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Long chain glycolipids with pentose head groups as biomarkers for marine endosymbiotic heterocystous cyanobacteria

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ABSTRACT

Marine endosymbiotic heterocystous cyanobacteria make unique heterocyst glycolipids (HGs) containing pentose (C_5) moieties. Functionally similar HGs with hexose (C_6) moieties found in free-living cyanobacteria occur in the sedimentary record, but C_5 HGs have not been documented in the natural environment. Here we developed a high performance liquid chromatography multiple reaction monitoring (MRM) mass spectrometry (HPLC–MS^2) method specific for trace analysis of long chain C_5 HGs and applied it to cultures of *Rhizosolenia clevei* Ostenfeld and its symbiont *Richelia intracellularis* which were found to contain C_5 HGs and no C_6 HGs. The method was then applied to suspended particulate matter (SPM) and surface sediment from the Amazon plume region known to harbor marine diatoms carrying heterocystous cyanobacteria as endosymbionts. C_5 HGs were detected in both marine SPM and surface sediments, but not in SPM or surface sediment from freshwater settings in the Amazon basin. Rather, the latter contained C_6 HGs, established biomarkers for free-living heterocystous cyanobacteria. Our results indicate that the C_5 HGs may be potential biomarkers for marine endosymbiotic heterocystous cyanobacteria.

1. Introduction

Cyanobacteria are cosmopolitan oxygenic photoautotrophs that play an important role in the global C and N cycles. Marine cyanobacteria are the major fixers of N_2 in modern tropical and subtropical oligotrophic oceans (Karl et al., 1997; Lee et al., 2002). Because N_2 fixation is sensitive to O_2, cyanobacteria have evolved a range of different strategies in order to combine the incompatible processes of oxygenic photosynthesis and N_2 fixation. One strategy found only in filamentous cyanobacteria is to fix N_2 in differentiated cells known as heterocysts (Wolk, 1973; Rippka et al., 1979). Heterocystous cyanobacteria can be the major source of N in eutrophic lakes and play a role in other freshwater and estuarine systems (Howarth et al., 1988). Free-living heterocystous cyanobacteria are rare in the open sea (Staal et al., 2003), but it recently has become evident that heterocystous taxa are abundant as endosymbionts in diatoms (Villareal, 1991, 2011, 2012; Foster et al., 2011; Luo et al., 2012). These heterocystous cyanobacteria provide the diatom with fixed N_2 (Foster et al., 2011) and can fully support the N needs of both host and symbiont (Villareal, 1990). This N subsidy, which explains the presence of these diatoms in low nutrient environments (Venrick, 1974) such as the tropical southwest North Atlantic Ocean where the symbiotic association produces nearly 70% of total N demand in the surface water (Carpenter et al., 1999).

In all free-living cyanobacteria, the heterocyst cell walls contain glycolipids (Nichols and Wood, 1968; Abreu-Grobois et al., 1977; Gambacorta et al., 1995; Bauersachs et al., 2009a). These heterocyst glycolipids (HGs) comprise a C_6 sugar head group glycosidically bound to long chain diols, triols, or hydroxyketones (**I–VI**; Fig. 1; Bryce et al., 1972; Gambacorta et al., 1998; Bauersachs et al., 2009b, 2011). Studies have found that C_6 HGs are not only biomarkers for heterocystous cyanobacteria and the N fixation process, but show structural diversity, depending on the family level within the divisions (Bauersachs et al., 2009a, 2014). The C_6 HGs are nearly exclusively from freshwater, free-living cyanobacteria. In contrast, the heterocystous cyanobacterium *Richelia intracellularis*, which lives endosymbiotically within the marine diatoms *Hemiaulus hauckii* and *Hemiaulus membranaceus* (Villareal, 1991)

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contained novel HGs with a C_5 sugar head group rather than a C_6 sugar (**VII–IX**; Fig. 1; Schouten et al., 2013). It was speculated (Schouten et al., 2013) that this might be an adaptation to the high O_2 concentration within their diatom host (Walsby, 1985). The only other report of HGs with a C_5 sugar moiety is that of Woermer et al. (2012), who tentatively assigned a compound with a C_5 head group and a shorter C_{26} carbon chain in a culture of the freshwater cyanobacterium *Aphanizomenon ovalisporum* UAM 290 and in suspended particulate matter (SPM) from three freshwater environments in Spain.

