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Occurrence, distribution and origin of C_{30} cyclobotryococcenes in a subtropical wetland/estuarine ecosystem

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ABSTRACT

A series of C_{30} branched isoprenoid hydrocarbons with a cyclobotryococcane skeleton were identified in the Florida Coastal Everglades Ecosystem. Nine such C_{30} alkenes were detected and identified as five-membered ring monocyclic hydrocarbons with an identical parent structure of 7,11-cyclobotryococcane based on their mass spectra and hydrogenation behavior. Previous reports have suggested *Botryococcus Braunii* or green sulfur bacteria (*Chlorobiaceae*) as likely sources of these 7,11-cyclobotryococcanes. However, their enriched $\delta^{13}C$ values (about -18%) determined in this study and their absence in freshwater marsh environments of the Everglades suggest that an origin from green sulfur bacteria or possibly marine diatoms is more likely. The depth profile of individual C_{30} cyclobotryococcenes in one sediment core from Florida Bay shows abundant variations in their historical deposition and may indicate long-term changes of their source strength due to variations in local environmental conditions.

1. Introduction

Since the first report of highly branched isoprenoids (HBIs) in sediments (Gearing et al., 1976), this class of isoprenoids has been the subject of extensive studies to determine their structures and sources (Rowland and Robson, 1990; Volkman et al., 1994; Sinninghe Damsté et al., 2004). HBI alkenes with 20, 25 and 30 carbon skeletons (see Appendix I–III) have been commonly observed in diverse environments including Shark Bay, Western Australia (Dunlop and Jefferies, 1985), the Tamar Estuary, UK (Hird and Rowland, 1995), Everglades National Park, USA (Jaffé et al., 2001) and the Arabian Sea (Massé et al., 2004a) among others. Volkman et al. (1994) first identified polyunsaturated C_{25} HBI alkenes in two marine diatom species *Rhizosolenia setigera* and *Haslea ostrearia* and several subsequent studies reported the occurrence of C_{25} or C_{30} HBIs in diatoms (Sinninghe Damsté et al., 1999a,b; Wraige et al., 1999; Allard et al., 2001; Belt et al., 2001a,b). To date the biosynthesis of C_{25} HBIs by diatoms has been firmly established (Volkman et al., 1994; Sinninghe Damsté et al., 2004), while the biological origin of the C_{20} and C_{30} HBIs and other branched isoprenoid alkenes remain to be fully determined. The greater Everglades ecosystem, including Florida Bay (FB), offers an ideal venue for investigating biogeochemistry of these classes of compounds, since relatively high abundance of C_{20}, C_{25} and C_{30} HBIs as well as unusual C_{32}–C_{34} botryococcanes have already been identified in this coastal wetland/estuarine environment (e.g. Jaffé et al., 2001; Xu et al., 2006; Gao et al., 2007).

Branched isoprenoids of the botryococcane family have been reported in environmental samples for quite some time (Maxwell et al., 1968). They represent a suite of cyclic and acyclic triterpenoids presumably biosynthesized by *Botryococcus braunii* race B (Metzger and Largeau, 1999). About 50 different compounds of this class have been identified in *B. braunii* race B, ranging from C_{30} to C_{37} usually with six degrees of unsaturation. Recently, novel C_{32}–C_{34} botryococcane hydrocarbons with lower degrees of unsaturation were reported for the Florida Everglades (Gao et al., 2007), and while *B. braunii* race B was suggested as a potential source, green algae were also considered for their origin. Thus, a great variety of HBIs and botryococcane type hydrocarbons have been detected in periphyton, floc, soils and sediments of the subtropical wetlands and estuaries of south Florida. Here we report on the presence of a series of C_{30}, monocyclic, branched isoprenoid hydrocarbons with a cyclobotryococcane skeleton in this ecosystem, and discuss their potential sources.

