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Composition and Sources of Sterols in Pulau Tinggi, Johor, Malaysia

(Komposisi dan Sumber Sterol di Pulau Tinggi, Johor, Malaysia)

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ABSTRACT

This study explores the role of sterols as lipid biomarkers to indicate their input which originates from various sources in the marine environment. Sterols and their ratios were investigated in sediments taken from sixteen sampling stations at Pulau Tinggi, Johor in order to assess the sources of organic matter. The compounds extracted from the sediments were quantified using a gas chromatography-mass spectrometry (GC-MS). The distributions of sterols indicated that organic matter at all sampling stations originated from a mixture of marine source and terrestrial origins at different proportions. A total of eleven sterols were quantified, with the major compounds being phytosterols (44% of total sterols), cholesterol (11%), brassicasterol (11%) and fecal sterols (12%).

Keywords: Cholesterol; fecal sterol; lipid biomarker; phytosterol

ABSTRAK

Kajian ini mengetengahkan peranan sterol sebagai penunjuk biolipid untuk menunjukkan input sebatian-sebatian sterol yang berpunca daripada pelbagai sumber di pesekitaran marin. Sterol yang hadir di dalam sampel sedimen yang diambil dari 16 stesen pensampelan di Pulau Tinggi dikaji dengan menggunakan nisbah tertentu untuk menentukan sumber bahan organik tersebut. Sebatian yang diekstrak daripada sedimen dianalisis menggunakan gas kromatografi-spektrometri jisim (GC-MS). Taburan sterol menunjukkan bahan organik di semua stesen pensampelan berpunca daripada campuran sumber marin dan terestrial pada jumlah yang berbeza. Sebanyak 11 sterol dikenalpasti hadir dengan fitosterol merupakan sebatian utama (44% daripada jumlah sterol), diikuti oleh kolesterol (11%), brasikasterol (11%) dan sterol daripada sisa kumbahan (12%).

Kata kunci: Fitosterol; kolesterol; penunjuk biolipid; sterol; sterol kumbahan

INTRODUCTION

Lipid biomarkers are reliable tools for assessing the input of organic matter originating from various sources (Logan et al. 2001; Méjanelle & Laureillard 2008; Zimmerman & Canuel 2001). Lipid compounds that have the potential to identify the major sources of organic matter and have been widely used are steroid alcohols (Froehner et al. 2008; Jardé et al. 2007; Marchand et al. 2005; Santos et al. 2008). Whilst lipid compounds are specific to a particular group of organisms, different classes of organisms are known to have different sterols patterns (Froehner et al. 2008; Logan et al. 2001; Puglisi et al. 2003). Therefore it was decided that lipid biomarkers would be used to identify and differentiate between inputs of organic matter in aquatic environments (Mudge & Duce 2005; Seguel et al. 2001).

Steroid alcohols, also known as sterols, are component of eukaryotic cells which are distributed widely in organisms which can be found in the fat storage areas of organisms (Logan et al. 2001; Mater et al. 2004; Puglisi et al. 2003). Sterols are hydrophobic compounds and as they are insoluble in water they tend to settle and be adsorbed by sediment (Froehner et al. 2008). Furthermore, sterols have a long residence time in the environment and do not degrade over a short period of time, if compared with other biological compounds such as amino acids and carbohydrates (Hyun et al. 2002; Mudge & Duce 2005; Seguel et al. 2001). The major sterols that can be found in the environment are cholesterol and its epimer, cholestanol, coprostanol and epicoprosanol, and phytosterols (Santos et al. 2008); their potential sources are estuarine phytoplankton and zooplankton, terrestrial plants and sewage (Fernandes et al. 1999; Mater et al. 2004).

In this study we investigated the composition of sterols and identified their sources in samples of surface sediment in Pulau Tinggi, Johor, Malaysia. Pulau Tinggi has an environment suitable to be used as the study area to review the presence of sterols that can be derived from variety of sources. The area is dotted with natural resources such as tropical forests and marine life such as fish and coral reefs. Other that that, Pulau Tinggi is also involved in turtle hatchery and giant clam breeding. These are major sources of sterols in the study area.

