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Microalgal lipid markers for paleoclimatic research

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ABSTRACT: Primary producers, mainly represented in the marine environment by microalgae, synthesize a wide variety of lipid constituents, some of which are recognized as 'biomarkers' that can be used to identify sources of organic matter. Furthermore, as phytoplankton rapidly reflect climate variations, the identification of organic matter in sediments may help to understand present or ancient specific environmental conditions. To highlight differences in some lipid constituents (hydrocarbons, sterols and fatty acids) of different taxa of microalgae, we determined these compounds in the diatom *Cylindrotheca closterium* and dinoflagellate *Scrippsiella trochoidea* isolated from the Adriatic Sea and grown in batch cultures. Total lipids were extracted in chloroform-methanol and, after separation, analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The only hydrocarbons present in these species were *cis*-3,6,9,12,15,18-heneicosahexaene (HEH) and squalene. Analysis of sterols in *C. closterium* revealed that only 4-desmethylsterols were present. Conversely, in *S. trochoidea*, 2 different groups of sterols (4-desmethylsterols and 4a-methylsterols) were identified. Besides the fatty acid 16:0, which predominated in both species, high percentages of 16:1n-7 and 20:5n-3 were observed in *C. closterium*, whereas 18:4n-3 and 22:6n-3 were detected in *S. trochoidea*. Differences in lipid patterns were observed between *C. closterium* and *S. trochoidea*. These differences might help understand the Adriatic Sea Basin's paleoclimatic history.

KEY WORDS: Lipid biomarkers · Cultured microalgae · Paleoclimatic research

1. INTRODUCTION

Marine coastal environments are characterised by high trophic production. The primary producers are mainly Bacillariophyta (diatoms) and Dinophyta (dinoflagellates), which are prominent constituents of 'microphytoplankton' and 'microphytobenthos'. These 2 classes of algae can form extensive blooms and are the main suppliers of organic matter in the marine food web.

Lipids are important constituents of this fraction and, due to their good preservation in marine sediments and to their specific structure, are widely used as biomarkers in biogeochemical studies (Volkman et al. 1998, Tonon et al. 2002) for determining the source, transformation and fate of organic matter and for determining specific environmental conditions (Grossi et al. 2003). Most of these lipids come from microalgae, which have a characteristic and distinct lipid composition: sterols, fatty acids, hydrocarbons, etc.

Phytoplankton rapidly reflect environmental changes and are thus a potentially interesting tool for the research on paleoclimates. Nevertheless, due to the difficulty in studying fossil phytoplankton, it may be convenient to identify alternate biogeochemical tracers that enable the identification of useful taxa as records of present or ancient specific environmental conditions.

In the present study we determined hydrocarbons, sterols and fatty acids in 2 cultured microalgal species that are widespread in the Adriatic Sea: the diatom *Cylindrotheca closterium* and the dinoflagellate *Scrippsiella trochoidea*. Our aim was to find differences in the lipid composition in these 2 important species that belong to different classes, in order to eventually use these differences as a tool for paleoenvironmental research.

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2. MATERIALS AND METHODS

2.1. Algal cultures

Algal species were isolated from the natural phytoplanktonic association of the Emilia-Romagna coast, Adriatic Sea, Italy. Strains were maintained in sterile f/10 medium at 20°C under a 16:8 h light:dark cycle at about 90 µmol m^{-2} s^{-1} by cool white lamps. For the experimental work, cultures were transferred into 2 l sterile Erlenmeyer flasks sealed with cotton plugs and containing 1 l f/2 medium (McLachlan 1973). All cultures were in 3 replicate flasks. Cell counts were made every 2nd day in settling chambers using the Utermöhl (1931) method. For lipid analysis, the cells of every flask were collected by centrifugation during the stationary phase of growth when the highest density was present. Analyses were performed in triplicate.