In contrast to the established paradigm that lipids with polar head groups are labile compounds which degrade upon cell death (White et al., 1979), C_6 HGs have been found to be preserved in ancient sediments of up to 49 Ma old (Bauersachs et al., 2010). Thus, they have been applied as unique biomarkers for N_2 fixation by heterocystous cyanobacteria in both present and past settings, such as microbial mats (Bauersachs et al., 2009b, 2011; Woermer et al., 2012; Bühring et al., 2014), freshwater lakes (Woermer et al., 2012), Pleistocene sediments from the eastern Mediterranean Sea and Eocene Arctic sediments (Bauersachs et al., 2010). However, it is not known whether C_5 HGs could be used as biomarker lipids in the same manner as C_6 HGs, as they have been only reported in two cultures and not in the natural environment.

In this study, we have developed a specific high performance liquid chromatography/multistage mass spectrometry (HPLC–MS^2) multiple reaction monitoring (MRM) method for trace analysis of long chain C_6 HGs to include analysis of C_5 HGs and carried out further screening of endosymbiotic cultures for the presence of these compounds. The method was then applied to suspended particulate matter (SPM) and surface sediments from the Amazon plume region in the southwest North Atlantic Ocean, which is known to host heterocystous cyanobacteria as endosymbionts with marine diatoms (Carpenter et al., 1999; Foster et al., 2007; Subramaniam et al., 2008). Examination of these and freshwater samples from the Amazon region allowed us to test the hypothesis that long chain C_5 HGs are specific biomarkers for endosymbiotic heterocystous cyanobacteria in the natural environment.

2. Methods

2.1. Culturing of diatoms containing endosymbionts

Rhizosolenia clevei Ostenfeld and its symbiont *R. intracellularis* Lemmerman were isolated from the coastal waters off Port Aransas, Texas in October 2010. Two strains were grown in MET-44 medium (Schöne and Schöne, 2009) as modified by Villareal (1990). Cells were grown in filter sterilized medium at 25 °C, 110 µmol quanta/m^2/s on a 12:12 light:dark cycle. Successive 2–4 l cultures were concentrated by sedimentation and centrifugation, the supernatant discarded, the resulting pellets frozen at −20 °C, and then shipped for analysis. In some cases, the culture was directly filtered onto a 10 µm polycarbonate filter, frozen and shipped. The host cells progressively decreased in diameter over the course of ca. 2 yr [MacDonald-Pfitzer rule MacDonald (1869), Pfitzer (1869)] and, after failing to produce auxospores, became moribund and died. The draft genome was reported by Hilton et al. (2013), NCBI accession number PRJEA104979.

2.2. Collection of environmental samples

Marine SPM and surface sediments from the Amazon shelf and slope encompassing the Amazon plume region were sampled on board of the R/V Knorr 197-4 between February and March 2010 (Table 1 and Fig. 2b; for details see Zell et al., 2014). SPM from the chlorophyll maximum was filtered on 0.7 µm glass fiber (GF) filters with an in situ pump. Sediment cores were collected using a box corer from which the top 1 cm was sub-sampled. In addition, surface sediments ($n = 6$) were collected using a grab sampler of

