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# Chemical fingerprinting of algaenans using $RuO_4$ degradation

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**Abstract**

The freshwater green microalgae, *Oocystus solitaria* Wittrock f. maior Wille, *Pediastrum braunii* Wartmann, *Pediastrum kawraiskyi* Schmidle, *Sorastrum spinulosum* Nägeli and *Coelastrum reticulatum* (Dangeard) Senn were investigated with respect to the presence of algaenan and the corresponding $RuO_4$ chemical fingerprint. All species, with the exception of *O. solitaria*, were shown to possess a cell wall resistant toward strong acid and base treatment. By analysis of the $RuO_4$ chemical degradation products from these materials a molecular fingerprint was obtained having similar features to the $RuO_4$ fingerprints of *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum* algaenans reported in earlier studies. Previously it had been shown that these $RuO_4$ fingerprints could also be obtained from ancient sediments, indicating the presence of algaenan as part of the organic matter. The results from this study show that the chemical composition of algaenans is possibly a chemotaxonomic feature and can be used to chemically explore the algal contribution to sedimentary organic matter on a family level. Furthermore, it illustrates that the use of $RuO_4$ degradation combined with gas chromatography is a suitable analytical tool for the purpose of chemical fingerprinting of algaenans.


## 1. Introduction

It is generally known that the cell walls of several algal species are able to resist diagenetic processes that recycle the organic matter in the bio- and geosphere (Blokker, 2000; de Leeuw and Largeau, 1993; Goth et al., 1988; Hutton, 1987; Largeau and de Leeuw, 1995; Tegelaar et al., 1989; van Bergen et al., 2004). There are also various reports of morphologically intact algal remains, whose presence supplies valuable information on the environmental conditions during the time of deposition (e.g. Jankovská and Komárek, 2000). More insight into the processes and chemistry involved in the preservation of these algal remains (Allard et al., 2002; Allard and Templier, 2001; Blokker et al., 2000; Blokker et al., 1998a; Gelin et al., 1997;

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Simpson et al., 2003) would not only help in obtaining a better understanding of past environmental settings, but also of the physiological function of these diagenetically resistant cell walls. The material comprising the resistant cell walls is termed algaenan and is often associated with the presence of a 10–30 nm thick trilaminar sheet (Corre et al., 1996; de Leeuw and Largeau, 1993; Derenne et al., 1992a). Under transmission electron microscopy (TEM) this trilaminar sheet consists of a middle electron-translucent layer and two opaque outer layers (Corre et al., 1996 and references therein). Operationally, algaenan is defined as an algal-derived material resistant towards treatment with strong acids and bases and insoluble in common organic solvents – in other words a three dimensional polymeric network composed of constituents that are interconnected by non-hydrolysable linkages. Though algaenan obviously has an important protective role (Corre et al., 1996), the exact physiological function remains unclear. It was suggested that algae living in relatively small habitats and relying on spreading via, for example, wind or on the feathers of birds might be favoured by having with a waterproof algaenan layer (Versteegh and Blokker, 2004).

Despite various studies and reports, the exact chemical composition of algaenan remains a point of debate, although there seems to be a general agreement on the highly aliphatic nature of the building blocks for a recent review see (Versteegh and Blokker, 2004). The use of various complementary analytical techniques such as analytical pyrolysis, controlled chemical degradation, infrared spectroscopy (IR) and solid state $^{13}C$ nuclear magnetic resonance techniques (NMR) can help in gaining a better insight into the chemistry and function of these biopolymers. Also, the algaenan of *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum* has been analyzed extensively using chemolytic methods (Blokker et al., 1998a; Schouten et al., 1998), which reveal that long chain ω-hydroxy fatty acids may play an important role as monomeric building-blocks. Analysis of sedimentary organic matter containing high amounts of algal remains revealed the presence of these ether-linked long-chain ω-hydroxy fatty acids, by way of $RuO_4$ chemolysis followed by determination of the distribution of the released compounds with gas chromatography-mass spectrometry (GC/MS; Blokker et al., 2000; Li et al., 2004; Yoshioka and Ishiwatari, 2004). The results can explain why algaenan is diagenetically resistant. It is noteworthy that the GC fingerprint of the $RuO_4$ degradation products of extant materials is comparable to that from fossil algae, suggesting that the chemical fingerprint stays virtually intact even after 50 million years of burial (Blokker, 2000; Yoshioka and Ishiwatari, 2004). This is particularly interesting considering the fact that the growth conditions of these fossilised algae are most likely not comparable to that of cultured algae, indicating that growth conditions do not appear to influence the $RuO_4$ fingerprint. These observations suggest that even when morphological recognition is impossible, it is be possible to recognize algal remains based on their chemical signature.