2. Experimental

2.1. Sample collection

Soil and sediment samples were collected along a North–South transect of the Taylor Slough/Pan Handle (TS/PH) portion of Everglades National Park, at combined sampling sites established for the Florida Coastal Everglades Long Term Ecological Research pro-

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gram (FCE-LTER; see http://fcelter.fiu.edu/) and the Paleoseagrass program (Xu et al., 2007). Those sites consist of the FCE-LTER Taylor Slough (TS/Ph) sites (1, 2, 3, 6 and 7), Shark River Slough (SRS) sites (2, 3, 4, 5 and 6) and the Paleoseagrass FB sites (TC, DK, BA, RK, RB and NB) which are subsequently referred to their corresponding station numbers/names (Fig. 1). Sediment/soil samples were collected with an Eckman dredge. Additionally, a 222 cm long sediment core from the Florida Bay site NB was analyzed to assess long-term changes in the abundance of the compounds studied here. This piston core had previously been dated using ^{210}Pb and estimated to cover the period from ca. 1850–2002 in its top 90 cm (Xu et al., 2007). Dates are not shown in the present study as ^{14}C dating needed to provide more accurate dates at depth deeper to those described previously was unavailable (Xu et al., 2007). All samples were collected in clean aluminum foil or glass jars and placed on ice for transportation to the laboratory, where they were kept in a freezer until analysis.

2.2. Analytical methods

The detailed analytical procedure has been described by Jaffé et al. (2001). Briefly, samples were freeze-dried at −40 °C and then Soxhlet extracted with pure methylene chloride (Optima, Fisher, USA) for 24 h. Activated copper was used to remove the elemental sulfur from the total extracts. Total extractable organics were saponified with 0.5 N KOH and separated into neutral and acid fractions. The neutral fraction was further separated by silica gel adsorption chromatography. The aliphatic hydrocarbon fraction containing the compounds of interest was eluted with pure hexane (Optima, Fisher, USA) and a known amount of squalane was added as the internal standard for quantitative analysis.

A Hewlett–Packard 5973 GC–MS system was used for the analyses of the isolated fractions and operated in the electron impact (EI) ionization mode at 70 eV, while the source temperature was kept at 200 °C. A DB5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; J&W; Flossom, California) was used and the GC oven was programmed from 60 to 180 °C at the rate of 6 °C after 1 min at the initial temperature; then increased up to 315 °C at the rate of 3 °C min^{-1} and kept for 15 min. Concentrations for the individual C_{30} alkenes and for comparative reasons those of the C_{20} and C_{25} HBIs and cyclobotryococcenes are presented in Table 1.

Hydrogenation of the aliphatic hydrocarbon fraction was carried out in hexane under mild pressure of hydrogen gas using palladium on charcoal as catalyst for 12 h. Resulting hydrogenation products were determined by GC–MS and tentatively identified using gas chromatographic retention indices, mass spectra interpretation and comparison with previously published data. Since temperature ramp instead of isothermal condition was applied for our GC–MS, the retention indices were defined as:

$$\mathrm{RI} = 100\left(\frac{\mathrm{RT}_i - \mathrm{RT}_n}{\mathrm{RT}_{n+1} - \mathrm{RT}_n} + n\right)$$

where RI is retention indices on DB5 column for compound of interest, RT is retention time in GC–MS chromatogram, and *i*, *n* and *n* + 1 are referred to compound of interest, carbon number of preceding *n*-alkane and carbon number of subsequent *n*-alkane, respectively.

Compound-specific ^{13}C stable isotope analyses were performed on a Finnigan Delta Plus coupled to a Hewlett–Packard 6890 gas chromatograph, following the procedures described by Hayes et al. (1990). A DB1 capillary column (30 m, 0.25 mm i.d., 0.25 μm; J&W; Flossom, California) was used for this purpose at the chromatographic conditions described above. A DB1 chromatographic phase was selected for compound specific isotope analysis as it was found generate the best chromatographic separation of the compounds of interest, and as such produced the most reliable isotope measurements.