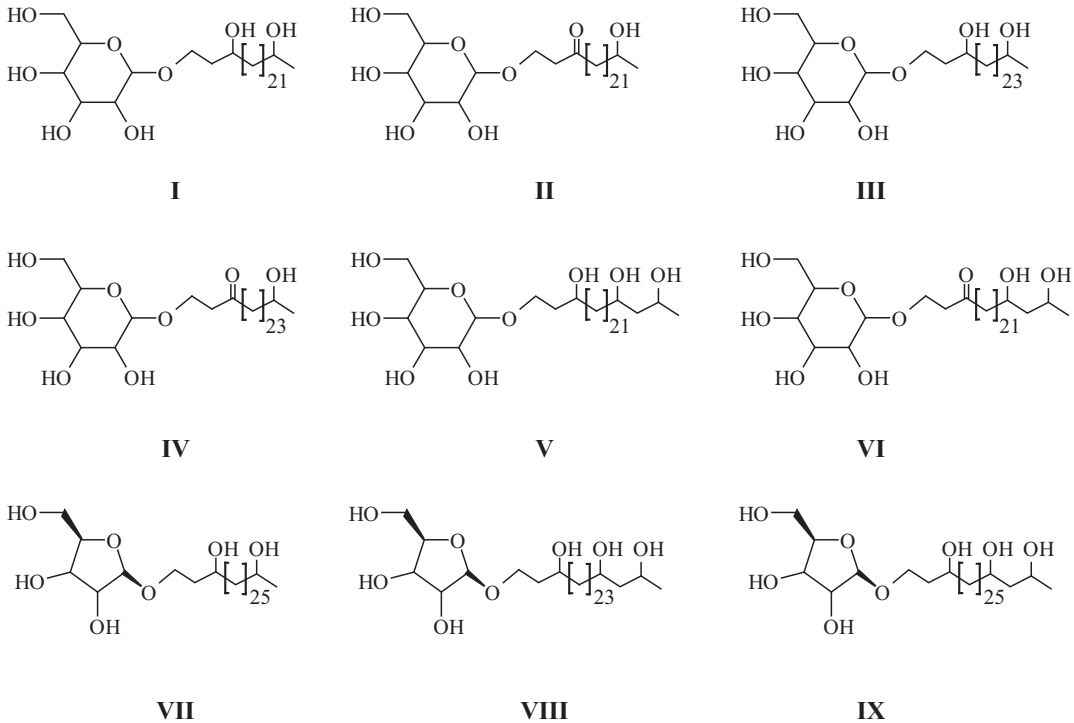

Fig. 1. Structures of heterocyst glycolipids. C_6 glycolipids: I 1-(O-hexose)-3,25-hexacosanediol; **II**, 1-(O-hexose)-3-keto-25-hexacosanol; **III**, 1-(O-hexose)-3,27-octacosanediol; **IV**, 1-(O-hexose)-3-keto-27-octacosanol; **V**, 1-(O-hexose)-3,25,27-octacosanetriol; **VI**, 1-(O-hexose)-3-keto-25,27-octacosanediol. C_5 glycolipids: **VII**, 1-(O-ribose)-3,29-triacontanediol; **VIII**, 1-(O-ribose)-3,29,31-dotriacontanetriol; **IX**, 1-(O-ribose)-3,27,29-triacontanetriol.

Table 1
Marine SPM and sediment samples and general properties (adapted from Zell et al., 2014).

	Sampling date (dd/mm/yy)	Longitude	Latitude	Sampling water depth (m)	Water temperature (°C)	TOC (wt%)
Marine SPM						
13	23/02/10	−48.61	4.46	85	27.2	0.9
20	24/02/10	−48.35	4.04	75	27.5	5.2
21	25/02/10	−48.54	3.96	85	27.8	6.0
22	25/02/10	−48.61	3.95	89	27.7	16.3
23	25/02/10	−49.06	3.68	75	27.9	13.7
25	26/02/10	−49.86	3.19	5	28.7	4.3
28	27/02/10	−48.17	3.78	100	27.4	0.5
32b	28/02/10	−47.64	3.07	100	26.3	1.4
42b	02/03/10	−47.74	2.85	90	27.0	1.4
42d	03/03/10	−47.85	2.57	60	26.9	0.8
43	03/03/10	−48.05	2.09	10	28.3	0.9
Marine surface sediments						
13	23/02/10	−48.61	4.46	1711	–	0.9
20	24/02/10	−48.35	4.04	1088	–	1.1
21	25/02/10	−48.54	3.96	738	–	1.1
22	25/02/10	−48.61	3.95	640	–	0.7
23	25/02/10	−49.06	3.68	107	–	0.6
25	26/02/10	−49.86	3.19	32	–	0.7
28	27/02/10	−48.17	3.78	1014	–	1.0
32b	28/02/10	−47.64	3.07	1028	–	0.7
32c	02/03/10	−47.31	3.65	2079	–	1.1
42b	02/03/10	−47.74	2.85	609	–	0.8
42d	03/03/10	−47.85	2.57	110	–	0.1
43	03/03/10	−48.05	2.09	65	–	0.0
44c	04/03/10	−46.25	3.39	3375	–	1.1
49	04/03/10	−45.36	1.64	2962	–	1.4
54	07/03/10	−44.35	0.66	2372	–	0.3
60	10/03/10	−42.74	−1.03	3113	–	1.0

100 cm^3 in the lakes Cabaliana, Janauaca, Marituba, Canaçari and Curuai in the low water season (October, 2009). These floodplain lakes are in the central Amazon basin between Manacapuru and Santarém (Table 2 and Fig. 2c; for details see Mortillaro et al., 2011; Zell et al., 2013b). SPM sampling was also conducted at Óbidos, the most downstream gauging station in the Amazon River in the rising water season (February, 2011; Table 2 and Fig. 2c; for details see Zell et al., 2013a). All samples were kept frozen (−20 °C) during the sampling campaign and transported frozen to NIOZ, where they were freeze-dried for analysis.