MATERIALS AND METHODS

STUDY AREA

Pulau Tinggi is one of Malaysia's tropical islands and was gazetted as a Marine Park of Malaysia in 1994. It is located about 37 km southeast of Mersing and the northeast coast of the State of Johor which has the highest residential population along the East Coast of Johor. The island is covered by the tropical forest that is rich with rattan, timber and other valuable plants. Its surrounding waters is rich with exotic marine life and beautiful underwater flora fringing its coral reefs, providing a breathtaking underwater view for divers and snorkeling enthusiasts. Other than that, the island has several Malay villages, holiday resorts and Marine Park Centre.

A total of sixteen sampling stations were established randomly around the coastal area of Pulau Tinggi (Figure 1 and Table 1). Surface sediment was collected from each of these sampling stations in July 2008 using a PONAR grab and samples were stored in a freezer at -4°C whilst they awaited further analysis.

TOTAL ORGANIC CARBON (TOC) ANALYSIS

The TOC content in the sediment samples was analyzed based on the different weight of the dry samples, which had been heated in a furnace. Sediment samples were dried in an oven at 60°C for 2 to 3 days until a consistent weight was gained. They were then crushed in a dry mortar with a porcelain pestle. Once again samples were oven-dried at 105°C for 24 hour and then underwent combustion in a furnace at 550°C for 4 hour, after which the TOC content was calculated using the following equation:

TABLE 1. Sampling locations

Station	Latitude (N)	Longitude (E)
PT1	02° 14' 32	104° 07' 33
PT2	02° 15' 32	104° 08' 39
PT3	02° 15' 33	104° 07' 01
PT4	02° 15' 51	104° 07' 59
PT5	02° 16' 09	104° 06' 09
PT6	02° 16' 29	104° 03' 29
PT7	02° 17' 14	104° 04' 43
PT8	02° 17' 19	104° 04' 59
PT9	02° 17' 05	104° 04' 56
PT10	02° 19' 27	104° 05' 03
PT11	02° 20' 34	104° 05' 55
PT12	02° 09' 39	104° 00' 43
PT13	02° 11' 24	104° 00' 43
PT14	02° 12' 00	104° 02' 05
PT15	02° 18' 37	104° 08' 34
PT16	02° 19' 00	104° 08' 09

$$\% \text{ TOC} = \frac{DW_{105}\,(\text{g}) - DW_{550}\,(\text{g}) \times 100,}{DW_{105}\,(\text{g})}$$

where DW_{105} represents the dry weight sample at 105°C and DW_{550} the dry weight of the samples after heating to 550°C.

