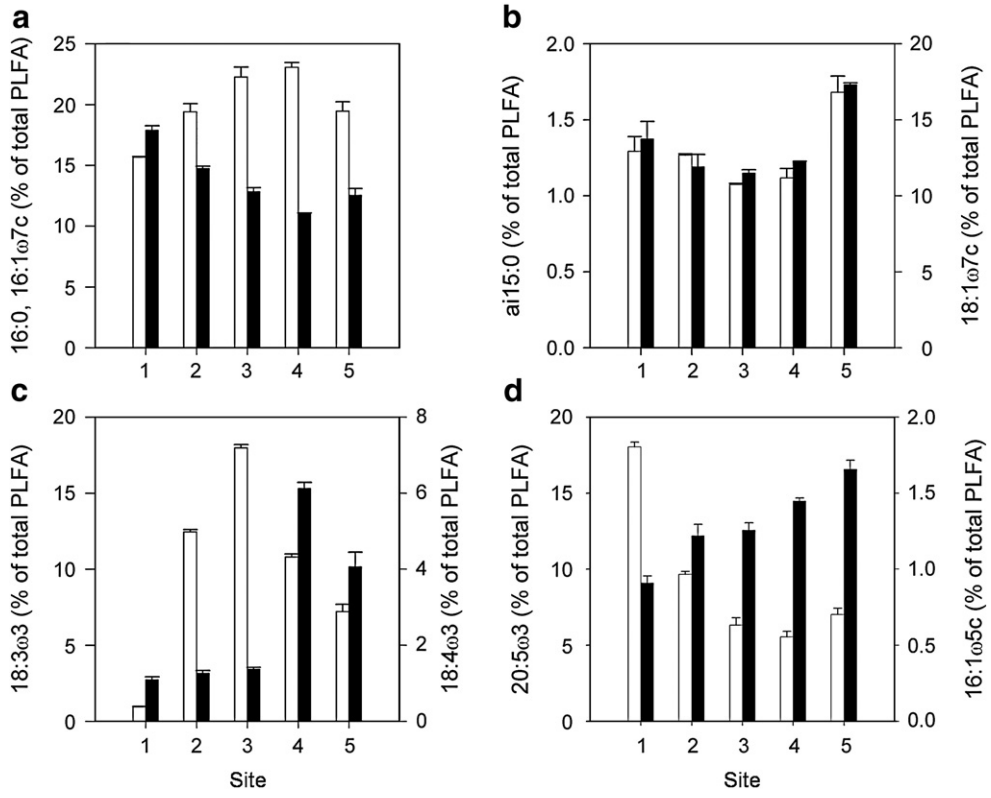

Fig. 3. Relative abundance (weight % of total PLFA) of several important PLFA in the five mat types. a. 16:0 (white bars) and 16:1ω7c (black bars) b. ai15:0 (white bars) and 18:1ω7c (black bars) c. 18:3ω3 (white bars) and 18:4ω3 (black bars) d. 20:5ω3 (white bars) and 16:1ω5c (black bars). Note the different scales on the *Y* axis. Absolute values for total PLFA are listed in Table 3.

Diadinoxanthin, another pigment typically found in diatoms, followed the same pattern (data not shown).

Our focus was on the oxygenic phototrophic community. Nevertheless, the HPLC chromatograms were checked for the presence of bacteriochlorophylls, as anaerobic phototrophic bacteria are a likely component of the mats. Bacteriochlorophylls were below the detection limit.

3.5. PLFA composition of the microbial mat

A large number of different PLFA were detected in the mats. In all samples, the same PLFA were present, but their relative abundance varied considerably among the sampling sites. The abundance of 16:0 varied between 15 to 23% of total PLFA. This PLFA increased from site 1 to site 4 and was slightly lower at site 5 (Fig. 3a). 18:2ω6c showed similar dynamics although with a much lower abundance of a maximum of 4.5% (data not shown). The content of 16:1ω7c varied between 11 and 18% and behaved opposite to 16:0 (Fig. 3a). 16:0 and 16:1ω7 were found in all taxa relevant to the microbial mat, but their relative abundances varied considerably (Table 2B). The ratio of 16:1ω7c over 16:0, which varied from a maximum of 1.1 at site 1 to a minimum of 0.5 at site 4, is quick indication for the relative abundances of diatoms and cyanobacteria, because this ratio is generally higher in diatoms (~1) than in cyanobacteria (~0.1–0.5).