2.3. Sample extraction

Extraction of freeze dried biomass, GF filters or sediment was carried out using a modified Bligh–Dyer extraction (Pitcher et al., 2011). The samples were extracted in an ultrasonic bath for 10 min with a known volume of MeOH:dichloromethane (DCM):phosphate buffer (2:1:0.8, v/v/v). After centrifugation (1000×*g*, 5 min) to separate the extract and residue, the solvent was collected in a separate flask. This was repeated 3× before DCM and phosphate buffer were added to induce phase separation, producing a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9, v/v/v). After centrifugation (1000×*g*, 5 min), the DCM phase was collected in a round bottom flask and the remaining MeOH:phosphate buffer phase was washed two additional times with DCM. Rotary evaporation was used to reduce the combined DCM phase before it was evaporated to dryness under a stream of N_2. Before analysis, the extract was re-dissolved in hexane, isopropanol and water (72:27:1, v/v/v) and aliquots were filtered through 0.45 μm mesh True Regenerated Cellulose syringe filters (4 mm diameter; Grace Alltech).

2.4. MRM method development

Analysis was accomplished using the same HPLC–MS system fitted with the same type of column as described by Bauersachs et al. (2010). The linear gradient, eluent composition and source settings were as reported by Bauersachs et al.(2010). Injection volume ranged from 1 μl for extracts of cultures to 10 μl for extracts of sediments.

In order to further develop the HPLC–MS^2 MRM method, designed for analysis of six C_6 HGs (Bauersachs et al., 2010), to include analysis of C_5 HGs, we first carried out direct infusion experiments with *H. hauckii* culture extracts, which had been described to contain three C_5 HGs (**VII**–**IX**; Schouten et al., 2013). A concentrated *H. hauckii* BD extract was infused continuously at 10 μl/min into a 0.2 ml/minstream of mobile phase (60% A, 40% B) using source conditions as per Bauersachs et al. (2010). HPLC–ESI-MS^2 was performed in product ion mode with 1.5 mTorr Ar as collision gas. For each individual C_5 HG, four diagnostic product ions were selected and the optimal collision energy for maximal abundance was determined for each parent-product transition, resulting in the settings reported in Table 3. The three C_5 HGs eluted at retention times < 15 min, whereas the C_6 HGs (**I**–**VI**) detected, using the MRM transitions described by Bauersachs et al.(2010), all eluted > 15 min (Fig. 3). Hence, we combined the two MRM methods into a single method with two segments, the C_5 HG segment from 0–15 min, followed by the C_6 HG segment from 15–42 min (Fig. 3). Since we did not have an authentic HG standard, both the C_5 and the C_6 HGs were quantified as the integrated peak area response (response unit, r.u.)/gram organic carbon (OC) for both water and sediment samples. In order to check that consistency in response was maintained between analyses two reference samples were run at regular intervals. These were an extract from an *Azolla* culture, which contained the six C_6 HGs (Bauersachs et al., 2010) examined by the MRM method and an extract from a culture of *H. hauckii* containing the endosymbiont *R. intracellularis* which contained the three C_5 HGs (Schouten et al., 2013). Response of the C_5 and C_6 HGs in these reference samples remained consistent during the period of method development and sample analysis showing no loss of sensitivity. A sub-set of 14 of the environmental samples were analyzed 2× to examine reproducibility. The variation in response was of an acceptable level for IPL analysis and was on average 12%.

