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The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*

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Abstract

Phosphate limitation caused significant changes in the fatty acid and lipid composition of *Monodus subterraneus*. With decreasing phosphate availability from 175 to 52.5, 17.5 and 0 μM (K_2HPO_4), the proportion of the major VLC-PUFA, eicosapentaenoic acid (EPA), gradually decreased from 28.2 to 20.8, 19.4 and 15.5 mol% (of total fatty acids), respectively. The cellular total lipid content of starved cells increased, mainly due to the dramatic increase in triacylglycerols (TAG) levels. Among polar lipids, cellular contents of digalactosyldiacylglycerol (DGDG) and diacylglyceroltrimethylhomoserine (DGTS) increased sharply from 0.29 and 0.19 to 0.60 and 0.38 fg cell^{-1}, respectively, while that of monogalactosyldiacylglycerol (MGDG) was not significantly changed. In the absence of phosphate, the proportion of phospholipids was significantly reduced from 8.3% to 1.4% of total lipids, and the proportion of triacylglycerols (TAG) increased from 6.5% up to 39.3% of total lipids. The share of MGDG was substantially reduced, from 35.7% to 13.3%, while that of DGDG and DGTS reduced less from 18.3% to 15.1%, and 12.2% to 8.6%, respectively. The most distinctive change in the fatty acid composition was noted in that of DGDG, where the proportion of EPA, located exclusively at the *sn*-1 position, increased from 11.3% to 21.5% at the expense of 16:0, 16:1 and 18:1. In MGDG, however, the proportion of EPA did not change appreciably. In contrast to higher plants, DGDG accumulated under P-deprivation in *M. subterraneus*, did not resemble PC and the positional distribution of its fatty acids was not altered, preserving the C20/C16 structure of its molecular species. We suggest that under phosphate starvation DGTS is a likely source of C_{20} acyl groups that can be exported to the *sn*-1 position of DGDG and can partially compensate for the decrease in PE, the apparent source of C_{20} acyl-containing diacylglycerols in this alga. Moreover, accumulation of non-esterified 18:0 indicates that no polar lipid can replace PC, which appears to be the only lipid capable of C_{18} desaturation in this alga.

Keywords: *Monodus subterraneus*; Eustigmatophyceae; PUFA biosynthesis; EPA; Phosphate starvation

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1. Introduction

The biosynthesis of polyunsaturated fatty acids (PUFAs) in higher plants proceeds via two pathways (Browse et al., 1986). In the prokaryotic pathway, which is entirely chloroplastic, C_{18} and C_{16} fatty acids are desaturated up to 18:3ω3 and 16:3ω3, while residing in the *sn*-1 and *sn*-2 positions, respectively, of chloroplastic lipids, resulting in the production of 18/16 type molecular species. In the eukaryotic pathway, which involves cytoplasmic and chloroplastic lipids, 18:1 is incorporated into phospholipids and further desaturated to 18:2. Diacylglycerols (DAG), mostly 18:2/18:2 and 16:0/18:2, are released from the phospholipids, exported into the chloroplast, incorporated into galactolipids and further desaturated. The obtained eukaryotic molecular species are thus either of the 18/18, or the 16/18, type.

Abbreviations: DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceroltrimethylhomoserine; EPA, eicosapentaenoic acid; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerols.

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In microalgae, which produce very long chain (C_{20}–C_{22}) polyunsaturated fatty acids such as arachidonic acid (20:4ω6, AA) and eicosapentaenoic acid (20:5ω3, EPA), the galactolipids were shown to contain prokaryotic-like and eukaryotic-like molecular species. Prokaryotic-like molecular species contain C_{20} acyl groups at the *sn*-1 position and a medium to long chain (C_{14}–C_{18}, MLC) acyl moieties at the *sn*-2 position (e.g., 20:4/16:0) and were designated by us as 20/MLC (Khozin-Goldberg et al., 2002). Eukaryotic-like molecular species (20/20) contain C_{20} acyl groups at both positions, e.g., 20:5/20:5. However, in the PUFA-rich algae studied so far, e.g., *Phaeodactylum tricornutum* (EPA, Arao et al., 1994), *Nannochloropsis* sp. (EPA, Schneider et al., 1995), *Porphyridium cruentum* (EPA and AA, Cohen et al., 1988; Khozin et al., 1997), *Monodus subterraneus* (EPA, Cohen, 1994; Khozin-Goldberg et al., 2002) and *Parietochloris incisa* (AA, Bigogno et al., 2002a), both AA and EPA were shown to be of extrachloroplastic origin and the resemblance of the molecular species of the galactolipids to that of higher plants appeared to be only in structure, not in biosynthesis.