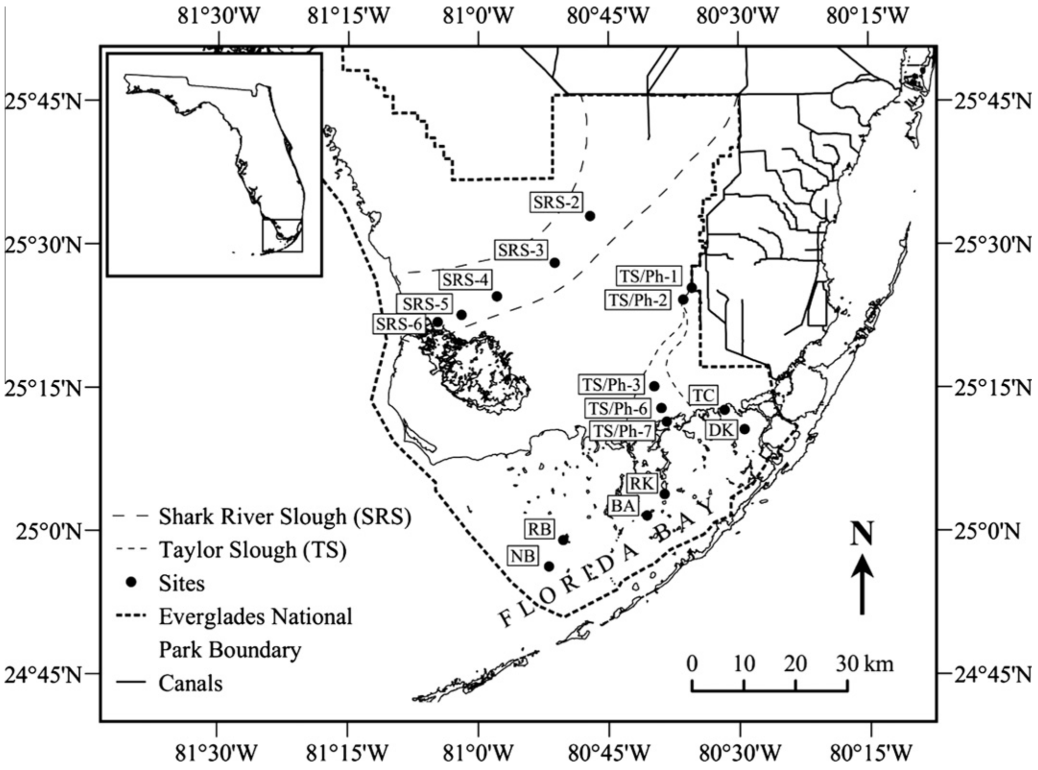


Fig. 1. Geographic location of sampling sites in the Florida Coastal Everglades.

Table 1
Concentrations (ng g^{-1} dw; dry weight) of selected C_{20} highly branched isoprenoids (HBIs), C_{25} HBIs, C_{30} cyclobotryococcenes and C_{32}–C_{34} botryococcenes in the Florida Coastal Everglades.

Compound RI[a]	Sampling site	C_{20} HBIs 1674	C_{25} HBIs	C30:3:1 2484	C30:3:1 2491	C30:2:1 2506	BC[b]
TS/PH*	1	308	<1	<1	<1	<1	<1
	2	1950	<1	<1	<1	<1	<1
	3	5080	<1	<1	<1	<1	<1
	6	204	<1	<1	<1	<1	<1
	7	209 000	<1	<1	<1	<1	<1
SRS**	2	128 000	<1	<1	<1	<1	36 740
	3	14 600	<1	<1	<1	<1	11 030
	4	6830	<1	<1	<1	<1	<1
	5	767	<1	<1	<1	<1	<1
	6	478	<1	<1	<1	<1	<1
FB***	TC	516	620	23	2	25	<1
	DK	358	56	11	<1	<1	<1
	BA	957	570	7	76	<1	<1
	RK	3440	187	3	1.4	<1	<1
	RB	223	1467	<1	<1	<1	<1
	NB	1440	450	681	787	<1	<1

[a] RI = Retention indices on DB5 column.
[b] BCs = C_{32}–C_{34} botryococcenes, data from Gao et al. (2007).
* TS/PH = Taylor Slough/Pan Handle.
** SRS = Shark River Slough.
*** FB = Florida Bay; the location of sampling sites is displayed in Fig. 1.