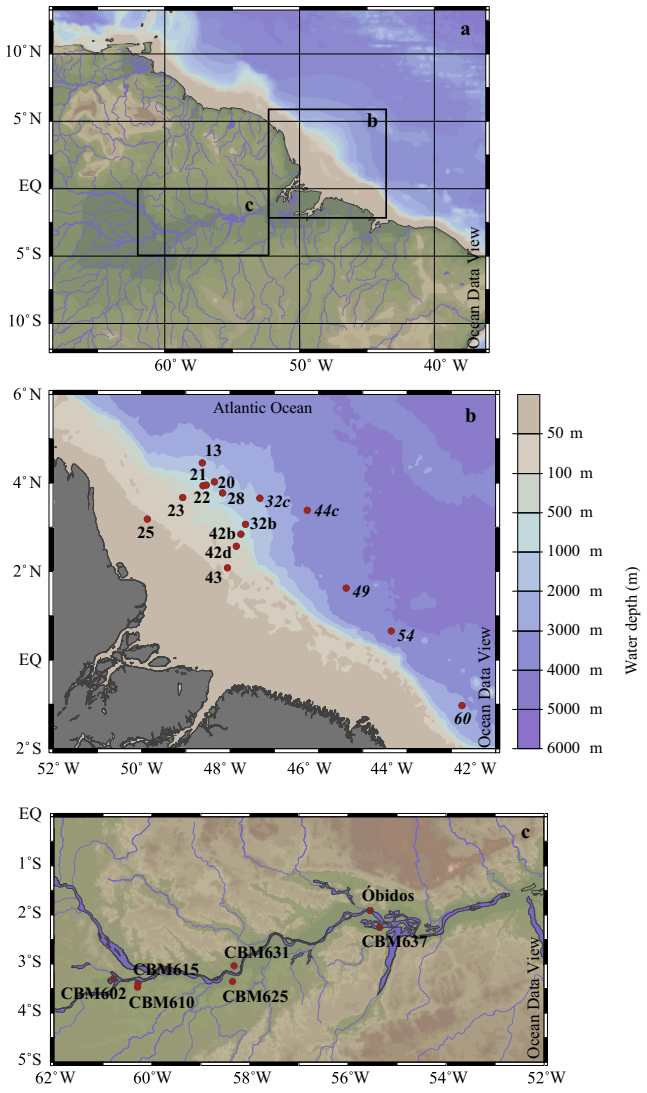

Fig. 2. (a) Overview map of study region. (b) Area of the Amazon plume with stations shown. (c) Map of Amazon River region with floodplain lakes and Óbidos sampling sites shown.

3. Results and discussion

3.1. Application of MRM method for analysis of C_5 HGs in heterocystous cyanobacterial cultures

To test the applicability of our method for analysis of heterocystous cyanobacterial cultures, we analyzed the HG composition of two strains of the marine diatom *R. clevei* containing the endosymbiotic heterocystous cyanobacterium *R. intracellularis* (Table 4). We could not detect any C_6 HGs in the *R. clevei–R. intracellularis* cultures, in agreement with a report for *R. intracellularis* in endosymbiosis with the marine diatoms *H. hauckii* and *H. membranaceus* (Schouten et al., 2013). However, using our MRM method we did detect the C_5 HG 1-(O-pentose)-3,27,29-triacontanetriol (**IX**) and relatively low amounts of 1-(O-ribose)-3,29,31-dotriacontanetriol (**VIII**). The C_5 HG, 1-(O-ribose)-3,29-triacontanediol (**VII**), previously detected in *H. hauckii* and *H. membranaceus*, was not detected in either strain of the *R. clevei–R. intracellularis* association.

The results provide further evidence that long-chain (30–32 carbons) HGs with a C_5 sugar head group are associated with marine endosymbiotic cyanobacteria. To date, they have been detected in 5 cultures of cyanobacterial endosymbionts that live within marine diatoms (Table 4) and not within any of the > 50 free-living cyanobacterial cultures analyzed (Bauersachs et al., 2009a,b, 2014; Woermer et al., 2012; Schouten et al., 2013). Although the symbionts in *Hemiaulus* and *Rhizosolenia* are described as the same taxon (*R. intracellularis*), molecular data suggest a high degree of host-symbiont specificity and support a taxonomic distinction between them (Foster and Zehr, 2006). In addition, the *Hemiaulus* symbiont also lacks a major N metabolism pathway found in the *Rhizosolenia* symbiont (Hilton et al., 2013; Hilton, 2014). The *Rhizosolenia* symbiont is also differentiated in that it lacks the C_5 HG, 1-(O-ribose)-3,29-triacontanediol (**VII**), noted in *Hemiaulus*.