In this study several Chlorophyte algae of the order Chlorococcales were cultured and investigated for the presence of resistant biochemicals in their cell walls. We describe investigation of the Chlorococcal algae *O. solitaria*, *C. reticulatum*, *P. kawraiskyi*, *P. braunii* and *S. spinulosum*. Both *Pediastrum* species were chosen to explore the inter-species variation in algaenan chemistry. *S. spinulosum* has various morphological similarities to *Pediastrum* species and also prefers eutrophic habitats, while *O. solitaria* is a widespread species living in habitats like small puddles in peatlands (Streble and Krauter, 1988). The aim of this study was, in the event of the presence of a resistant material, to obtain a chemical fingerprint by using $RuO_4$ degradation. In combination with pyrolysis(py)-GC/MS, it would then possible to obtain insight into the variations in chemical structure and the possibility for application of this information to sedimentary organic matter. Furthermore, the results should provide a basis for further research on the chemistry of algaenan, its function in algae and the processes involved in its preservation.

## 2. Experimental

### 2.1. Culture conditions and algaenan isolation

*O. solitaria* Wittrock f. maior Wille (SAG 83.80), *P. braunii* Wartmann (SAG 43.85), *P. kawraiskyi* Schmidle (SAG 35.81), *S. spinulosum* Nägeli (SAG B40.81) and *C. reticulatum* (Dangeard) Senn (SAG 8.81) were obtained from the Sammlung von Algenculturen at the University of Göttingen (SAG) and were cultured in 1 L batches cultures using a Kates and Jones medium at 19 °C, aerated with normal air supplemented with 2% $CO_2$. The cultures were har-

vested at the end of the log growth phase and worked up as previously described (Blokker et al., 1998a,b) by using respectively mechanical, enzymatic and chemical steps. A small fraction of the culture was freeze dried to determine the dry weight biomass.

### 2.2. FTIR

A Bruker Vector 22 was used, scanning over a frequency range of 500–3400 $cm^{-1}$ and using KBr pellets containing 1 mg of dry material.

### 2.3. $RuO_4$ treatment

The treatment was modified from a procedure reported earlier (Blokker et al., 1998a). In short, the final residue (ca. 5 mg) was ultrasonically suspended in a mixture of 1 mL chloroform, 1 mL acetonitrile and 2 mL of an aqueous solution of $NaIO_4$ (0.2 M, pH 3–4). After addition of 6 mg $Ru(III)Cl_3$ the two phase system was allowed to react in an ultrasonic bath. After 4 h, 3 mL water and 2 mL hexane were added. The residual material was removed by centrifugation and the organic layer was transferred into methanol (MeOH) (0.5 mL). The aqueous layer was extracted with 2 mL hexane (1×) and 2 mL dichloromethane (DCM) (2×) and the organic layers combined with the first organic extract. The black Ru salts were precipitated from the organic layer by centrifugation. The remaining supernatant was washed with 0.5 mL $Na_2S_2O_3$ solution (5% in $H_2O$). The extract was dried over $Na_2SO_4$, evaporated to dryness under a nitrogen flow and derivatized with diazomethane prior. Deuteriated dodecane (10 μg/mg algal material) was used as an internal standard.

### 2.4. GC/MS, GC/flame ionisation detection (FID) and Curie-point py-GC/MS

GC/MS, GC/FID and py-GC/MS equipment and conditions were as previously described (Blokker et al., 1998a).

GC/FID was performed with a Carlo Erba GC-8000 series instrument equipped with a flame ionisation detector (FID), an on-column injector and a 25 m fused silica capillary column coated with CP-Sil 5 (0.32 mm ID; film thickness 0.12 μm). Helium was used as the carrier gas and a temperature of the FID was 320 °C. The oven was typically programmed from 70 °C to 130 °C at 20 °C/min, then to 320 °C at 6 °C/min and held at this temperature for 15 min. For the analysis of the $RuO_4$ product mixture the following temperature programme was used: 1 min at 40 °C and subsequently heated to 320 °C at 4 °/min. The final temperature was held for 10 min.

GC/MS was conducted with a Hewlett-Packard 5890 series II gas chromatograph fitted with a 25 m × 0.32 mm CP-Sil 5 (film thickness 0.12 μm) fused silica capillary column coupled to the electron ionisation source of a VG Autospec Ultima mass spectrometer (mass range $m/z$ 50–800 at a resolution of 1000; cycle time 1.8 s; ionisation energy 70 eV). Helium was used as the carrier gas and the temperature programme was similar to that used for the FID method described above.

The py-GC/MS was conducted with a VG Autospec Ultima mass spectrometer connected to a Hewlett-Packard 5890 series II gas chromatograph equipped with a FOM-4LX pyrolysis unit. Samples were pressed on a flattened ferromagnetic wire with a Curie point temperature of 610 °C. The wire was inserted into a glass liner, introduced into a FOM-4LX pyrolysis unit and inductively heated for 10 s. The desorbed fragments were flushed to the capillary column using helium as carrier gas. The gas chromatograph was equipped with a cryogenic unit and programmed from 0 °C (5 min) to 320 °C (hold time 10 min) at 3 °C/min. Helium was used as carrier gas. Compounds were ionised at 70 eV and analysed over a range of $m/z$ 50–800 at a resolution of 1000 and a cycle time of 1.8 s.