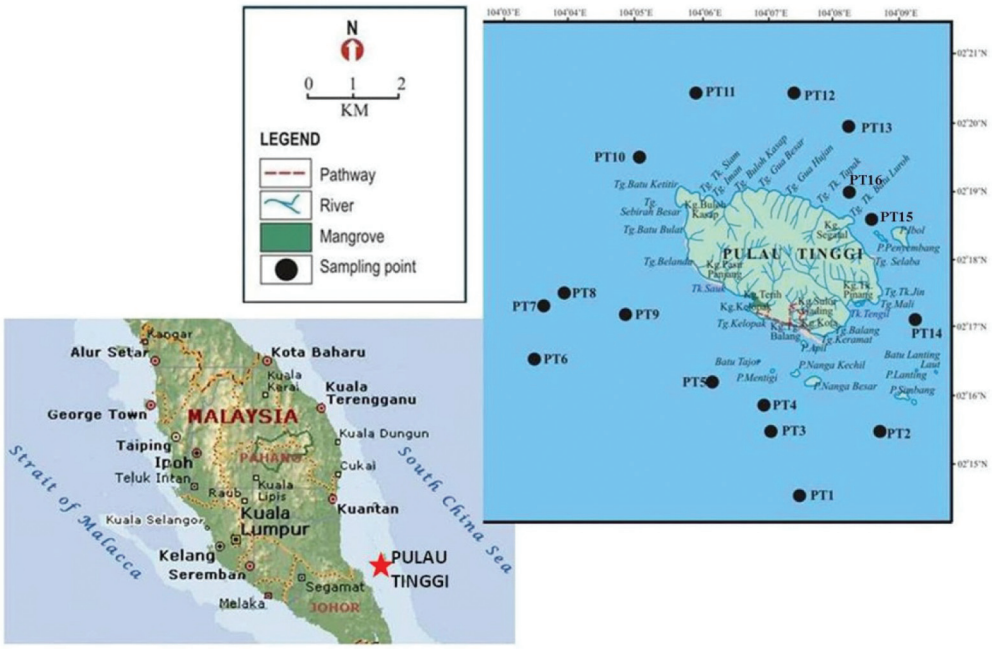

FIGURE 1. Location of sampling sites

STEROL ANALYSIS

The methods used for preparation and analysis, followed the extraction procedures used by Mudge and Norris (1997) and Masni and Mudge (2006). Approximately 30-40 g wet weight of sediment was hydrolyzed with 50 mL of 6% potassium hydroxide in methanol. The samples were refluxed for 4 h and centrifuged at 2500 rpm for 5 min. The supernatant was then funneled into the separating flask.

Non-polar lipids were extracted from the supernatant by liquid-liquid separation. About 20 mL of hexane and 10 mL of double distilled water were added to the supernatant. The mixture was then shaken vigorously and the top layer of the samples, which contained the non-polar fraction, was transferred to a florentine flask. The whole procedure was repeated once again to maximize extraction. The non-polar fraction of the samples was evaporated at 40°C in a rotary evaporator, re-dissolved in 2-3 mL of hexane and transferred to the 14 mL vial. Anhydrous sodium sulphate was added to remove any water and the polar compounds were left in the samples. The remaining solution was then filtered through filter paper and blow-dried under oxygen free nitrogen (OFN).

Samples derivatisation had to be undertaken in order to permit analysis of compounds with the Gas-Chromatograph (GC). About 2-3 drops of bis-(trimethylsilyl) triflouroacetamide (BSTFA) were added to the samples which were then heated in a heating block for 10 minutes at 60°C. Finally, they were evaporated to dryness under OFN, and then re-dissolved in 1 mL of hexane.

A computerized gas chromatography-mass spectrometry (GC-MS) (Perkin Elmer Clarus 500) was used to analyze the sterols in the samples. The temperature program used started at 80°C, increasing at 15°C min^{-1} to 300°C, then at 5°C min^{-1} to a maximum of 350°C for 10 minutes. Spectrum of each sterol compounds recorded directly into a computer program, Turbo Mass. This software used to identify compounds by their primary and secondary mass spectrum (Table 2).

QUALITY ASSURANCE PROCEDURES

Standard method and techniques were adopted whenever possible during this work. In the laboratory, analyses were carried out in Decon-90 washed glassware. The efficiency of the whole extraction process was confirmed by repeat reflux of some sediment samples; no further sterols could be detected in these later extractions. Blanks and calibration standards were used throughout the GC injections. A blank was injected first and followed by the calibration standard. Five samples were injected afterwards, and followed by the blank and calibration standard again. GC-MS injector is also cleaned with methanol in dichloromethane after each sample injected for analysis.

Random samples were extracted three times to test the reproducibility of the extraction. The mean and standard deviation of the cholesterol was 191.21 $\pm$ 7.65 ng g^{-1} dry weight of sediment. Therefore, the reproducibility of the extraction has been found to be better than 90%. Procedural blanks were also analyzed and no compounds of interest were measured in any sample. All glassware and Teflon-lined caps used in these analyses were rinsed with organic solvents prior to work.