This difference in head group between marine endosymbiotic cyanobacteria and free-living cyanobacteria is supported by the lack of a *hglT* gene – which encodes the glycosyltransferase responsible for glycosylation of the aglycone moiety of glycolipids in the draft genome of *R. intracellularis* (Hilton et al., 2013). Further work is necessary to find out the effect of different culturing conditions on the distribution of the C_5 HGs as a previous study found a temperature effect on the amount of ketone C_6 HGs relative to the

Table 3
Optimal collision energy for diagnostic parent-product transition for each C_5 HG.

C_5 glycolipid	$[M+H]^+$ *m/z*	Product *m/z*	Collision energy (V)
VII	603.6	417.4	18
		435.4	17
		453.4	14
		471.4	8
VIII	619.6	415.4	20
		433.4	20
		451.4	20
		487.4	11
IX	647.4	443.4	20
		461.4	19
		479.4	20
		515.4	11

Table 2
Amazon Varzea sediments and Amazon River SPM samples and their general properties.

	Sampling date (dd/mm/yy)	Longitude	Latitude	Water depth (m)	Temperature (°C)	TOC (wt%)
Amazon floodplain lake sediments						
CBM602Cabaliana	04/10/09	−60.780	−3.298	1.8	34.3	2.2
CBM610Janauacá	06/10/09	−60.289	−3.467	6.0	32.7	2.9
CBM615Janauacá	07/10/09	−60.283	−3.400	5.8	32.7	n.a.
CBM625Mirituba	11/10/09	−58.354	−3.352	3.0	30.1	1.6
CBM631Canaçari	13/10/09	−58.324	−3.032	6.1	30.9	1.7
CBM637Curuai	17/10/09	−55.351	−2.251	2.5	32.1	1.8
Amazon River SPM						
CBM8 Óbidos	01/02/11	−55.5535	−1.9115	Surface	28.7	1.0

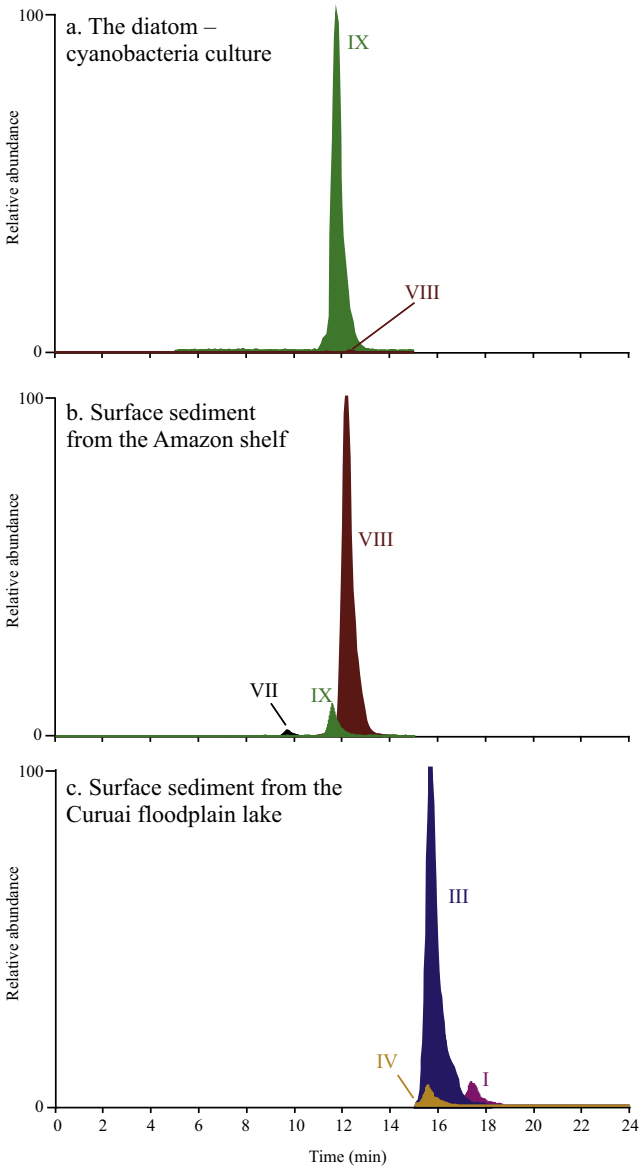

Fig. 3. Examples of summed ion chromatograms of MRM transitions produced using the C_5/C_6 MRM method for the analysis of HGs for (a) the diatom–cyanobacteria culture *Rhizosolenia–R. intracellularis*#136, (b) surface sediment from the Amazon shelf, station 50 and (c) surface sediment from the Curuai floodplain lake. Roman numerals refer to structures in Fig. 1.

amount of alcohol C_6 HGs (Bauersachs et al., 2009a), while Woermer et al. (2012) suggested that growth phase may also play an effect in the varying distribution of ketone and alcohol C_6 HGs.