3. Results

3.1. Identification of C_{30} alkenes

Several C_{30} alkenes eluting between the C_{24} and C_{26} *n*-alkanes (Fig. 2) were observed in some of the extracts from Florida Bay sediments, but not at any of the freshwater sites. Their mass spectra show a molecular ion at *m/z* 414 ($C_{30}H_{54}$) or 416 ($C_{30}H_{56}$), implying three or four degrees of unsaturation. The hydrogenation of the aliphatic hydrocarbon fractions yielded two products both of which have molecular ion at 420 ($C_{30}H_{60}$), suggesting one cycle and two or three double bonds for the original alkene structures. As a result, nine of the C_{30} alkenes detected in this study (six compounds are shown in Fig. 2) were referred to as C30:3:1$_{DB5}$ 2425, 2434, 2469, 2484, 2491, 2513, 2583, 2589 and C30:2:1$_{DB5}$ 2507, respectively. In the nomenclature used 'C' denotes a cyclic skeleton followed by the number of carbon atoms, the number of double bonds, the number of rings and the retention index on a DB5 column. Belt et al. (2003) and Massé et al. (2004a) reported the occurrence of six-membered ring monocyclic C_{30} HBI alkenes in the marine diatom, *R. setigera*. However, their RI values (2568 and 2605 on DB5 column) and their mass spectra (see Fig. 3 and Massé et al., 2004a) are quite different from those of the C_{30} alkenes reported here, suggesting structural differences among these compounds. Actually, one of the C_{30} hydrocarbon alkenes (C30:3:1$_{DB5}$ 2491; compound 5) was tentatively identified as the five-membered ring monocyclic compound (7,11-cyclobotryococca-5,12,26-triene, see Appendix IV) based on its retention time and mass spectrum (Fig. 3) (Behrens et al., 2000a; Zhang et al., 2004). Its characteristic molecular ion (*m/z* 414) in addition to fragment ions at *m/z* 343, 288, 259, 231, 191, 163, 107 and 81 are in agreement with a report by Behrens et al. (2000a) who observed this compound in sediments from Lake Cadagno, Switzerland, and first established its structure using a combined approach of mass spectrometry, catalytic hydrogenation, and heteronuclear NMR.

Although the location of double bonds in the other monocyclic C_{30} alkenes was not unequivocally assigned, their hydrocarbon skeletons were determined. The hydrogenation of the C_{30} alkenes consistently yielded only two products which were identified as 7,11-cyclobotryococcane (V) diastereomers (Fig. 3; Behrens et al., 2000a). Their identical mass spectra with characteristic ions of *m/z* 420, 336, 266, 210 and 111 and easily resolved gas chromato-

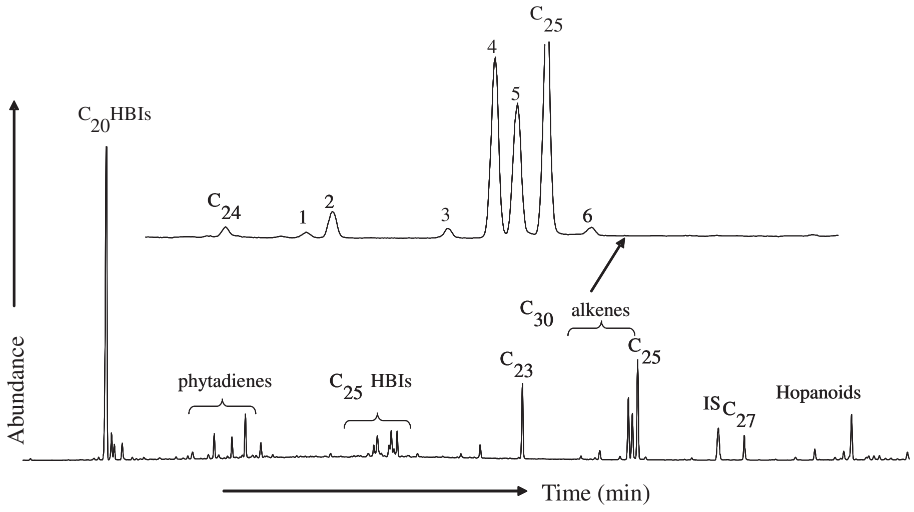


Fig. 2. Partial GC/MS total ion current (TIC) chromatogram for aliphatic hydrocarbons in surface sediments of the NB core. IS: internal standard; *n*-C_{2X} = *n*-alkanes.