The abundance of ai15:0 decreased from site 1 to site 4 and increased to a maximum of 1.7% at site 5 (Fig. 3b). i15:0 and i16:0 followed similar dynamics (data not shown). These branched fatty acids are characteristic for the domain Bacteria (e.g. Kaneda, 1991), but are absent from cyanobacteria. For instance, sulfate-reducing bacteria contain large amounts of branched fatty acids (Grimalt et al., 1992). In the mats, the abundance of 18:1ω7c was approximately 8 times higher than the abundance of ai15:0, but the pattern of variation between the categories was similar to ai15:0 (Fig. 3b). 18:1ω7c has been found in high amounts in purple sulfur bacteria (Grimalt et al., 1992), as well as in many other Gram-negative bacteria (Ratledge and Wilkinson, 1988). However, small amounts are also present in cyanobacteria and diatoms (Table 2). The relative abundance of 18:1ω7c followed the same pattern as PLFA unique to bacteria, which was different from the variations observed for PLFA originating in diatoms or cyanobacteria (see below), suggesting that in our samples 18:1ω7c was mainly derived from bacteria.

18:3ω3 contributed only 1% to total PLFA at site 1, but was much more abundant at the other sites. The highest abundance of 18:3ω3 was 13% and 18% of total PLFA at the sites 2 and 3, respectively (Fig. 3c). Diatoms contain only a small fraction of 18:3ω3, and therefore most of this PLFA in the mat types 2–5 probably originated from cyanobacteria. 18:4ω3 constituted an almost identical fraction of 3.5% in the sites 1, 2 and 3, but was 3 to 5 times higher at the sites 4 and 5 (Fig. 3c). 18:4ω3 is present in both diatoms and cyanobacteria, but is more important in the latter.

Diatoms usually contain a large amount of 20:5ω3. The highest abundance of this PLFA was measured at site 1 and amounted 18% of total PLFA. 20:5ω3 decreased with increasing distance from the waterline to as low as 5% of total PLFA at site 4. It was slightly higher at site 5 (Fig. 3d). This pattern of variations reflected that of the diatom biomarker fucoxanthin (Fig. 2b). 16:2ω4, 16:3ω4, 20:4ω6 and 22:6ω3 all showed similar variations to 20:5ω3, but were less abundant (data not shown). These PLFA all occur in diatoms.

16:1ω5c increased from around 0.9% at site 1 to 1.6% at site 5 (Fig. 3d). Although 16:1ω5c was not detected in any of our cyanobacterial strains, it has been reported in several cyanobacteria (Gugger et al., 2002). This PLFA is also common in many other bacteria (Ratledge and Wilkinson, 1988). A low contribution of 16:3ω3 was detected at site 2 to 5 (<1%, data not shown). The fraction of 16:4ω3 was even lower and more variable. 16:3ω3 and 16:4ω3 are typical for green algae (Dijkman and Kromkamp, 2006) but may also occur in some cyanobacteria (Table 2B).

3.6. Group abundance in the microbial mats

To obtain an estimation of the contribution of each taxonomic group to biomass we used the matrix factorization program CHEMTAX (Mackey et al., 1996). This program requires a data file containing the sample data and an input ratio file containing the biomarker composition of the taxonomic groups present in the samples. The program was designed to use pigments as biomarkers, but as suggested by Mackey et al. (1996) PLFA can also be used as biomarkers (Dijkman and Kromkamp, 2006). The groups taken into account in our analysis were bacteria, diatoms and three groups of cyanobacteria. Group 1 contained cyanobacteria without PUFA like the strain of *Synechococcus* sp. that we analyzed. Group 2 comprised cyanobacteria with 18:3ω3 as the longest, most unsaturated PLFA (e.g. *Microcoleus* and *Lyngbya*), and the cyanobacteria of group 3 possessed both 18:3ω3 and 18:4ω3 (e.g. *Calothrix* and *Nodularia*). The PLFA composition for the three cyanobacterial groups and the diatoms were calculated from the data shown in Table 2. The group 'bacteria' comprised all bacteria other than cyanobacteria. The PLFA composition for this group was taken from (Grimalt et al., 1992). These authors studied the depth distribution of fatty acids in *Phormidium* and *Microcoleus* mats. The data used for the input ratio file was the fatty acid composition between 5.5 and 20 mm depth, where living cyanobacteria were absent. Table 4 shows the input file with the PLFA ratios of the taxa considered in the analysis.