## 3. Results

### 3.1. Workup procedure

To isolate the acid and base resistant algaenan, all cell contents must be removed so that no secondary reactions can take place between the algaenan and/or released cell constituents such as amino acids and carbohydrates (Allard et al., 1997, 1998). This prevents formation of artefacts (e.g. Maillard reaction products) that can contaminate the algaenan. Since such an isolation process requires a mild treatment on the one hand and more vigorous conditions to remove all other cell polymers like proteins and polysaccharides on the other hand, a series of mechanical, enzymatic and chemical methods was used. Since after the mechanical, enzymatic and mild

trifluoroacetic acid (TFA) treatment almost all hydrolysable cell polymers like polysaccharides and proteins are removed, a more artefact-prone treatment with sulfuric acid can be used safely to remove the last traces of residual hydrolysable material. A final saponification yields a material resistant to acids and bases and insoluble in common organic solvents. All algae investigated in the present study, except *O. solitaria*, yield a final residue. *P. braunii* provided 1% (dry wt.), *P. kawraiskyi* 6% (dry wt.), *S. spinulosum* 10% (dry wt.) and *C. reticulatum* 2% (dry wt.) of residue. Only *P. braunii* and *P. kawraiskyi* yielded enough material for FTIR analysis (Fig. 1) as a result of ensuring that an artefact-free algaenan was obtained. Absorption peaks around 3000–2800 $cm^{-1}$, 1466 $cm^{-1}$ ($CH_2$ asymmetric bending) and 720 $cm^{-1}$ [$(CH_2)_{n \geqslant 4}$ skeletal vibrations] are typical for aliphatic material such as algaenan (Blokker et al., 1998a). Bands between ~1550 and ~1700 $cm^{-1}$ are typical for carbonyl groups and indicative for the presence of carboxylic acid groups. Bands around 1100 $cm^{-1}$ originate from C–O vibrations, possibly reflecting ether bonds or hydroxyl groups (C–O stretching and OH deformation C–O stretching).

Earlier investigations (Blokker et al., 1998a) showed that lipids removed during the workup procedure with the different treatments may also provide valuable information about the final residue. The final saponification releases algaenan monomers that are not part of the core of the ether-linked non-hydrolysable algaenan, but likely represent peripheral constituents (Table 1). Species like *T. minimum*, *P. boryanum* and *S. communis* released long-chain ω-hydroxy fatty acids on saponification. Analysis of the final residues obtained after acid and base treatment suggested that these compounds, although released as peripheral compounds, are the precursors of the algaenans in which they are present in an ether-linked polymerised form (Blokker et al., 1998a). In this study *P. kawraiskyi*, *P. braunii* and *S. spinulosum* to contain ester-linked long-chain ω-hydroxy fatty acids. Although only traces of the $C_{30:1}$ ω-hydroxy fatty acid were detected in *P. kawraiskyi*, the distribution of the ω-hydroxy fatty acids in the *S. spinulosum* product mixture is similar to that observed previously in the base hydrolysis product mixture of *P. boryanum* (Blokker et al., 1998a), consisting of the $C_{30:1}$, $C_{30:2}$,

Table 1
ω-Hydroxy fatty acids in base hydrolysis fraction

| *Species* | Chain length | Double bond no. | Amount |
|---|---|---|---|
| *O. solitaria* | – | – | – |
| *P. kawraiskyi* | 30 | 1 | Trace |
| *P. braunii* | 30 | 0 | High |
| | 32 | 0 | High |
| *C. reticulatum* | n.d. | – | – |
| *S. spinulosum* | 30 | 1 | High |
| | 30 | 2 | High |
| | 32 | 1 | High |
| | 32 | 2 | High |

n.d., not detected.

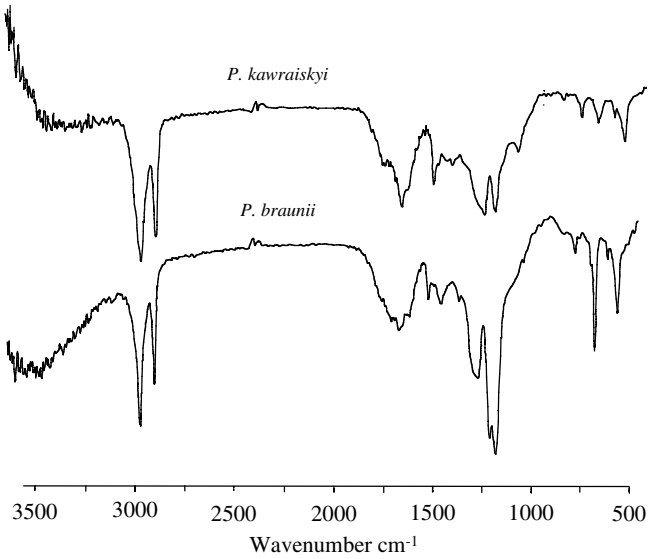



Fig. 1. FTIR spectra of algaenans.