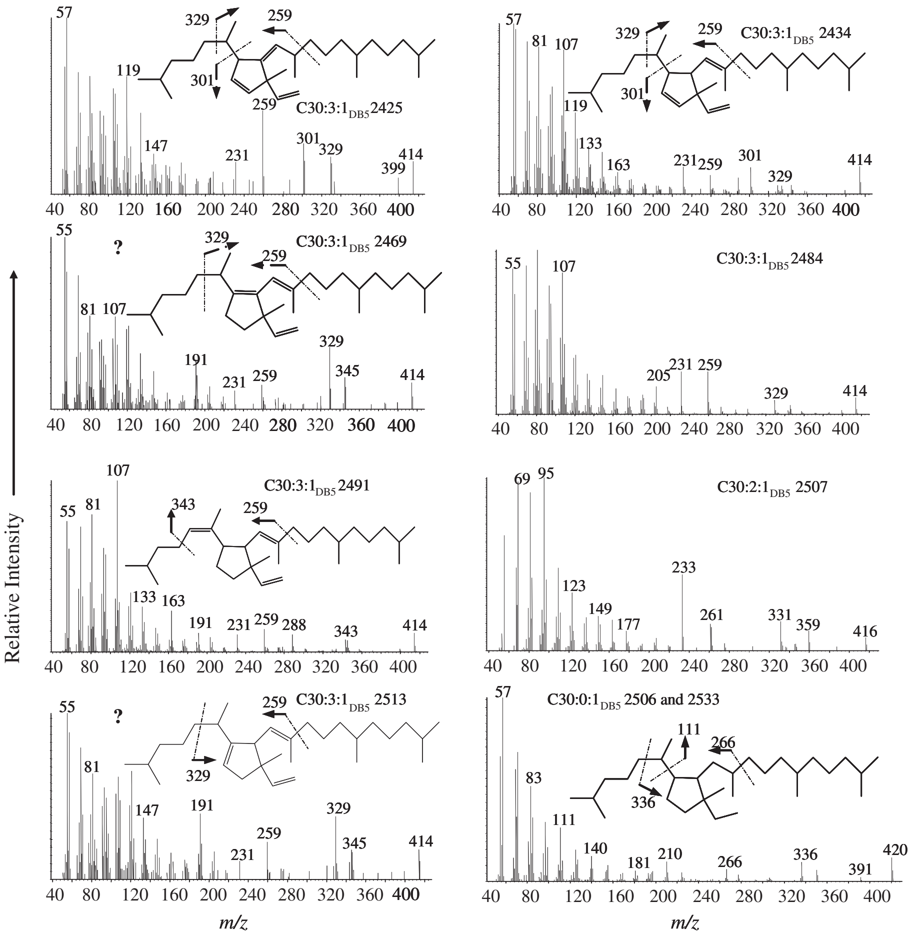


Fig. 3. Mass spectra, retention indexes and tentative structures for selected C_{30} cyclobotryococcenes and related hydrogenation products in Florida Bay.

graphic characteristic (RI_{DB5} 2506 and 2533) suggest that an asymmetric carbon atom most likely occurs within the ring system, specific at C-7, C-10 or C-11 (see V), resulting in α and β configurational isomerism. Therefore, all the detected C_{30} alkenes must have the same parent hydrocarbon skeleton of 7,11-cyclobotryococcane (V) but different locations and/or numbers of double bonds and are referred to as C_{30} cyclobotryococcenes. Requejo and Quinn (1983) reported the presence of C_{30} HBIs in sediments of Narragansett Bay. In their study, catalytic hydrogenation produced two isomers with the molecular weight at 418 instead of 420 and hence they assigned those compounds as bicyclic hydrocarbon skeletons. However, those compounds have very similar mass spectra to that of the 7,11-cyclobotryococca-5,12,26-triene. Based on comparisons of hydrogenation conditions in different literature reports, Rowland and Robson (1990) proposed that some double bonds within HBI structures are highly hindered from reaching saturation. More recently, Massé et al. (2004a) reported that an incomplete hydrogenation of C_{30} HBI alkenes could result in the production of intermediates with two degree of unsaturation. Therefore, it is quite possible that some monocyclic C_{30} alkenes have erroneously been assigned a bicyclic structure in previous reports.