The results of the CHEMTAX analysis are presented as the contribution of each taxonomic group to 16:0 (Fig. 4). 16:0 was chosen because this PLFA is present in all taxonomic groups. Bacteria contributed between 11 and 18% of 16:0, with the lowest contribution occurring in type 3 and 4 mats. Diatoms dominated in mat type 1 where they contributed 74% of 16:0, but were also an important fraction in mat types 2–5 where this group contributed between 23 and 37%. Cyanobacteria of group 1 were a minor group in most mat types with abundance varying between 0 and 7%. Cyanobacteria of group 2 were a minor component of the microbial community in mat type 1. This group contributed between 41 and 56% of 16:0 in mat types 2 and 3 respectively and around 20% in mat type 4 and 5. Cyanobacteria of group 3 contributed 4 and 6% in type 2 and 3 mats, respectively and made a major contribution of 43 and 30% of 16:0 type 4 and 5 mats respectively.

Green algae were omitted from the analysis, since their PLFA composition resembles the PLFA composition of cyanobacteria too closely to add them as a separate group. *Enteromorpha* sp., which was the filamentous green algae species seen in low numbers in most samples, contains among others the fatty acids 16:0, 18:2ω6, 18:3ω3

Table 4
CHEMTAX input file used to estimate group abundance from the PLFA data of the microbial mat.

PLFA	Cyanobacteria group 1	Cyanobacteria group 2	Cyanobacteria group 3	Diatoms	Bacteria
i15:0	0	0	0	0	0.354
ai15:0	0	0	0	0	0.270
15:0	0	0	0	0.033	0.064
i16:0	0	0	0	0	0.198
16:0	1	1	1	1	1
16:1ω7c	2.534	0.391	0.232	1.070	0.235
18:1ω9c	0.430	0.077	0.025	0.104	0.672
18:1ω7c	0.250	0.070	0.081	0.006	0.890
18:3ω6	0	0	0.028	0.204	0
18:3ω3	0	1.404	0.339	0.012	0
18:4ω3	0	0	0.564	0.190	0
20:4ω6	0	0	0	0.293	0
20:5ω3	0	0	0	1.228	0
22:6ω3	0	0	0	0.122	0

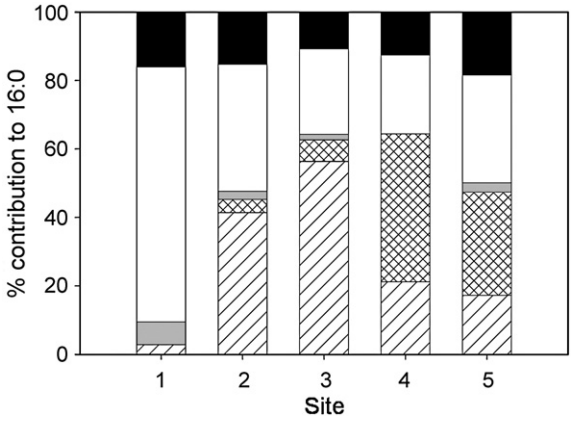

Fig. 4. Group abundance in the five mat types, estimated from the PLFA composition using the matrix factorization program CHEMTAX. Grey: group 1 cyanobacteria, hatched: group 2 cyanobacteria, cross-hatched: group 3 cyanobacteria, white: diatoms, black: bacteria other than cyanobacteria. Absolute values for 16:0 were 3.1, 5.4, 10.8, 22.5 and 22.6 (μg g^{-1} sediment) in the mat types 1–5 respectively.

and 18:4ω3 (Wahbeh, 1997). Hence, the PLFA composition of this species resembles the PLFA composition of group 3 cyanobacteria and will be included in this group in the group abundance derived using CHEMTAX. The low content of Chlorophyll *b* indicates that the error introduced by this is small.