2.2. Lipid analyses

The extraction of total lipids was carried out in chloroform-methanol (Bligh & Dyer 1959). Fatty acid methyl esters (FAMEs) were obtained by a sodium methoxide-catalysed transmethylation carried out on a fraction of the total lipid extract, while hydrocarbons and sterols were separated from total lipids by thin-layer chromatography (TLC); the sterol fraction was treated with N,O-*bis*-(trimethylsilyl)-acetamide (BSA) to obtain the O-(trimethylsilyl)-derivatives (OTMS). Hydrocarbons, sterols, and fatty acids (FAs) were then analysed by gas chromatography (GC). Sterols and hydrocarbons were processed by a Carlo Erba HRGC 5160 MEGA gas chromatograph equipped with a flame ionization detector (FID) and a fused silica capillary column coated with OV1 (25 m × 0.32 mm, film thickness = 0.4 µm). For hydrocarbon determination, oven temperature was increased from 120 to 295°C at the rate of 3.5°C min^{-1} and the final isothermal was held for 4 min. For sterol-derivatives, the same fused silica capillary column of the hydrocarbons was used and the same general conditions were maintained an oven isotherm temperature of 280°C. Hydrocarbon and OTMS sterol mass spectra were obtained by gas chromatography-mass spectrometry (GC-MS) using a Fisons MD 800, 70 eV coupled to a Fisons 8000 gas chromatograph equipped with a fused silica capillary column coated with Rtx-5MS (30 m × 0.25 mm; film thickness = 0.25 µm).

FAMEs were analysed by a Fisons instrument 9000 gas chromatograph equipped with an FID and a fused silica capillary column coated with Supelco Omega Wax 320 (30 m × 0.32 mm; film thickness = 0.25 µm); oven temperature was increased from 180 to 200°C at the rate of 2.0°C min^{-1} and the final isothermal was held for 45 min. FAMEs were identified by comparison of retention times with various SUPELCO and SIGMA standards.

2.3. Statistical analysis

Values were expressed as means ± SD. Results obtained were compared using Student's *t*-test for independent samples (STAT software); values of $p < 0.05$ were considered significantly different.

3. RESULTS AND DISCUSSION

3.1. Cell growth

Growth of *Cylindrotheca closterium* and *Scrippsiella trochoidea* in conditions described in 'Materials and methods' was followed up to Day 18. *C. closterium* exhibited exponential growth until Day 4, followed by a stationary phase from Day 6 to Day 18, with a maximum cell density of 2 000 000 cells ml^{-1}. *S. trochoidea* showed a lag phase until Day 5, exponential growth until Day 13 (with maximum cell density 36 000 cells ml^{-1}), and a very short stationary phase followed by a rapid decline in the culture.

3.2. Total lipids and hydrocarbons

The total lipid content of *Cylindrotheca closterium* (34.8 ± 3.8% dry wt) was in accordance with or slightly higher than values obtained by other authors (Brown et al. 1997, Renaud et al. 1999). On the contrary, that of *Scrippsiella trochoidea* (10.0 ± 1.2% dry wt) was lower than those recently determined in other species of dinoflagellates (Mansour et al. 2003).

The total hydrocarbon fraction was 3.2 ± 2.1 µg g^{-1} dry wt in *Cylindrotheca closterium* and 1.1 ± 0.4 µg g^{-1} dry wt in *Scrippsiella trochoidea.* In both species, a prevalence of the *cis*-3,6,9,12,15,18-heneicosahexaene (HEH) was observed, which is typical of many phytoplankton classes, especially diatoms and dinoflagellates. This olefine represented 86.1 ± 1.6% of total hydrocarbons in *C. closterium* and 62.3 ± 2.5% in *S. trochoidea*. Due to its wide diffusion, HEH may be regarded as an important indicator of present and ancient trophic conditions in environments from where it was isolated (Volkman et al. 1989, 1998). Squalene, interesting as a metabolic precursor of sterols, was the other hydrocarbon found, at levels of 13.8 ± 1.6% and 37.7 ± 2.5% of total hydrocarbons in *C. closterium* and *S. trochoidea*, respectively.

3.3. Sterols

Analyses of sterols in *Cylindrotheca closterium* revealed that only 4-desmethylsterols, a group of sterols lacking the methyl group in C-4, were present in this species, with high percentages of 22-dehydrocholesterol and cholesterol (amounts of these 2 sterols were significantly different to those of other sterols, $p < 0.05$) followed by low percentages of stigmasterol, β-sitosterol and campesterol (Table 1).