$C_{32:1}$ and $C_{32:2}$ ω-hydroxy fatty acids. The ester-linked lipids of *P. braunii* are $C_{30:0}$ and $C_{32:0}$ ω-hydroxy fatty acids and a $C_{30}$ β-hydroxy fatty acid.

In addition to these specific algaenan precursors, the ester-linked fractions of all the algae investigated here also contain some $C_{16}$ up to $C_{30}$ long-chain fatty acids, with the highest concentrations in *P. braunii*.

### 3.2. Flash pyrolysis

Fig. 2 shows the flash pyrolysates of the four isolated final residues. Although all these residues are clearly composed of algaenan as revealed by the abundance of *n*-alkenes and *n*-alkanes in their flash pyrolysate (Goth et al., 1988; Tegelaar et al., 1989), some variations in chain-length distributions and

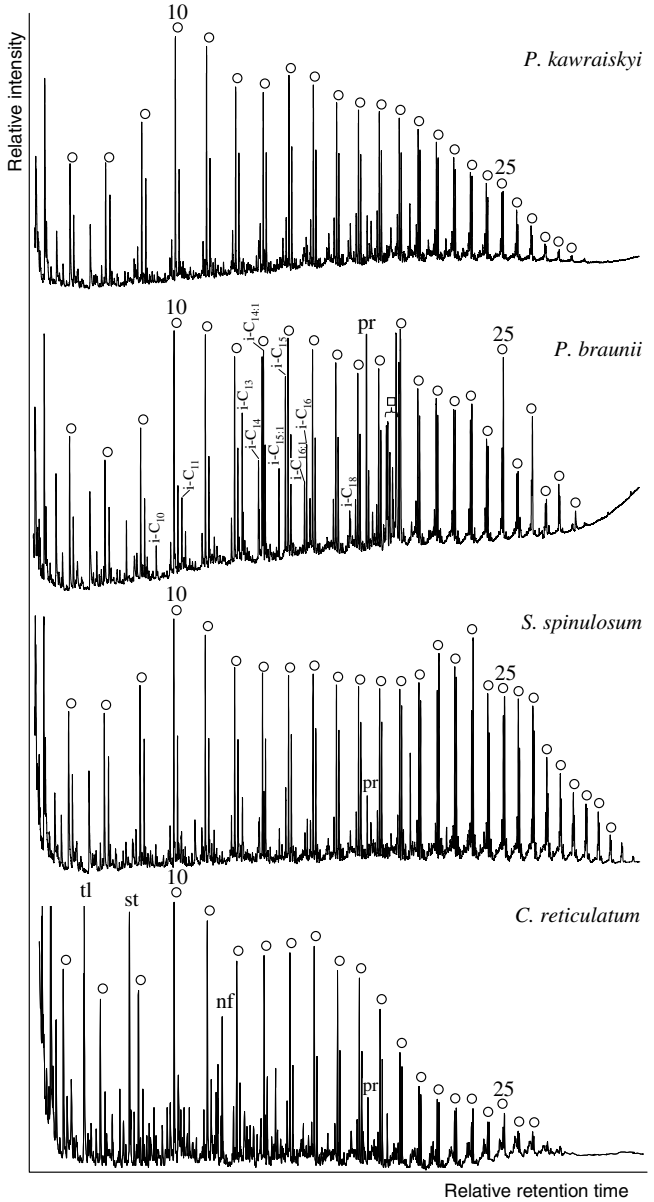



Fig. 2. Gas chromatograms of Curie-point (610 °C) flash pyrolysates of isolated final residues. Circles indicate *n*-alkene/*n*-alkane doublets, *i* = isoprenoids, open squares phytadienes and numbers chain length. Isoprenoid alkanes and alkenes are denoted by *i*-$C_{x:y}$, where *x* is the number of carbon atoms and *y* is the number of double bonds. tl, toluene; st, styrene; nf, naphthalene; pr, prist-1-ene.

peak intensities are observed. Most distinct is the pyrolysate of *P. braunii* algaenan, which displays a second series of peaks next to the *n*-alkene/*n*-alkane doublets. This second series consist of saturated and unsaturated $C_{10}$, $C_{11}$, $C_{13}$–$C_{16}$ and $C_{18}$ isoprenoids dominated by the $C_{14:1}$ homologue, prist-1-ene and a series of phytadienes. Another distinct feature is the odd over even carbon number predominance of the $C_{23}$–$C_{29}$ *n*-alkanes, which is dominated by $C_{25}$.