Characteristic fragment ions reveal valuable information on the location of double bonds in this series of C_{30} cyclobotryococcenes. Compared to 7,11-cyclobotryococca-5,12,26-triene (C30:3:1_{DB5} 2491; compound 5), compound 1 (C30:3:1_{DB5} 2425) and compound 2 (C30:3:1_{DB5} 2434) have relatively high abundant ions at m/z 301 likely due to β-cleavage of double bound at C8(9). Additionally the relative intensity of m/z 259 is remarkably higher for compound 1 than compound 2, suggesting one double bond at C11(12) instead of C12(13) for compound 1. Based on these interpretations, we tentatively assign C30:3:1_{DB5} 2425 and C30:3:1_{DB5} 2434 as 7,11-cyclobotryococca-8,11,26-triene and 7,11-cyclobotryococca-8,12,26-triene, respectively. Compounds 3 and 6 (C30:3:1 2469 and 2513) present quite similar mass spectra with enhanced abundance of m/z 329, suggesting that a double bond occurs at C7(8) or C7(11). One compound (not shown in Fig. 2; C30:2:1_{DB5} 2507) only occurring at the TC site displays characteristic ions at m/z 233 and 261 in addition to molecular fragment ion of 416. A difference of 2 mass units for characteristic fragment ions between this unknown compound and 7,11-cyclobotryococca-5,12,26-triene, e.g. 416(414), 331(329), 233(231) and 261(259), strongly implies that the former has an identical hydrocarbon skeleton to the latter but one degree lower saturation.

3.2. Potential sources of C_{30} cyclobotryococcenes

The presence of C_{30} HBIs including polyunsaturated acyclic alkenes (Volkman et al., 1994; Belt et al., 2001a, 2002) and six-membered ring monocyclic alkenes (Belt et al., 2003; Massé et al., 2004b) has been reported in the marine diatom, *R. setigera*. However, other algae may also contribute to the C_{30} alkene pool. Behrens et al. (2000a) assigned the source of the 7,11-cyclobotryococca-5,12,26-triene(IV) to green alga *B. braunii* or green sulfur photosynthetic bacteria (*Chlorobiaceae*) based on its similar structure to botryococcene hydrocarbons and relatively enriched $\delta^{13}C$ values. In this case, it is unlikely that 7,11-cyclobotryococca-5,12,26-triene and related C_{30} alkenes are derived from *B. braunii* as C_{32}–C_{36} botryococcene hydrocarbons (Maxwell et al., 1968; Huang et al., 1996), the predominant compounds in *B. braunii*, were not detected at the Florida Bay sites where C_{30} cyclobotryococcenes were present (see Table 1). In addition, C_{32}–C_{34} botryococcene structures were reported in the freshwater marsh environments of the Everglades (ca. 36.7 μg g^{-1} in surface soil; Gao et al., 2007) where C_{30} cyclobotryococcenes were not detected. Therefore, based on our data and the suggestions by Behrens et al. (2000a), green sulfur bacteria are a more likely source to account for the occurrence of C_{30} cyclobotryococcenes in Florida Bay. This seems to be supported by relatively high abundance of sulfur bacterial mats in this region (Carlson et al., 1994). Two C_{30} cyclobotryococcenes (br30:3:1 2484 and 2491) were sufficiently abundant in sediments of NB to examine their ^{13}C isotopic composition, which resulted in highly enriched values of −18.7‰ and −19.0‰ respectively. These values are significantly enriched relative to the compounds reported in sediments from Lake Cadagno (−27‰) (Behrens et al., 2000a). We attribute this difference to different environmental conditions between two systems. Lake Cadagno is a closed system with an important recycling of organic carbon and a dense population of sulfate-reducing bacteria which influence significantly the carbon isotope compositions (Putschew et al., 1996; Behrens et al., 2000b). A permanent anoxic zone is formed below 10–12 m of water depth in Lake Cadagno (Behrens et al., 2000b). Both Putschew et al. (1996) and Schaeffer et al. (1997) found depleted ^{13}C-contents for bacterial lipids due to the low ^{13}C-content of the dissolved inorganic carbon in Lake Cadagno. In contrast, Florida Bay is a shallow, subtropical coastal ecosystem with a mean depth of less than 3 m, where dense seagrass beds are an important organic matter source that is characterized by exceptionally heavy $\delta^{13}C$ values (−7.2‰ to −10.4‰ for bulk organic carbon and −13‰ to −20‰ for *n*-alkanes) (Anderson and Fourqurean, 2003; Mead et al., 2005). Combining the fact that the C_{30} cyclobotryococcenes in this study were only detected in marine sediments that they presented close $\delta^{13}C$ values to those of the C_{25} HBIs for this region (−17.62‰ to −21.92‰; Xu and Jaffé, unpublished) and that C_{30} HBI alkenes have been reported in diatoms, these C_{30} cyclobotryococcenes may be also derived from marine diatoms. Indeed, polyunsaturated C_{30} alkenes from diatoms can undergo isomerization and cyclization reactions to yield cyclic compounds under acid-clay catalytic conditions (Belt et al., 2000), and thus, similar processes may account for the formation of these C_{30} cyclobotryococcenes.