RESULTS AND DISCUSSION

TOTAL ORGANIC CARBON (TOC)

The concentration of TOC in the surface sediment samples of Pulau Tinggi is relatively low, with the minimum of 2% and the maximum of 13% with a mean value of 0.04% (Figure 2). The highest content of TOC is found at station PT16 followed by station PT15 (10%). Both of these sampling stations are located at a river mouth where the riverside is taken up by residential areas and mangroves. As a consequence of this, both locations received an input of riverine organic matter and have a high amount of terrestrial organic matter while the other sampling stations most probably only received organic matter from marine organisms.

TABLE 2. Mass spectrum of sterols

Sterol compounds	Molecule mass	Mass diagnostic	Secondary mass diagnostic
Coprostanol	460	370	75 215 257 355
Epicoprostanol	460	370	215 257
Cholesterol	458	329	129 329 368 443
Cholestanol	460	370	215 305 384 403 445
Brassicasterol	470	380	69 129 255 341 365 380
Ergosterol	468	337	337 363
Campesterol	472	382	129 343 472
Stigmasterol	484	394	83 129 255 351
β-sitosterol	486	396	129 275 296 357 381
Dinosterol	500	388	359 388
24-nor-cholesta-22(E)-en-3β-ol	442	352	97 129 255 313 442
24-nor-cholesta-5,22(E)-dien-3β-ol	444	374	252 345 444

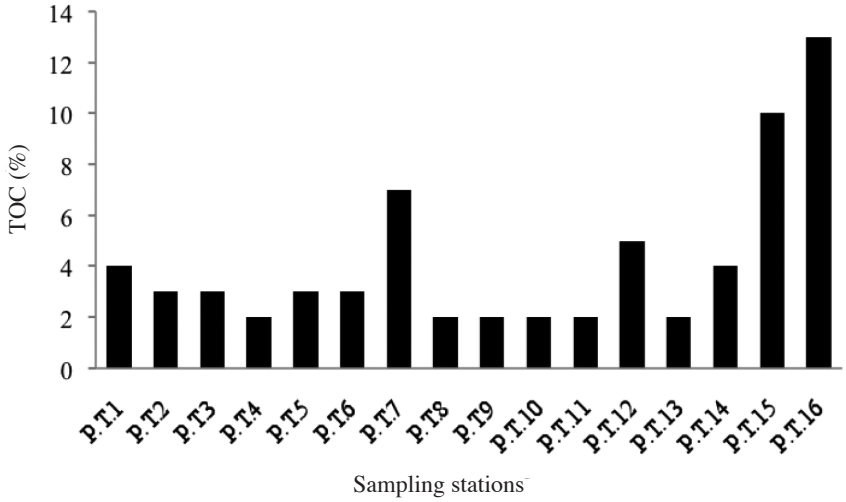

FIGURE 2. TOC content of each sampling station

Marine sediments act as sinks for organic carbon which is comprised of dissolved organic carbon (DOC) and particulate organic carbon (POC) and has the ability to indicate organic contamination and to estimate the quality level of aquatic environments (Ni et al. 2008; Smith & Hollibaugh 1993). Sterol compounds also contribute a small fraction of TOC in sediments (Mudge et al. 1998). Organic carbon in newly formed sediments are affected by factors such as biological productivity, the natural condition of sediments, the sedimentation rate, surrounding environments and the diagenesis process (Duan 2001). Organic carbon occurs naturally in the environment, and is produced by marine phytoplankton, particularly from high phytoplankton growth rate and high diatoms productivity, input from terrestrial higher plants or from anthropogenic inputs (Méjanelle & Laureillard 2008; Ni et al. 2008; Pagani et al. 1999).

STEROLS DISTRIBUTION

Individual sterols identified in the surface sediments of Pulau Tinggi are listed in Table 3 and their individual percentages of total sterols are plotted in Figure 3. Mixtures of sterols from marine and terrestrial sources were observed through eleven quantified sterol compounds.