4. Discussion

4.1. Pigments and PLFA as biomarkers

Pigments are the most used biomarkers for oxygenic phototrophs, but many groups of oxygenic phototrophs can be differentiated by their PLFA composition as well. PLFA are present in all eukaryotes and bacteria, so information on both pigmented and non-pigmented taxa can be derived. This was an advantage in the microbial mats that we studied, since bacteria, both heterotrophic and autotrophic, are an important component of the system. Simultaneously, the fact that PLFA can originate in so many different organisms obviously complicates the interpretation of the data and a careful assessment of all species present in the samples is required.

Diatoms display a typical pigment- and PLFA profile, with variations usually limited to changes in the relative abundances of pigments and PLFA rather than their absence or presence (Table 2; Gugger et al., 2002; Dijkman and Kromkamp, 2006; Jeffrey and Wright, 2006). In contrast, within the cyanobacteria different pigment and PLFA types are found.

Large differences were observed in the carotenoid composition between cyanobacterial species. In addition to species-specific differences, variations may be caused by environmental conditions and stress such as high irradiance, UV-radiation or desiccation (e.g Potts et al., 1987; Ehling-Schulz et al., 1997). Sheath pigments such as scytonemin are also synthesized in response to UV-radiation (Ehling-Schulz et al., 1997). The high abundance of scytonemin in mat type 4 coincided with the observation of colored sheaths under the microscope. Variations in pigment composition among species as well as in response to changes in environmental conditions are known for eukaryote algae as well. However, the large differences between species grown under identical conditions, such as the seven-fold difference in zeaxanthin Chl a^{-1} ratio observed between cyanobacterial strains, are rare in eukaryotes.

The high variability in carotenoid composition in cyanobacteria is probably related to the function of the carotenoids in the cell. Cyanobacteria mainly rely on water-soluble phycobiliproteins for light harvesting. Many carotenoids are present in the cellular membranes and serve a photoprotective function rather than being involved in light harvesting (Hirschberg and Chamowitz, 1994), which probably allows for more flexibility in the relative pigment abundance. Some carotenoids are essential for normal cell wall structure and thylakoid organization (Mohamed et al., 2005).

Zeaxanthin is often used as the biomarker pigment for cyanobacteria (e.g. Pinckney et al., 1995; Jeffrey and Wright, 2006). However, many strains typically found in microbial mats were found to lack zeaxanthin (Table 2A; Potts et al., 1987). Hence, applying only zeaxanthin as biomarker for cyanobacteria will obviously underestimate them. The strains tested in this study contained either zeaxanthin or canthaxanthin, but there are also reports on cyanobacteria that contain both (e.g. Hertzberg et al., 1971; Hirschberg and Chamowitz, 1994; Schagerl and Donabaum, 2003). For example, *Nostoc* sp. was reported to contain both zeaxanthin and canthaxanthin by Schagerl and Donabaum (2003) whereas we detected only canthaxanthin in our strain *Nostoc* sp.. Canthaxanthin was found in all heterocystous cyanobacteria that were tested, but more strains need to be analyzed before it can be concluded that this is a general phenomenon for this group.

Cyanobacteria have been classified into five groups based on their capability to synthesize certain PUFA (Kenyon, 1972; Kenyon et al., 1972; Wood, 1974; Cohen et al., 1995; Gugger et al., 2002), three of which were detected in the microbial mats on Schiermonnikoog. These five groups unfortunately do not represent phylogenetic coherent taxa. They are, however, well documented in literature (see references above) and are referred to as the Kenyon–Murata classification system (Gugger et al., 2002). The ability of cyanobacteria to synthesis either 18:3ω3 or 18:3ω6, or both, in which they can also synthesize 18:4ω3, is determined by the presence of specific enzymes (Cohen et al., 1995) and is thus not merely due to a response to changes in environmental parameters. The PLFA composition within a group varied much less than was the case with the carotenoid composition of cyanobacteria.