### 3.3. $RuO_4$ degradation

The gas chromatograms of the $RuO_4$ degradation product mixtures of the four isolated algaenans are shown in Fig. 3. The yields are relatively low; ~8% is removed from the biopolymer upon oxidation. Despite the low yield, the products are identical to those found earlier for extant and fossil algaenans and can be used to reconstruct the polymer structure (Blokker et al., 2000, 1998a; Gelin

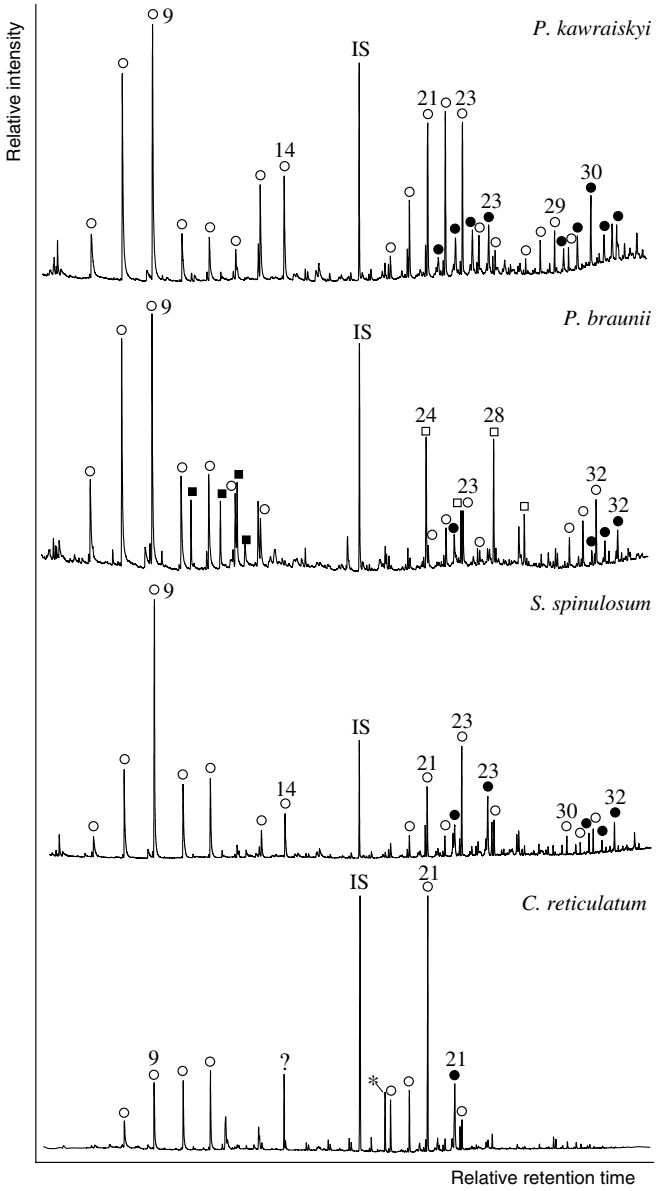



Fig. 3. Gas chromatograms of $RuO_4$ degradation product mixtures. Open circles indicate dicarboxylic acids, closed circles oxo-dicarboxylic acids, open squares fatty acids, closed squares isoprenoid fatty acids and numbers chain length.

et al., 1997; Schouten et al., 1998). All product mixtures are dominated by the presence of $C_8$–$C_{32}$ dicarboxylic acids. Like the results obtained in an earlier study (Blokker et al., 1998a), the dicarboxylic acids show a bimodal distribution maximising around $C_9$ and around $C_{22}$ depending on the species. Furthermore, *P. kawraiskyi*, *P. braunii* and *S. spinulosum* even display a third maximum around $C_{30}$–$C_{32}$. All product mixtures also reveal a series of oxo-dicarboxylic acids, which in most cases follow the distribution of the dicarboxylic acids in the range $C_{20}$–$C_{32}$. Although coelution obscures much detail, a specific feature of the oxo-dicarboxylic acids is the position of the carbonyl group, with a Gaussian-like distribution around the ω9 and ω10 positions in the case of the $C_{30}$–$C_{32}$ homologues and ranging from ω4 to approximately ω14 in the case of the shorter oxo-dicarboxylic acids. Although all product mixtures contain fatty acids, the *P. braunii* $RuO_4$ product mixture clearly contains the highest relative concentration and is dominated by the $C_{24}$ and $C_{28}$ homologues. Furthermore, a series of isoprenoids is observed in this product mixture. It is dominated by 4,8,12-trimethyl tridecanoic acid, 5,9,13-trimethyl tetradecanoic acid and 6,10,14-trimethylpentadecan-2-one.

## 4. Discussion

### 4.1. O. solitaria

Since no final residue was obtained after the work up procedure it is clear that this alga does not contain an insoluble non-hydrolysable biopolymer in its cell wall. This absence also shows that the isolation procedure followed seems not to generate artificial algaenan-type residues, so that these results can be seen as the outcome of a pseudo-blank analysis.