The prevalence of 22-dehydrocholesterol is common in diatom species belonging to the order Bacillariales, such as *Cylindrotheca closterium* (Barrett et al. 1995, Serrazanetti et al. 2002a). Conversely, 24-methylenecholesterol or other desmethylsterols prevail in diatom species belonging to the order of Biddulphiales (Gladu et al. 1991, Barrett et al. 1995). The presence of 22-dehydrocholesterol and 24-methylenecholesterol is considered to be a traditional indication of marine algal input in sediments (Hudson et al. 2001). Cholesterol used to be regarded as an animal sterol, but is now described as one of the common sterols found in diatoms (Tsitsa-Tzardis et al. 1993), dinoflagellates (Volkman et al. 1999) and the majority of members of the Rhodophyceae (Patterson 1991, Serrazanetti et al. 2002b). The minor sterols found in *C. closterium*, in particular the β-sitosterol, used to be considered as unique to vascular plants, but some diatoms also produce these sterols in appreciable quantities (Barrett et al. 1995, Volkman et al. 1998). However, in the marine environment it is possible to establish the marine or terrestrial origin of these latter sterols by comparison of individual sterol abundances with concentrations of total lignin-derived phenols and 3,4-dimethoxybenzoic acid methyl ester (Hudson et al. 2001).

The sterol composition of *Scrippsiella trochoidea* was very different to that of *Cylindrotheca closterium* (Table 1). Two different groups of these compounds were isolated: desmethylsterols (1-5) and 4α-methylsterols; the latter with the methyl group at C-4 (Withers 1987).

The 4α-methylsterols were the most abundant; among all the determined sterols, dinosterol (28.5 ± 1.7%; present in significantly higher amounts than other sterols, $p < 0.05$), together with 4α-24-dimethyl-5α-cholest-22-en-3β-ol, 4α-24-dimethyl-5α-cholestan-3β-ol and 2 epimers of dinostanol (Volkman et al. 1999, Mansour et al. 2003), accounted for >70% of total sterols. The abundance of 4α-methylsterols in *Scrippsiella trochoidea* is common in many dinoflagellates (Withers 1987, Volkman et al. 1998).

The main desmethylsterol isolated in *Scrippsiella trochoidea* was 5α-cholestanol-3β-ol (significantly different with respect to other desmethylsterols, $p < 0.05$). Sterols with a fully saturated ring system such as 5α(H)-stanols often occur in dinoflagellates, but are uncommon in other marine microalgae (Robinson et al. 1984, Volkman et al. 1998).

Our results on the sterol composition of this dinoflagellate are different from those reported by Harvey et al. (1988) for the same species, but are fairly similar to those obtained by Mansour et al. (1999) from the *Scrippsiella* sp. CS-295/c strain.

A wide variety of desmethylsterols was previously isolated in both diatom and dinoflagellate species; in contrast, 4α-methylsterols and 5α(H)-stanols were detected only in dinoflagellates. The same sterols have been identified by us (Piretti et al. 1997, Serrazanetti et al. 2002c) and by other authors working on other algal species (Barrett et al. 1995, Mansour et al. 2003), in mucilaginous aggregates of the Adriatic Sea, as well as in recent and fossilised terrestrial and marine sediments and in settling particles (Hudson et al. 2001, Pinturier-Geiss et al. 2002, Pistocchi et al. 2005).

Table 1. *Cylindrotheca closterium* and *Scrippsiella trochoidea*. Sterol composition (percentage of total sterols, mean ± SD)

	Sterol	*C. closterium*	*S. trochoidea*
1	24-nor-5α-cholest-22-en-3β-ol	–	0.5 ± 0.2
2	Cholesta-5,22-dien-3β-ol (22-dehydrocholesterol)	49.5 ± 0.9[a]	–
3	Cholesta-5-en-3β-ol (cholesterol)	35.3 ± 1.6	4.2 ± 0.8
4	5α-cholestan-3β-ol (cholestanol)	–	7.9 ± 1.3[b]
5	24-methylcholest-5,22-dien-3β-ol	–	1.5 ± 0.4
6	24-methylcholest-5-en-3β-ol (campesterol)	2.3 ± 0.5	–
7	24-ethylcholest-5,22-dien-3β-ol (stigmasterol)	6.6 ± 1.0	–
8	24-ethylcholest-5-en-3β-ol (β-sitosterol)	6.4 ± 0.8	–
9	24-ethyl-5α-cholestan-3β-ol	–	2.1 ± 0.3
10	4α-methyl-5α-cholestan-3β-ol	–	1.3 ± 0.3
11	4α,24-dimethyl-5α-cholest-22-en-3β-ol	–	12.5 ± 1.6
12	4α,24-dimethyl-5α-cholestan-3β-ol	–	12.4 ± 1.1
13	4α,23,24-trimethyl-5α-cholest-22-en-3β-ol	–	28.5 ± 1.7[c]
14	4α-methyl-24-ethyl-5α-cholest-8(14)-en-3β-ol	–	3.8 ± 0.5
15	4α,23S,24R-trimethyl-5α-cholestan-3β-ol	–	11.7 ± 1.2
16	4α,23R,24R-trimethyl-5α-cholestan-3β-ol(-13) = dinosterol, (-15 & -16) = dinostanol epimers	–	13.5 ± 1.1