The surface sediment samples reveal a predominance of phytosterols. These consist of campesterol, stigmasterol and β-sitosterol which are present in large quantities in higher terrestrial plants and have been used to indicate their input in aquatic sediments. Such compounds accounted for 44% of total sterols with a higher amount at station PT16. However, among these three compounds, campesterol is the major phytosterol found in all the samples, followed by stigmasterol and then lastly β-sitosterol. The high amount of phytosterols at station PT16 is fundamentally as a result of its location at the river mouth, where an input

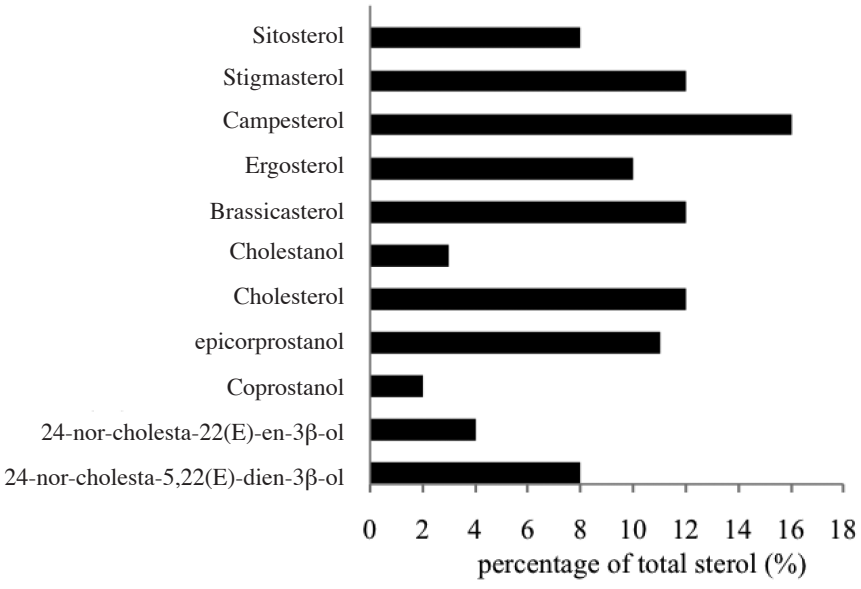

FIGURE 3. Individual percentages of sterols

TABLE 3. Sterols concentrations of each sampling stations (ng g^{-1} dry weight sediment)

	PT1	PT2	PT3	PT4	PT5	PT6	PT7	PT8	PT9	PT10	PT11	PT12	PT13	PT14	PT17	PT16
24-nor-cholesta-5,22(E)-dien-3β-ol	260.05	42.99	363.60	51.19	20.52	109.04	157.79	17.91	17.89	98.14	nd	305.52	nd	138.43	nd	2926.60
24-nor-cholesta-22 (E)-en-3β-ol	17.00	161.41	415.74	89.62	78.35	97.73	88.16	122.58	497.15	129.81	113.45	127.99	89.06	214.99	69.06	55.44
Coprostanol	24.99	187.98	28.20	24.67	10.54	30.58	20.40	15.70	9.80	222.13	59.59	25.04	46.57	13.74	38.45	89.17
Epicoprostanol	152.10	314.36	2235.42	371.41	246.12	190.95	176.24	121.82	1425.42	259.16	62.95	386.37	49.94	25.49	39.78	147.53
Cholesterol	697.96	199.08	299.85	442.28	313.70	628.32	2000.35	24.11	104.94	341.52	69.09	480.06	12.91	706.37	44.43	333.50
Cholestanol	237.50	19.07	60.22	86.66	108.92	171.56	414.11	72.51	20.28	66.40	11.64	212.39	8.60	159.28	9.04	81.16
Brassicasterol	555.82	236.63	212.71	279.24	109.33	383.95	792.18	55.85	1377.15	1381.68	60.04	433.49	45.51	424.87	2.84	40.33
Ergosterol	12.66	269.53	2067.33	82.89	285.30	83.34	nd	147.22	1726.37	168.35	62.64	270.38	49.65	117.63	23.81	225.74
Campesterol	1701.87	9.78	172.78	44.58	220.88	523.92	1080.28	68.35	231.34	162.40	12.63	1507.93	nd	511.82	nd	2757.55
Stigmasterol	139.90	195.90	33.38	167.16	522.07	366.75	760.99	1127.04	286.22	213.76	nd	97.41	nd	418.09	nd	2499.41
Sitosterol	83.17	170.74	1618.15	71.95	103.65	612.57	29.69	37.75	1002.23	99.13	34.49	186.46	32.29	62.55	30.43	192.40

nd - not detected

of terrestrial higher plants are received into the river and then transported to this sampling station by currents.