### 4.2. P. kawraisky

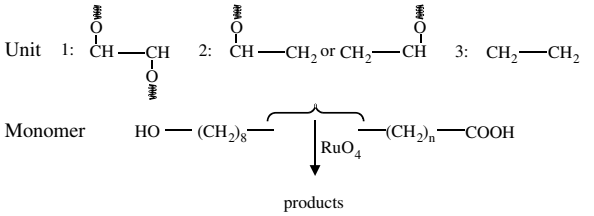
The amount of final residue obtained from this alga is about 6% (dry wt. biomass). Although only a trace amount of the $C_{30:1}$ ω-hydroxy fatty acid is observed in the base hydrolysis fraction of the workup procedure, the yield of ω-hydroxy fatty acids upon saponification probably strongly depends on the degree of ether linkages in the algaenan. In other words, if all monomers were ether bound, no ω-hydroxy fatty acid building blocks would be released upon saponification. Therefore, the low amounts, or even the lack, of these compounds does not imply that algaenan is absent. The products of the $RuO_4$ degradation of this algaenan clearly illustrate the idea that *P. kawraiskyi* uses the same set of building blocks as *P. boryanum* (Blokker et al., 1998a). A combination of ether linked $C_{30}$ and $C_{32}$ ω-hydroxy fatty acids produces the specific fingerprint formed by the dicarboxylic acids (Table 2). The presence of oxo-dicarboxylic acids in the $RuO_4$ product mixture is indicative of polymer fragments linked by a single ether linkage (Blokker et al., 1998a). The presence of a relatively abundant $C_{22}$ dicarboxylic acid compared to other *Pediastrum* species investigated can be the result of a different double bond position in the monomers or may possibly be due to a different distribution of ether linkages in the algaenan around the original double bonds of the monomers.

### 4.3. P. braunii

The amount of final residue obtained from *P. braunii* is about 1% of its biomass (dry wt.). The pyrolysate of this residue is highly aliphatic, but contains some additional compounds absent from the pyrolysates of the other algaenans. The presence of some extra isoprenoids in the pyrolysate and the

Table 2
$RuO_4$ degradation of ω-hydroxy fatty acid-based algaenans and main dicarboxylic acids formed[a]

| Monomer length | Unit | Formed dicarboxylic acids | |
|---|---|---|---|
| $C_{30}$ ($n = 19$) | 1 | $C_9$ | $C_{21}$ |
| $C_{32}$ ($n = 21$) | 1 | $C_9$ | $C_{23}$ |
| $C_{34}$ ($n = 23$) | 1 | $C_9$ | $C_{25}$ |
| $C_{30}$ ($n = 19$) | 2 | – | $C_{30}$ ω9 and ω10 oxo |
| $C_{32}$ ($n = 21$) | 2 | – | $C_{32}$ ω9 and ω10 oxo |
| $C_{34}$ ($n = 23$) | 2 | – | $C_{34}$ ω9 and ω10 oxo |
| $C_{30}$ ($n = 19$) | 3 | – | $C_{30}$ |
| $C_{32}$ ($n = 21$) | 3 | – | $C_{32}$ |
| $C_{34}$ ($n = 23$) | 3 | – | $C_{34}$ |




[a] It should be noted that ether-linkage positions are distributed around the position depicted in the table in a normal distributed fashion (Blokker et al., 1998a). This explains the formation of dicarboxylic acids shorter and longer than the $C_9$ upon $RuO_4$ degradation.

relative dominance of the $C_{23}$, $C_{25}$, $C_{27}$ and $C_{29}$ *n*-alkanes suggests that the algaenan as obtained was not as pure as the other algaenans investigated here. This is also suggested by the relatively high abundance of fatty acids and isoprenoids in the $RuO_4$ product mixture. Despite this, the involvement of $C_{30}$ and $C_{32}$ ω-hydroxy fatty acids as building blocks of the algaenan is clear from the composition of the saponified fraction. This is supported by the presence of long-chain $C_{30}$–$C_{32}$ oxo-dicarboxylic acids and dicarboxylic acids in the $RuO_4$ product mixture. Furthermore, although the $C_{21}$–$C_{25}$ dicarboxylic acids are dominated by long chain fatty acids, the product still shows an increase in the intensity of the $C_{21}$–$C_{23}$ dicarboxylic acids as observed in the $RuO_4$ product mixture of *P. boryanum* (Blokker et al., 1998a) and *P. kawraiskyi*, indicating the involvement of the same algaenan building blocks.