[a] $p < 0.05$ between this and all other sterols in *C. closterium*
[b] $p < 0.05$ between this and other desmethylsterols (1, 2, 3, 5) in *S. trochoidea*
[c] $p < 0.05$ between this and other sterols in *S. trochoidea*

3.4. Fatty acids

The FA composition of *Cylindrotheca closterium* and *Scrippsiella trochoidea* is given in Table 2. The

Table 2. *Cylindrotheca closterium* and *Scrippsiella trochoidea*. Fatty acid composition (percentage of total FAs, mean ± SD)

Fatty acid	*C. closterium*	*S. trochoidea*
14:0	8.7 ± 0.8	9.8 ± 0.8
15:0	1.1 ± 0.6	0.2 ± 0.1
16:0	20.3 ± 0.1[a,c]	26.1 ± 1.1[b,c]
18:0	4.8 ± 0.3	3.8 ± 0.4
24:0	–	0.2 ± 0.1
Total saturated	34.9 ± 0.1	40.1 ± 0.9
14:1n-5	0.4 ± 0.1	0.1 ± 0.1
16:1n-7	17.9 ± 1.0[d]	3.6 ± 0.2[d]
18:1n-9	4.5 ± 1.1	7.8 ± 0.7
18:1n-7	1.2 ± 0.1	1.6 ± 0.3
20:1n-9	0.4 ± 0.1	0.2 ± 0.1
Total monosaturated	24.4 ± 0.9	13.2 ± 0.7
16:2n-7	1.2 ± 0.1	–
16:2n-4	1.9 ± 0.3	–
16:3n-4	5.8 ± 0.1[e]	1.1 ± 0.3
16:4n-1	1.8 ± 0.1	–
18:2n-6	4.1 ± 0.3	0.6 ± 0.2
18:3n-6	2.9 ± 0.5	1.2 ± 0.2
18:3n-3	0.3 ± 0.1	–
18:4n-3	1.9 ± 0.3	8.1 ± 0.8[f]
18:5n-3	–	11.2 ± 0.9[g]
20:4n-6	2.7 ± 0.4	–
20:4n-3	0.7 ± 0.1	–
20:5n-3	9.5 ± 0.3[e,h]	5.7 ± 0.6
22:6n-3	4.8 ± 1.2	17.9 ± 1.2[f,g,i]
Total polyunsaturated (PUFA)	37.6 ± 0.5	45.8 ± 0.9
Others	3.1 ± 0.6	0.9 ± 0.2

[a] $p < 0.05$ between 16:0 and other FAs in *C. closterium*
[b] $p < 0.05$ between 16:0 and other FAs in *S. trochoidea*
[c] $p < 0.05$ between 16:0 in the 2 species
[d] $p < 0.05$ between 16:1n-7 in the 2 species
[e] $p < 0.05$ between 20:5n-3 and 16:3n-4 in *C. closterium*
[f] $p < 0.05$ between 22:6n-3 and 18:4n-3 in *S. trochoidea*
[g] $p < 0.05$ between 22:6n-3 and 18:5n-3 in *S. trochoidea*
[h] $p < 0.05$ between 20:5n-3 and other PUFA in *C. closterium*
[i] $p < 0.05$ between 22:6n-3 and other PUFA in *S. trochoidea*

proportions of the major FA classes were quite similar between the two species: the polyunsaturated (PUFA) class prevailed, followed by the saturated and monounsaturated FAs respectively. However, when the distribution of single FAs was compared between *C. closterium* and *S. trochoidea*, remarkably different patterns became apparent, which reflect the different taxonomic position of the 2 species. Only the pattern of saturated FAs was similar between *C. closterium* and *S. trochoidea*; saturated FAs were present in relatively high total amounts, with similar percentages of 14:0 and 18:0 and with a prevalence of 16:0 (20.3 ± 0.1 and 26.1 ± 1.1%, respectively), when compared to all other FAs ($p < 0.05$).