The fatty acid composition of microorganisms is known to vary in response to e.g. temperature changes (Sushchik et al., 2003; Teoh et al., 2004), irradiance conditions (Brown et al., 1996) or with growth phase (Brown et al., 1996; Gugger et al., 2002). However, in most cases the changes are limited to variations in relative abundance and do not change the main characteristics of the various groups. Some species that are capable of accumulating large amounts of triglycerols are storage lipids which may lead to large changes in the total fatty acid composition and abundance (Piorreck et al., 1983). As mentioned in the introduction, using PLFA instead of total fatty acids, minimizes this type of variation. It should be noted that this is less of a concern with cyanobacteria, as these do not tend to accumulate storage lipids in the form of triacylglycerols (Hu et al., 2008).

4.2. Estimating group abundance from biomarker composition

Biomarker abundance can be linked to species or taxon abundance as done in the Results section. Estimating relative group abundance from the data is an additional step. As most PLFA (and pigments as well) are not unique to a single group, the whole biomarker pattern of each group has to be used as opposed to single biomarkers. CHEMTAX was specifically designed for this purpose (Mackey et al., 1996). It should also be noted that, as most PLFA are not unique to a single group, microscopic analysis will remain very important as a means to determine which species are present and are the origin of specific PLFA.

Analysis of the PLFA data using the software program CHEMTAX provided an estimation of the relative abundance of the groups present in the microbial mat. Varying the PLFA composition of the groups in the input ratio file within reasonable limits (i.e. within the range of variation found in culture data and literature data) had little effect on the estimated group abundance. The variations in pigment composition and occurrence of species agreed well with the group abundance derived from the PLFA composition. For instance, the estimated abundance of

group 2 cyanobacteria matched the observations of *Microcoleus* sp. and *Lyngbya* sp. and the presence of zeaxanthin.

Despite the observed agreement between variations in pigments and PLFA composition, estimating group abundance from the pigment composition did not lead to reliable results due to the large variations in pigment ratios between the various cyanobacterial species. Manipulation of the ratios of canthaxanthin Chl a^{-1} and zeaxanthin Chl a^{-1} in the input ratio file well within the range found in individual species led to major changes in the relative group composition as estimated with CHEMTAX.

Groups without unique biomarkers, such as cyanobacteria group 1, can be recognized using CHEMTAX (Schlüter and Møhlenberg, 2003), although it should be noted that the estimated values for such groups are less reliable than for those containing unique biomarkers. We also ran the analysis omitting cyanobacteria group 1 as a separate group. In this case, this cyanobacterial group appeared to be included in the group 'bacteria' and very little change in the relative abundance of the other groups was observed.

The combined group 'bacteria' included a wide range of species, which can have a widely varying fatty acid composition (Ratledge and Wilkinson, 1988). The PLFA composition of this group is not easily composed from data on individual species, and we used literature data from the deeper layers of microbial mats (Grimalt et al., 1992). On one hand this can be expected to induce bias, as the biomarker composition was based on data from a hypersaline mat and total fatty acids, whereas we studied the PLFA composition of mats on coastal sandy beaches. On the other hand, a comparison with the fatty acid composition of other microbial communities such as marine sediments or the microbial community in drinking water shows that the variation in fatty acid composition of such bacterial communities is limited (see Dijkman and Kromkamp, 2006). Moreover, during the fitting procedure, the PLFA composition of each group is adapted to best fit the data, which is one of the advantages of using CHEMTAX over conversion algorithms based on single biomarkers (Mackey et al., 1996).