The $C_{23}$, $C_{25}$, $C_{27}$ and $C_{29}$ alkanes in the pyrolysis product mixture illustrate that the final residue isolated from *P. braunii* probably contains some residual long-chain fatty acids. These long-chain alkanes are probably formed upon thermal decarboxylation during pyrolysis from the $C_{24}$, $C_{26}$, $C_{28}$ and $C_{30}$ fatty acids, respectively, a process already observed by Hartgers et al. (1995) for shorter fatty acids in their salt form. Indeed, $C_{24}$, $C_{26}$, $C_{28}$ and $C_{30}$ fatty acids are present in the alga and dominate the base hydrolysis fraction of the workup procedure and the $RuO_4$ degradation products. Although biosynthetic intermediates normally do not accumulate, it is possible that these fatty acids represent the precursors of the algaenan monomer, which are probably formed upon chain elongation of shorter fatty acids, followed by ω-hydroxylation. This could explain the presence of the $C_{30}$ β-hydroxy fatty acid in the base hydrolysis fraction, which may be an intermediate in the $C_2$ chain elongation from the $C_{28}$ to the $C_{30}$ fatty acid. Although β-hydroxy fatty acids have never been reported in Chlorophytes they are present in some species of Eustigmatophytes (Volkman et al., 1999), a class of algae with algaenan-containing members (see also Gelin et al., 1997).

Another rather dominant group of compounds in both the pyrolysate and $RuO_4$ product mixture is represented by the isoprenoids. The phytadienes in the pyrolysate are probably derived from the phytol side chain of chlorophyll-*a* (van de Meent et al., 1980), which would produce the *i*-$C_{18}$ ketone upon $RuO_4$ degradation. The $C_{10}$–$C_{18}$ isoprenoid alkenes and alkanes in the pyrolysate most likely are derived from the same source as the $C_{10}$, $C_{11}$, $C_{13}$–$C_{16}$ isoprenoids in the $RuO_4$ product mixture, suggesting a second origin of such compounds apart from chlorophyll *a*. Comparison of these data with those obtained earlier from analysis of an insoluble non-hydrolysable polymer isolated from the green microalga *Botryococcus braunii* (Bertheas et al., 1999; Gelin et al., 1994; Metzger et al., 1991; Schouten et al., 1998) could suggest the presence of functionalized lycopadiene-type compounds as a possible source of these isoprenoids in the $RuO_4$ and pyrolysis product mixtures of *P. braunii*.

### 4.4. S. spinulosum

*S. spinulosum* is a microalga closely related to *Pediastrum* and belongs to the same family. About 10% of the biomass dry wt. represents resistant cell wall biopolymer. The lipid fraction obtained upon the final base hydrolysis in the workup procedure shows, apart from a series of even carbon chain length $C_{16}$–$C_{30}$ fatty acids, some $C_{30:1}$, $C_{30:2}$, $C_{32:1}$ and $C_{32:2}$ ω-hydroxy fatty acids identical to those from the base hydrolysis lipid fraction of *P. boryanum* (Blokker et al., 1998a). In addition the $RuO_4$ degradation reveals an algaenan signal identical to this alga, based on the typical dominance of the $C_9$, $C_{21}$ and $C_{23}$ dicarboxylic acids. The involvement of $C_{30}$ and $C_{32}$ ω-hydroxy fatty acid monomers in this algaenan is also expressed by the presence of $C_{30}$–$C_{32}$ oxo- and normal dicarboxylic acids in the product mixture (Blokker et al., 1998a).

### 4.5. C. reticulatum

About 2% of the biomass (dry wt.) of *C. reticulatum*, an alga from the family Coelastraceae, consists of a non-hydrolysable insoluble product. Although the base hydrolysis fraction from the work up procedure does not reveal the presence of the typical algaenan monomers as seen in the other algae described in this study, the final residue is clearly highly aliphatic in nature as shown by pyrolysis and is typical for an algaenan. The presence of toluene, styrene and naphthalene in the pyrolysates might be the effect of incomplete removal of some cell contents, or just be an artefact of pyrolysis, which generally will give a plethora of different products. The $RuO_4$ product mixture shows that *C. reticulatum* algaenan is highly comparable to that of *S. communis*, which is built up predominantly from ω9 $C_{30:1}$ ω-hydroxy fatty acid monomers.

Consequently, the $C_9$ and $C_{21}$ dicarboxylic acids in the $RuO_4$ product mixture can be ascribed to cleavage of vicinal ether linkages or a double bond at the ω9 position. The $C_{21}$ ω4–ω14 oxo-dicarboxylic acids were also observed in the $RuO_4$ degradation product mixture of *S. communis* (Blokker et al., 1998a).

These results illustrate the value of $RuO_4$ chemical degradation in resistant aliphatic biopolymer research. Although the lipids released by the acid and especially base treatment often provide clues about the algaenan structure, by revealing the presence of precursors, the results presented here point out that this is not always the case, as illustrated by *C. reticulatum*. In such cases only methods like $RuO_4$ chemical degradation can assist in revealing the biopolymer structure.