The mixing process of freshwater and seawater can cause flocculation and coagulation, which results in a rapid deposition of organic matter into the sediment (Dai & Sun 2007). In other sampling stations, which were farther from the terrestrial input however, the phytosterols found are most likely to be from the algae group and phytoplankton, as is reported in literature by Mudge et al. (1998), Volkman et al. (1998), Giner et al. (2001), Méjanelle and Laureillard (2008) and Santos et al. (2008).

The next most abundant compound identified was cholesterol which has a large concentration range within 12.91 to 2000.35 ng g^{-1} d.w. and constitutes 11% of total sterols identified. Cholesterol is the main sterol commonly quantified in sediments and could originate from a variety of sources such as sewage disposal, terrestrial enrichment and contribution by algae and zooplankton (even though it is the main higher animal sterol) (Puglisi et al. 2003; Seguel et al. 2001;Volkman et al. 1999). Consequently, this ubiquitous sterol has limitations in being used as an independent marker, although it can be applied in the form of a ratio with other sterols. In the marine environment, cholesterol is mainly derived from diatoms, microbial, aquatic macrophytes, zooplankton and benthic invertebrates (Hyun et al. 2002; Logan et al. 2001; Pratt et al. 2008; Reeves & Patton 2001; Shi et al. 2001). One of the cholesterol epimers, cholestanol, which is found in all samples, accounted for only 3.19% of the total sterol. Cholestanol is the most thermodynamically stable of all cholesterol epimers (Leeming et al. 1996). It can either be derived from the bacterial hydrogenation of cholesterol or biosynthesized by phytoplankton, zooplankton and aquatic plants (Devane et al. 2006; Froehner et al. 2008; Pittet et al. 1990). In an earlier study by Teshima and Kanazawa (1978), it is reported that in a marine environment, cholestanol also occurs among marine organisms in invertebrates such as sponges.

Other cholesterol epimers quantified in the samples were coprostanol and the coprostanol isomer, epicoprostanol, which are widely used to assess sewage contamination in aquatic environments. Coprostanol is produced in the digestive tracts of humans and higher vertebrates by the microbial reduction of cholesterol, while epicoprostanol is produced during sewage degradation and used as an indicator of sewage treatment level or old sewage in environments (Mudge & Norris 1997; Froehner et al. 2008). The amount of coprostanol determined in the samples is relatively low at only 1% of the total sterol, compared to epicoprostanol which accounted for 11% of the total. In the case of this sampling location, which is not located near to a river system into which sewage is released, it is almost impossible for coprostanol to be derived from sewage sources. A study undertaken by Venkatesan and Santiago (1989), shows that coprostanol can also be originated from biogenic sources such as cyanobacteria, microalgae, phytoplankton and zooplankton. Another way to confirm the occurrence of sewage contamination in an aquatic environment is through the sterols ratio. The ratio of coprostanol to cholesterol has been used to differentiate between biogenic and sewage sources (Patton & Reeves 1999). In Figure 4, it can be seen that none of the sampling stations reach the ratio value of one which indicates a biogenic source.

Meanwhile, epicoprostanol is an indicator of treated or old sewage in the environment (Froehner et al. 2008; Mudge & Seguel 1999; Mudge & Duce 2005). The compound is formed from the microbial conversion from cholesterol to coprostanol and lastly to epicoprostanol during sewage treatment, thus the compound is found in high levels in treated sewage but in lower levels in fresh sewage (Bull et al. 2002; Devane et al. 2006; Seguel et al. 2001). In this study, however, the epicoprostanol quantified does not originate from sewage matter or coprostanol, and therefore does not indicate the degree of treated sewage in the study area. According to Thoumelin et al. (1997), epicoprostanol is also formed from the reduction of phytosterols.