Based on inhibitor and radiolabelling studies, we have recently shown (Khozin-Goldberg et al., 2002) that in *M. subterraneus*, 20:3ω6, the first C_{20} PUFA produced in the biosynthetic pathway, is mostly incorporated into two extrachloroplastic lipids, phosphatidylethanolamine (PE) and diacylglyceroltrimethylhomoserine (DGTS), where it can be stepwise desaturated by the Δ5 and the Δ17 desaturases, leading to the production of AA and finally EPA. We have hypothesized that PE is the donor of the 20:5/20:5 DAG that is imported to the chloroplast to form the eukaryotic-like molecular species of monogalactosyldiacylglycerol (MGDG), whereas DGTS is the source of the C_{20} acyl groups, mostly EPA, that are incorporated to the *sn*-1 position of digalactosyldiacylglycerol (DGDG).

In animals and yeast, phospholipids are the most abundant membrane lipid class. The availability of phosphate in the plant cell is often limited and indeed in plants and algae, galactolipids predominate. Dörmann and Benning (2002) suggested that the large amount of galactolipids serve to reduce the dependence of photosynthetic organisms on phosphate. Indeed, under phosphate deprivation of *Arabidopsis thaliana*, the galactolipid DGDG is accumulated, compensating for the decrease in phosphatidylcholine (PC) (Essigmann et al., 1998; Härtel et al., 2000). The increase in DGDG is governed by several DGDG synthases (Kelly and Dörmann, 2002; Klaus et al., 2002). The over-accumulated DGDG is localized in extraplastidic membranes and resembles PC in the positional distribution of its fatty acids. Similarly, DGDG replaced up to 70% of the plasma membrane phosphoglycerolipids in oat (*Avena sativa*) cultivated under severe phosphate limitation (Andersson et al., 2003). In the photosynthetic bacterium, *Rhodobacter sphaeroides*, sulfoquinovosyldiacylglycerol (SQDG) and the betaine lipid DGTS are accumulated under phosphate starvation replacing PG and PC, respectively (Benning et al., 1993; Benning et al., 1995). Similar findings were recently reported for the freshwater alga *Desmodesmus subspicatus* and the soil alga *Chlamydomonas reinhardtii* (Lind et al., 2004).

In this report, we show that under phosphate deprivation, cells of the fresh water eustigmatophyte *M. subterraneus* increase the share of both DGTS and DGDG, in keeping with the postulated DGTS–DGDG pathway.

2. Results and discussion

We studied the redistribution of lipids and fatty acids in *M. subterraneus* under different levels of phosphate deprivation, to assess the role of DGTS and DGDG in the biosynthesis of PUFAs in this alga. Before the onset of the experiment, cultures were daily diluted to a relatively high chlorophyll concentration (11–15 μg ml^{-1}). Under these conditions, the biomass of *M. subterraneus* was previously shown to have a maximal content of MGDG and EPA (Cohen, 1994) and a high proportion of the eukaryotic-like molecular species 20:5/20:5 in MGDG (Khozin and Cohen, 1996).

Under phosphate limitation, cell division (Table 1) and chlorophyll synthesis (data not shown) were severely retarded. However, even in phosphate-free medium, cell number doubled after 4 days of cultivation, from 2.50×10^7 up to 4.65×10^7 cell ml^{-1}, indicating that intracellular phosphate was sufficient for at least one cell division. Cell dry weight gain in phosphate-deprived cultures was lower than in the control (Table 1). The cellular fatty acid content, at 52.5 and 17.5 μM phosphate, was twice as high as that of the control (1.6 fg cell^{-1}) and much higher (4.4 fg cell^{-1}) in the absence of phosphate (Table 1).

The phospholipid content of phosphate-deprived cells was drastically reduced while the content of triacylglycerols (TAG) increased dramatically from 0.1 to 1.7 fg cell^{-1} (Fig. 1). Two glycerolipids, DGDG and DGTS increased sharply from 0.29 and 0.19 to 0.60 and 0.38 fg cell^{-1}, respectively, while the cellular content of MGDG was not significantly changed.

On a relative basis, in the absence of phosphate, the proportion of TAG was increased from 6.5% up to 39.3% of total lipids. A similar phenomenon is obtained under nitrogen starvation (data not shown). The proportion of phospholipids was significantly reduced from 8.3% of total

Table 1
Effect of phosphate deprivation on cell number, dry weight (DW), volumetric and cellular total fatty acid (TFA) content in batch cultivated *Monodus subterraneus*

P concentration (μM)	Cell number (10^7 cell ml^{-1})	DW (mg ml^{-1})	TFA (mg ml^{-1})	TFA (fg cell^{-1})
175	16.0	1.95	0.25	1.6
52.5	8.4	1.97	0.27	3.2
17.5	6.3	1.44	0.19	3.1
0	4.7	1.37	0.21	4.4

Cells were maintained at different levels of phosphate and harvested after 4 days.