3.2. Heterocyst glycolipids in SPM and surface sediments from the Amazon plume

We applied our MRM method to SPM from the Amazon shelf and slope, which extends from the river mouth into the tropical Atlantic Ocean (cf. Fig. 2). This region of the Atlantic affected by the Amazon plume has been shown to support high numbers of *H. hauckii* in association with its symbiont *R. intracellularis* (Carpenter et al., 1999; Foster et al., 2007; Subramaniam et al., 2008). Indeed, SPM collected from the chlorophyll-*a* maximum depth always contained at least one of the three C_5HGs, and the majority contained all three (Table 4; for individual samples see Table S1). Their relative distribution was quite distinct from that of any of the C_5 HG containing cultures analyzed to date (Table 4), i.e. **VIII** was more abundant than **VII** and **IX**. We extended the study of the Amazon plume to surface sediment, collected from the same stations as the SPM, plus five additional stations (Fig. 2b and Table 1). The C_5 HGs were also present in the surface sediments from underneath the plume, in a similar distribution to that in the SPM (Table 4; for individual samples see Table S2), suggesting they are transported from the water column to the sediment, a process which is probably enhanced by the association with the diatom silica skeleton, which provides both mineral ballast as well as matrix protection. Their presence in sediment and association with diatom frustules suggests that they have the potential to be preserved in sediments.

The abundance of the three C_5 HGs showed that in the SPM, the concentration was low (1.0×10^3 to 2.9×10^3 r.u./g OC) at the four stations closest to the river mouth (25, 43, 42d and 42b; Fig. 4a) and higher at the seven stations further from the river mouth (0.7×10^4 to 7.7×10^4 r.u./g OC). However, there was neither a significant correlation between distance from the river mouth and the SPM C_5 HG concentration nor between the SPM C_5 HG concentration and the concentration of chlorophyll in the water (data not shown). Interestingly, the spatial distribution of the total C_5 HG concentration in surface sediments was different from that from the overlying SPM (Fig. 4). While the highest sediment concentration was at a station further away from the river mouth (32b, 6.9×10^8 r.u/g OC) the second highest was relatively close to the river mouth at station 25 (5.4×10^8 r.u./g OC^{-1}). This difference between the SPM and the surface sediment is to be expected due to annual changes in the position and extent of the plume-related

Table 4
Distribution of C_5 HGs and C_6 HGs in cyanobacterial biomass, SPM and sediments (–, not detected; +, minor amount; ++, intermediate amount; +++, major amount; Tr., trace.

$[M+H]^+$	C_5 HGs			C_6 HGs					
	VII *m/z* 603	VIII *m/z* 619	IX *m/z* 647	I *m/z* 577	II *m/z* 575	III *m/z* 605	IV *m/z* 603	V *m/z* 621	VI *m/z* 619
Culture									
R. clevei–R. intracellularis #132	–	Tr.	+++	–	–	–	–	–	–
R. clevei–R. intracellularis #136	–	Tr.	+++	–	–	–	–	–	–
H. hauckii–R. intracellularis #81[a]	++	+++	+++	–	–	–	–	–	–
H. hauckii–R. intracellularis #83[a]	++	+++	+++	–	–	–	–	–	–
H. membranaceous–R. intracellularis[a]	+	+++	+++	–	–	–	–	–	–
Environmental samples									
Marine SPM (sum of all)	Tr.	++	+	–	–	–	–	–	–
Marine sediment (sum of all)	+	+++	++	Tr.	–	+	Tr.	–	–
Óbidos Amazon River SPM	–	–	–	+	Tr.	++	Tr.	–	–
Amazon floodplain lake sediment (sum of all)	–	–	–	Tr.	Tr.	++	+	Tr.	Tr.

[a] Data from Schouten et al. (2013).