### 4.6. Chemotaxonomic considerations

Although *O. solitaria* lacks an algaenan containing cell wall, the results in this study illustrate that more members of the Chorococcales than previously thought contain algaenan (Table 3). Based on literature data (Blokker et al., 1998a; de Leeuw and Largeau, 1993) and our findings it can be expected that more trilaminar sheet-containing members of the genera investigated here contain algaenan and use the same suite of building blocks to synthesise this biopolymer. Since the chemical fingerprint of *S. spinulosum* algaenan is identical to that of the *Pediastrum* species (Table 3), it seems that the chemical composition of algaenan is family related. It is worthwhile noting that *C. reticulatum* and *S. communis* are nowadays classified as algae from two different families on the basis of morphological features (Komárek and Fott, 1983; Table 3), whereas they were formerly placed within the same family (Scenedesmaceae; Fritsch, 1935). Although this remains speculative, the similarities between their algaenan chemical compositions would suggest that the latter classification is more correct. It seems probable that the algaenans of unknown chemical composition observed in some *Chlorella* species (Chlorellaceae) (Allard and Templier, 2001; Derenne et al., 1992a,b) are synthesised from the same building blocks as *T. minimum* (Chlorellaceae) algaenan, although recent taxonomic investigations have shown that the algaenan-containing *Chlorella fusca* is in fact a *Scenedesmus* species (Huss et al., 1999). The ω-hydroxy fatty acid profile of *C. emersonii* indeed is more similar to that of *Scenedesmus* than that of *Tetraedron*, according to Allard and Templier (2001).

### 4.7. Implications for sedimentary organic matter

Chlorococcales are amongst the most abundant planktonic organisms in eutrophic lakes and are often important contributors to the benthic flora in these lakes (Komárek and Fott, 1983). Furthermore, this order of algae has an important, but still often neglected, indicative value in palaeoecology (Jankovská and Komárek, 2000). Since the order Chlorococcales contains a significant number of algae that biosynthesise algaenan, the relative dominance of these algae in sediments in such

Table 3
Overview of freshwater algae from order Chlorococcales, which have been fingerprinted using $RuO_4$, taxonomy (according to Komárek and Fott, 1983), presence of algaenan and the algaenan monomer composition

| Order | Family | Species | Algaenan | Monomers |
|---|---|---|---|---|
| Chlorococcales | Hydrodictyaceae | *P. boryanum* | ✓[a] | $C_{30}$ and $C_{32}$ ω-OH FA[a] |
| | | *P. kawraiskyi* | ✓[b] | $C_{30}$ and $C_{32}$ ω-OH FA[b] |
| | | *P. braunii* | ✓[b] | $C_{30}$ and $C_{32}$ ω-OH FA[b] |
| | | *S. spinulosum* | ✓[b] | $C_{30}$ and $C_{32}$ ω-OH FA[b] |
| | Scenedesmaceae | *S. communis*[c] | ✓[a] | $C_{30}$ ω-OH FA[a] |
| | Coelastraceae | *C. reticulatum* | ✓[b] | $C_{30}$ ω-OH FA[b] |
| | Chlorellaceae | *T. minimum* | ✓ | $C_{30}$, $C_{32}$ and $C_{34}$ ω-OH FA[a] |
| | Oocystaceae | *O. solitaria* | – | – |
| | Botryococcaceae | *B. braunii*[d] | ✓ | $C_{32}$ dialdehyde[e] |

[a] Blokker et al. (1998a).
[b] This study.
[c] Formerly classified as a member of the Coelastraceae (Fritsch, 1935).
[d] Metzger and Largeau (1999).
[e] Blokker et al. (2000).

environments (e.g. Hooghiemstra and Ran, 1994; Van Geel and Van der Hammen, 1973) can be explained. These findings explain the specific, often highly aliphatic nature of lacustrine sediments (e.g. Hutton, 1987) and illustrate that algaenans are probably a major source for the formation of lacustrine petroleum. Insight into the sources of sedimentary organic matter will help determine past communities of organisms and thus assist the reconstruction of palaeo-environments and climates.

It was shown previously that $RuO_4$ degradation can be used to reveal the presence of algaenan in lacustrine sediments (Blokker et al., 2000; Grice et al., 2003; Yoshioka and Ishiwatari, 2004) and to determine the presence and chemical nature of such biopolymers in sedimentary organic matter. This implies that the chemical structure of algaenans can be used to evaluate the contribution of certain algae to sedimentary matter, serving a biomarker purpose. This is especially useful since these algae cannot always be morphologically recognised in sediments and, furthermore, often do not have specific lipids that could fulfil a biomarker function.

### 4.8. Future research

To date the presence and distribution of algaenan within the order of the Chlorococcales and algae in general is still unclear. Most often the amount of material obtained from cultures does not allow extensive analysis. $RuO_4$ can be applied to small sample sizes and reveals useful information on the chemical structure of the algeanan, but more importantly the $RuO_4$ degradation GC fingerprint is preserved in sedimentary organic matter. To date this is the only method that can reveal the presence of algaenan-containing algae chemically to the species level. Further improvements to the degradation protocol as suggested by Dragojlovic et al. (2005) will improve the applicability of the method. Culturing and analysing various algae for the presence and $RuO_4$ fingerprint will be a starting point for further research.

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