3.3. Spatial and depth profiles of C_{30} cyclobotryococcenes

The spatial distribution of C_{30} cyclobotryococcenes identified above was basically restricted to marine environments. Contrary to C_{32}–C_{34} botryococcene hydrocarbons (Gao et al., 2007), they were not observed at any of the freshwater sites. Even for most sites in FB their concentrations were quite low, with the notable exception of the NB site where their total concentration was determined at 2.4 μg g^{-1}. The depth profile for these components in the sediment core collected at this site was quite variable in both concentration and composition (Fig. 4). Zhang et al. (2004) have reported the occurrence of 7,11-cyclobotryococca-5,12,26-triene during the main glacial section (35 600–11 800 year BP) in a sediment core of Lake Heqing, southwestern China, suggesting an applicability of C_{30} cyclobotryococcenes as a paleo-environmental proxy. In the case of Florida Bay, the entire core was divided into three zones based on the characteristics of the C_{30} cyclobotryococcenes abundance, namely Z-1 (upper zone; 0–60 cm), Z-2 (middle zone; 60–180 cm), and Z-3 (bottom zone; 180–220 cm). The 7,11-cyclobotryococca-5,12,26-trienes were absent in the bottom zone. Starting from 180 cm, their concentrations slightly increased to 265 ng g^{-1} at 100 cm, and then gradually decreased up-core. After a period of absence during (60–40 cm), they dramatically increased to about 2.4 μg g^{-1} in the surface sediment.

Individual C_{30} cyclobotryococcenes displayed significantly different depth profiles. Overall, a total of eight C_{30} cyclobotryococcenes were identified in this core which was dominated by two compounds (C30:1:3_{DB5} 2484 and 2491) both of which had the maximum concentration of ~800 ng g^{-1}, followed by three isomers (C30:1:3_{DB5} 2434, 2469 and 2513) of around 340 ng g^{-1}, and another three C_{30} cyclobotryococcenes namely C30:1:3_{DB5} 2425, 2583 and 2589 which occurred at concentrations below 100 ng g^{-1}. No C_{30} cyclobotryococcenes were detected in the bottom zone (Z-3). Given the present day presence of C_{30} cyclobotryococcenes only under marine conditions, this region may be indicative of historical brackish or freshwater conditions in the bay. In fact, fossil-diatom species analysis showed the existence of a predominantly freshwater environment for Z-3 (Xu et al., 2006). In Z-2, C30:1:3_{DB5} 2583 and 2589 reached their maximum concentration (<54 ng g^{-1}). At the same period, other C_{30} compounds were either absent or present at relatively low abundance. In Z-1, C30:1:3_{DB5} 2583 and 2589 first remained absent from 60 to 50 cm and then increased toward surface until 6 cm after which they disappeared again. The other six cyclobotryococcenes, however, presented a consistent trend in Z-1 where they first remained at relative low concentration or were absent and then markedly increased toward surface and reach the peak at the top surface sediment. Similar trends of increased abundance of microbial biomarkers in surface core sections have been reported across the Everglades (Saunders et al., 2006; Xu et al., 2006, 2007; Gao et al., 2007) and have been suggested to be the result of increased