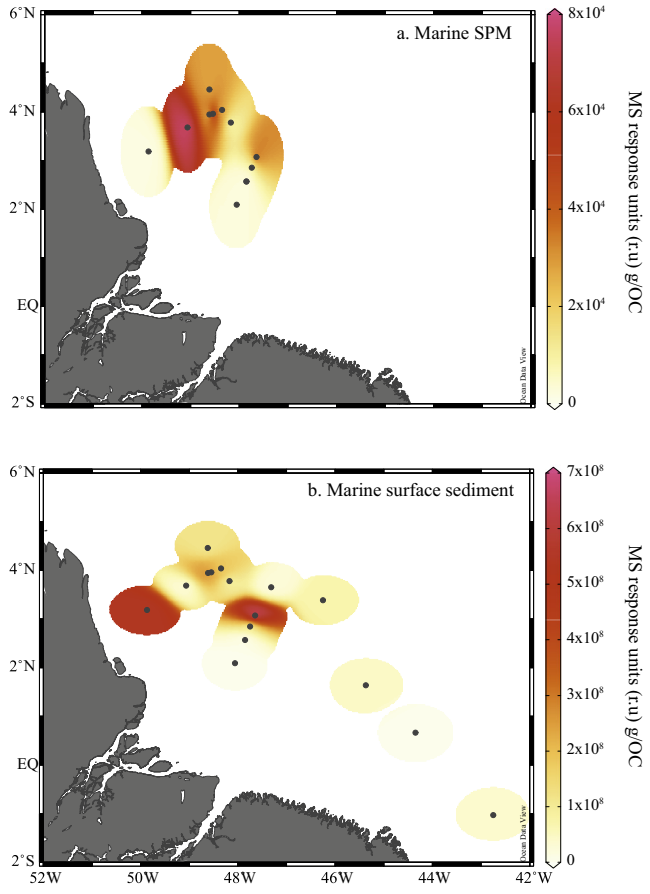

Fig. 4. Sum of the three C_5 HGs (r.u./g OC) in (a) SPM from the chl-*a* max depth and (b) surface sediment. For station labels see Fig. 2c. Note the different scales of the two graphs.

diatom bloom, with the sediment providing an 'integrated' multi-decadal record of the deposition of C_5 HGs.

3.3. Potential freshwater sources of C_5 HGs

A HG tentatively assigned as containing a C_5 headgroup and a C_{26} carbon chain (pentose hexacosane-diol) was recently detected in a culture of the freshwater cyanobacterium *A. ovalisporum* UAM 290 and in three lakes in Spain (Woermer et al., 2012), suggesting that C_5 HGs can be produced in freshwater environments. To exclude the possibility that the C_5 HGs we detected in the Amazon plume SPM and sediment were delivered there from the continent via the Amazon River, we analyzed surface sediments from six floodplain lakes and SPM from the Amazon River obtained at a sampling station at the town of Óbidos (Table 2 and Fig. 2c). Both the Amazon floodplain lake sediments and the Amazon River SPM contained between three and six C_6 HGs (Table 4; for individual samples see Table S3) which have all been described in cyanobacterial cultures and sediments from freshwater environments (Bauersachs et al., 2009a,b, 2011; Woermer et al., 2012). However, C_5 HGs were not detected in any of these freshwater settings. Low levels of three of the C_6 HGs were detected in some of the surface sediments from the Amazon plume region, while C_6 HGs were not detected in the plume SPM (Table 4). This suggests that C_5 HGs are produced in situ in the marine environment and are not derived from the continent, while C_6 HGs seem to be restricted mainly to freshwater environments. The results suggest that C_5 HGs are potential biomarkers for marine endosymbiotic heterocyst cyanobacteria.

4. Conclusions

C_5 HGs occur in cultures of marine endosymbiotic heterocystous cyanobacteria. We have also shown that they occur in SPM and surface sediment from the Amazon plume, a region known to contain heterocystous cyanobacteria as endosymbionts with marine diatoms. These compounds have not been reported in the marine environment. Our results also indicate that the C_5 HGs are transported to marine surface sediment and thus have the potential to be preserved in the geological record. It is also possible that the alkyl moieties of the C_5 HGs may be of chemotaxonomic or biomarker importance due to the varying C_5 HG distribution observed in different cultures of marine diatoms containing the endosymbiotic heterocystous cyanobacteria (Table 4). Further work would be necessary to ascertain whether the alkyl chains are preserved without the pentose head group, presumably as C_{30}–C_{32} triols and tetraols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.orggeochem.2015.01.004.

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