4.3. Composition of the microbial mats of Schiermonnikoog

The PLFA composition of the microbial mat of Schiermonnikoog is in general consistent with the total fatty acid composition measured on several other cyanobacterial mats (Grimalt et al., 1992; Wieland et al., 2003; de Oteyza et al., 2004). Exceptions are 18:3ω3, which was not previously found, and 18:4ω3, which was only reported once (Wieland et al., 2003). These PLFA are present in most filamentous cyanobacteria that are common in microbial mats (Table 2, Gugger et al., 2002) and 18:4ω3 is an important PLFA in diatoms (Volkman et al., 1989; Dijkman and Kromkamp, 2006). Therefore, their presence is expected and it is odd that they haven't been reported in other studies. This might have been due to the chromatographic method used. The fatty acids 18:3ω3 and 18:4ω3 are poorly separated from other PLFA with a chain length of 18 carbon atoms when using a non-polar chromatographic column. We used a polar column that separates polyunsaturated fatty acids such as 18:3ω3 and 18:4ω3 very well.

Microbial mats on sandy intertidal sediments are usually dominated by cyanobacteria. However, as revealed from pigment- and PLFA composition, diatoms made a considerable contribution to the biomass in the Schiermonnikoog mats. This was also found by Pinckney et al. (1995) who observed a seasonal variation in diatom abundance in a microbial mat, with the highest diatom abundance during winter. Our measurements were done during late spring/early summer and, hence, we expect that the diatoms would decrease in importance later in the season. Moreover, severe rain showers occurred a few days prior to our sampling. The rain fully flooded the beach, which might have favored growth of the diatoms.

The relative abundance of bacteria (other than cyanobacteria) was relatively constant among the mat types. This seemed somewhat surprising since the presence of bacteria was much more conspicuous in the higher mat types than in the low mat types due to the black sediment below the former, the black color being the result of the activity of sulfate-reducing bacteria. This bacterial abundance was, however, strongly supported by bacterial markers (i15:0, ai15:0, i16:0 and 18:1ω7c). Moreover, the results were reported as relative abundance. As biomass increased from low to high mat type, absolute numbers of bacteria did increase with mat type.

The ratio PLFA:Chl *a* ratio (w/w) varies between 1 and 2.5 in cultures grown under identical conditions and does not show a systematic difference between diatoms and cyanobacteria (unpublished data). The relative abundance of bacteria remained more or less constant among the mat types (see Fig. 4). Hence, the change in PLFA/Chl *a* as observed in the field samples is probably not caused by a change in species composition. More likely, Chl *a* increased due to photoacclimation. Algae and cyanobacteria are known to respond to low light conditions by increasing Chl *a* content on a per cell basis (Post et al., 1985; Müller et al., 1993) and light attenuation will be higher in dense, mature mats than in the loose mats close to the waterline.

The C:N ratios were well above the Redfield ratio of 6.6 in all mat types. These high ratios indicate a considerable contribution of extracellular carbon in all mat types, which is well known for microbial mats. These ecosystems are usually strongly nutrient limited, although in most microbial mats N_2 fixation might alleviate nitrogen limitation. Many mat organisms, cyanobacteria as well as other bacteria and archaea, are capable of N_2 fixation (Omoregie et al., 2004). The mat types 4 and 5 are higher in biomass and presumably older than the mat types 1–3. The decreased C:N ratios in the mat types 4 and 5 are likely the result of the high rates of N_2 fixation that were measured in these mats (unpublished data).

The mat types in the present study were defined based on the macroscopic structure of the mat. A larger survey indicated that the change in mat type from waterline to the edge of the dunes was a general trend. Microscopic observation, pigment data and PLFA data all show a change in community composition of the mats from the waterline to the edge of the dunes, and not just an increase in biomass. Nutrient concentrations in the pore water did not show a consistent relation to the position on the beach (A. Ernst et al., unpublished data). This leaves desiccation time, which increased from the water line to the edge of the dunes, and pore water salinity, which decreased from the water line to the edge of the dunes, as the two environmental variables most likely determining the composition of the mats. Rothrock and Garcia-Pichel (2005) found that desiccation time is an important factor shaping the community structure along the tidal gradient on an intertidal sand flat microbial mat. We can not exclude, however, that the different types of mats represent in fact different stages of development. Further study is required to investigate the perennial development of the microbial mats under a given environmental setting. We have shown here that a detailed analysis of the pigment and PLFA biomarkers, accompanied by microscopic observations, provides sufficient resolution for such studies.

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