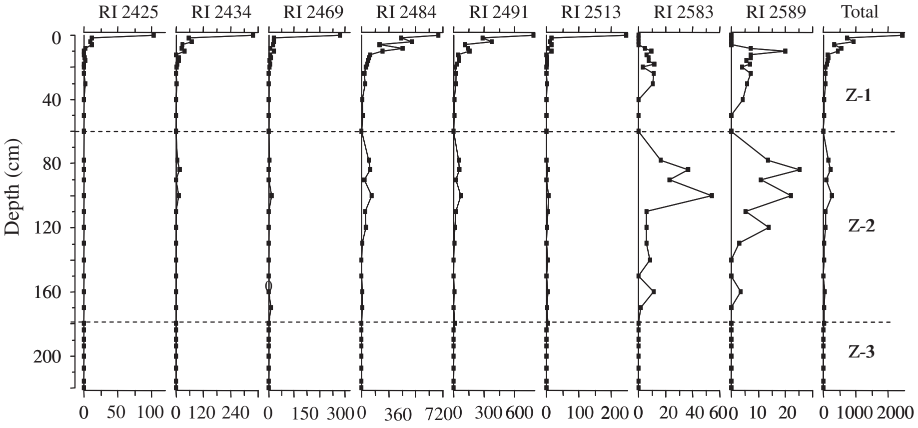


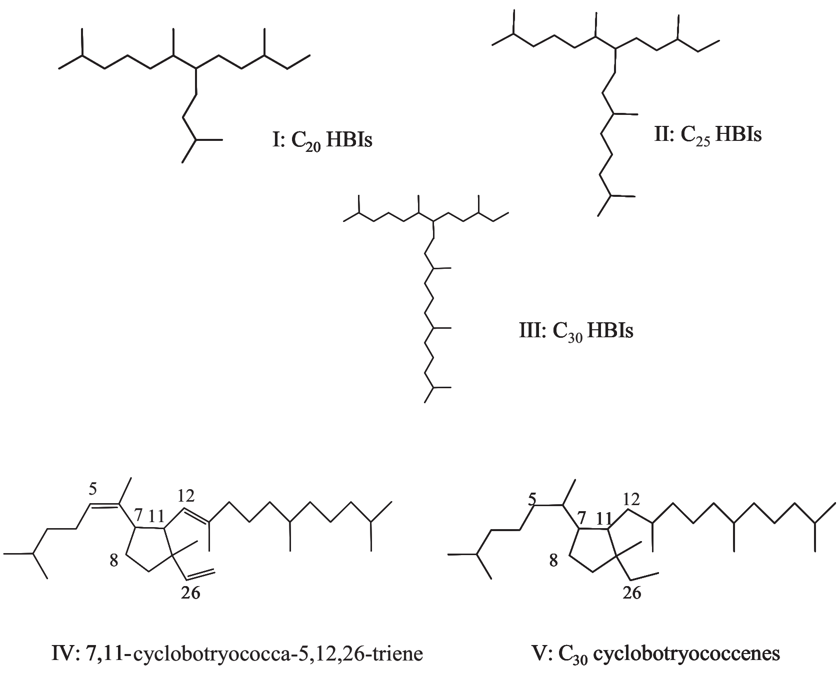
Fig. 4. Depth profiles of C_{30} cyclobotryococcenes in a sediment core from station NB, Florida Bay (concentrations in ng g^{-1} dw; dry weight).

nutrient levels of this ecosystem due to its recent history in water management, regional urbanization and changes in land-use.

A strong coupling was observed between most of the C_{30} cyclobotryococcenes. For example, significant correlation ($p < 0.01$) was observed between C30:3:1_{DB5} 2425 and 2434 ($r = 0.98$), between C30:3:1_{DB5} 2425 and 2469 ($r = 0.99$), and between C30:3:1_{DB5} 2484 and 2491 ($r = 0.95$). This kind of strong coupling between individual C_{30} cyclobotryococcenes suggests that they originate from a similar source and/or undergo similar diagenetic processing. Although a high correlation coefficient was observed between C30:3:1_{DB5} 2583 and 2589 ($r = 0.70$), they do not show a strong coupling with other C_{30} cyclobotryococcenes. Interestingly, quite similar depth profiles as for these two compounds were observed when compared with previously reported depth profiles for C_{25} HBIs in a sediment core from RK (Fig. 1). The highest abundance of these C_{30} cyclobotryococcenes occurred during a period of high organic matter inputs from some specific benthic and particularly epiphytic marine diatoms (Xu et al., 2006), providing some indication that a marine diatom origin for these compounds is possible.

4. Conclusions

Nine alkenes were detected in sediments from Florida Bay and identified as five-membered ring monocyclic compounds with an identical parent structure of 7,11-cyclobotryococcane. Although the origin of similar structures had previously been assigned to *B. braunii* or green sulfur bacteria, their highly enriched δ^{13}C values and spatial distributions suggests the latter as most likely source. However, biosynthesis by marine diatoms and catalyzed rearrangement processes cannot be excluded as a potential source. The depth profile of C_{30} cyclobotryococcenes may reflect historical variations of their source strength to sediments and therefore C_{30} cyclobotryococcenes are a potential valuable proxy for paleo-environmental studies.



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Appendix A

Structures of C_{20}, C_{25} and C_{30} HBIs as well as C_{30} cyclobotryococcenes.

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