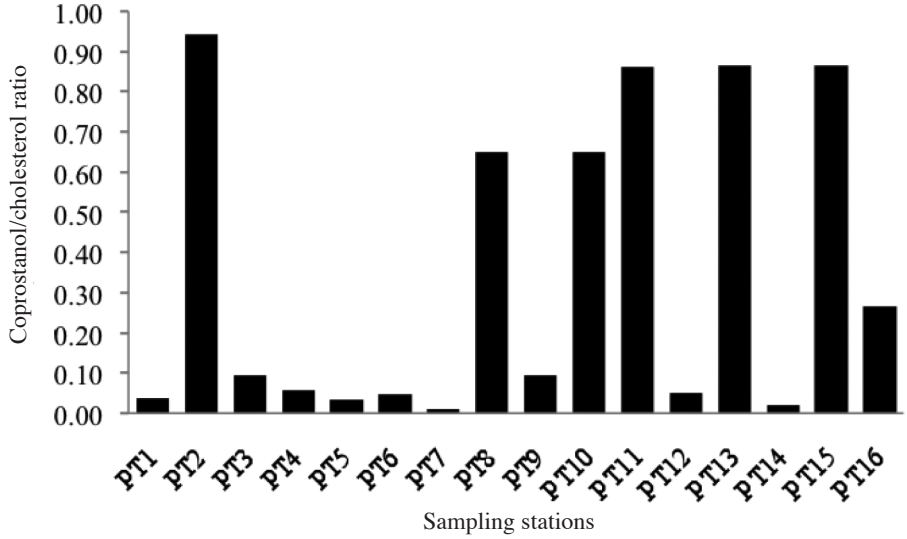

FIGURE 4. Coprostanol/cholesterol ratio in Pulau Tinggi

Brasiccasterol and ergosterol are other compounds which are found in high concentrations, constituting 11% and 10% of the total sterol, respectively. Generally, brasiccasterol is mainly used as an indicator of marine inputs, particularly those found in diatoms, while ergosterol is an endogenous sterol of fungi and some microalgae (Fahl & Stein 1999; Puglisi et al. 2003). The remaining sterols found in the samples are 24 nor-cholesta-5,22(E)-dien-3β-ol and 24 nor-cholesta-22(E)-en-3β-ol and according to Mudge et al. (1998), based on Ponomarenko et al. (1995) literature, the presence of these compounds are used to indicate oxic marine conditions, and they are commonly found in sponges and selected marine worms which are unusual in freshwater systems.

CONCLUSION

Sterols have been successfully used to assess organic matter input into marine sediments and to identify their sources. Our study on the concentration levels of sterols in the marine surface sediments of Pulau Tinggi, Johor, Malaysia showed that the distribution of the lipid compounds are derived from a variety of sources; from marine organisms such as phytoplankton, microalgae, zooplankton, sponges to higher terrestrial plants. Phytosterols as the most abundant compounds quantified followed by cholesterol, brassicasterol and fecal sterols. However, in this study area phytosterols cannot originate from higher terrestrial plants due to the fact that most sampling stations are located far from a terrestrial input, except for station PT15 and PT16 which receive input from the river nearby. This result was also reflected in the TOC content which is the highest at these stations. The concentration of the main fecal sterol, coprostanol only contributed to 1% of the total sterols identified but its epimer, epicoprostanol accounted for 11%. However, the coprostanol/cholesterol ratio indicates that coprostanol originated from biogenic sources, such that epicoprostanol is not formed from treated or old sewage but is instead a product of the microbial conversion of coprostanol and of the reduction of phytosterols. In conclusion, results from this study are fascinating as they presented various sources of different sterols quantified in the marine sediments.

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