Total saturated FA levels in the diatom *Cylindrotheca closterium* and in the dinoflagellate *Scrippsiella trochoidea* was of the same order of magnitude as previously reported in other species belonging to the same classes (Nichols et al. 1983, Volkman et al. 1989, Renaud et al. 1999) or, for *S. trochoidea*, higher than those determined in other species and in *Scrippsiella* sp. (Harvey et al. 1988, Mansour et al. 1999). High levels of the ubiquitous 16:0 were previously determined in diatoms (Pernet et al. 2003) and dinoflagellates (Mansour et al. 1999, 2003), concurrent with high levels of 14:0 (Volkman et al. 1989, Renaud et al. 1999, 2002). High percentages of these 2 FAs might explain the relative abundance of total saturated FAs in several species of algae, as in our samples.

The percentage of monounsaturated FAs found in *Cylindrotheca closterium* (24.4 ± 0.9%) was almost twice that determined in *Scrippsiella trochoidea* (13.2 ± 0.7%), and was due to significantly higher levels of 16:1n-7 (17.9 ± 1.0%) in the former species ($p < 0.05$). High percentages of this FA (sometimes higher than that obtained by us for *C. closterium*) are usual in diatoms, but not in dinoflagellates (Volkman et al. 1998). The low amounts of the bacterial 18:1n-7 detected in both species confirmed that the analysed cultures were axenic (Volkman et al. 1989).

In addition, the composition of PUFAs was particularly different and characteristic between the species. Several PUFAs were identified in *Cylindrotheca closterium*, but only 20:5n-3 (9.5 ± 0.3%) and 16:3n-4 (5.8 ± 0.3%) with percentages >5% (these values were significant different with respect to other PUFAs; $p < 0.05$). In this species, C16 PUFAs were particularly diverse when compared with our *Scrippsiella trochoidea* samples and with species belonging to other classes. The presence of these C16 PUFAs in diatoms, derived from a further specific desaturation of 16:1n-7, might explain the lower presence of n-3 and n-6 FAs when compared with, for example species belonging to other divisions such as Chlorophyta. This difference in C16 PUFA composition (including differences in double-bond positions) can be used in environmental studies to estimate the presence of diatoms. The FA 20:5n-3 has been reported often in diatom species, but in a wide range of concentrations. The percentage of this FA in our *C. closterium* samples was rather low. On the other hand, the percentage of 22:6n-3, which is usually found in low amounts in diatoms (Volkman et al. 1989, Zhukova & Aizdaicher 1995, Renaud et al. 2002, Rousch et al. 2003), turned out to be rather high in our samples (4.8 ± 1.2%).

The PUFA composition in *Scrippsiella trochoidea* was simple and dominated by only a few high unsaturated fatty acids (HUFAs). Among these, 22:6n-3 was the most abundant (17.9 ± 1.2%), and 18:5n-3, 18:4n-3 and 20:5n-3 were present at rates of 11.2 ± 0.9%, 8.1 ± 0.8% and 5.7 ± 0.6%, respectively. In *S. trochoidea*, we detected C18 PUFAs (namely 18:5n-3 and 18:4n-3) at concentrations ranging from that measured by Harvey

et al. (1988) in the same species and that recorded by Mansour et al. (1999) in *Scrippsiella* sp. CS-295/c strain. High percentages of these C18 PUFAs, of 22:6n-3 and sometimes of 20:5n-3 are typical in dinoflagellates and can represent chemotaxonomic markers of this class (Volkman et al. 1998).

4. CONCLUSIONS

Our results show that high percentages of specific desmethylsterols, 22-dehydrocholesterol and cholesterol, and FAs 16:1n-7, C16 PUFAs and 20:5n-3, which are common in diatoms, were present in *Cylindrotheca closterium*. The specific 4α-methylsterols, C18 PUFAs and 22:6n-3 isolated in *Scrippsiella trochoidea* are common in dinoflagellates. Although this study concerns only 2 cultured species of algae, results underline the high degree of variability and peculiarity in the composition of some lipid constituents in marine microalgae. This confirms that some of these compounds are characteristic of the different plant classes and may be useful as biochemical indicators: it is known that lipid composition is strictly dependent on the availability of nutrients, temperature levels, light intensity or light:dark cycles and culture phases (Piretti et al. 1997, Volkman et al. 1998, 1999, Rousch et al. 2003).

Knowledge of the natural lipid composition of different species gives us a better understanding of the impact of algal constituents on ocean biogeochemical cycles and of the vertical flux of organic matter from surface to seabed (Hayakawa et al. 1996). Further in-depth studies on lipid patterns in single algal species, in different marine samples and in recent and ancient sediments will provide useful information for better understanding trophic and climatic factors of the past and present.

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