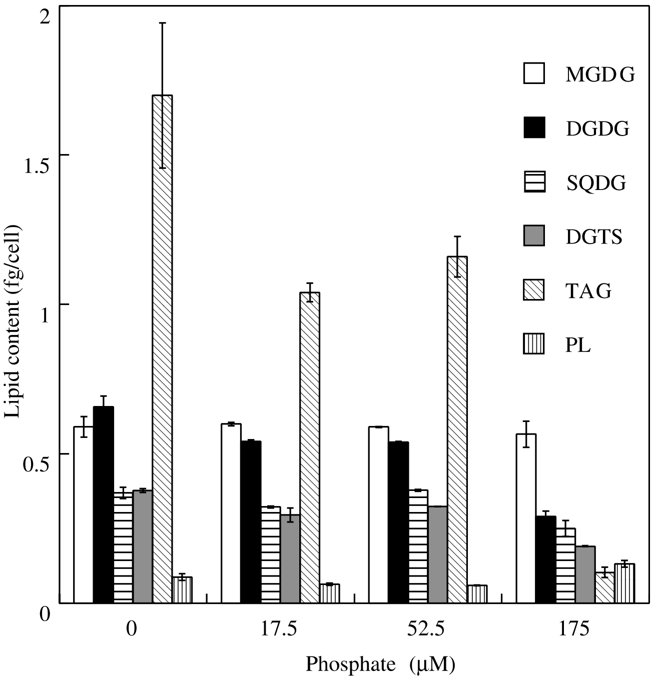

Fig. 1. Effect of phosphate deprivation on cellular content of major lipids of *M. subterraneus*.

lipids to 1.4%. The share of MGDG was drastically reduced, from 35.7% to 13.3% of total fatty acids (TFA), while that of DGDG and DGTS were only slightly reduced from 18.3% to 15.1%, and 12.2% to 8.6%, respectively, in accord with the increase in their cellular content (Fig. 1, Table 3).

Phosphate deprivation caused significant changes in the fatty acid composition of *M. subterraneus* in a manner commensurate with phosphate concentration. With decreasing phosphate availability from 175 to 52.5, 17.5 and 0 μM, the proportion of the major VLC-PUFA, EPA, significantly decreased from 28.2% to 20.8%, 19.4% and 15.5% (mol% of TFA), respectively (Table 2). We also observed a substantial increase in 18:0, 18:1ω9 and 20:3ω6, indicating a decrease in both C_{18} and C_{20} desaturations. In complete absence of phosphate the proportion of 18:0 increased substantially up to 10%, comprising 80% of the free fatty acids.

In DGTS, EPA decreased from 21.8% to 16.4%, while the saturated fatty acids, 14:0, 16:0 and 18:0 increased by a total of about 11% (Table 3). We thus infer an increase in the proportion of the 14:0–18:0/20:5 molecular species at the expense of that of the 20:5/20:5 and 16:1/20:5 species (Khozin-Goldberg et al., 2002). The proportion of EPA in DGDG increased from 11.3% to 21.5%, at the expense of 16:0 and 16:1, which decreased from a total of 75.9% to 65.6%. We have recently shown (Khozin-Goldberg et al., 2002) that under control conditions DGDG is almost exclusively made of two types of molecular species, 16/16 and 20/16. Under P starvation, the share of the 16/16 molecular species decreased from 71.2% to 45.9% whereas that of the 20/16 molecular species increased from 8.9% to 50.7% (Table 4). The content of phospholipids decreased sharply. In PE, decreased C_{20} desaturation was indicated by the lower proportion of AA and EPA which decreased from a total of 46.9% to 24.9%, whereas in PC there was a decrease in the proportion of 18:2 and 18:3ω6 from 12.5% to 2.1%, demonstrating reduced C_{18} desaturation (Table 3). In TAG, the proportion of EPA decreased from 10.7% to 4.4%, yet, due to the sharp increase in the content of TAG, the EPA content of TAG was higher than under control conditions.

We have suggested that PE, a substrate for C_{20} desaturations, is the donor of the diacylglycerols that are incorporated into the chloroplast to produce the 20/20 molecular species of MGDG. DGTS, which contains C_{20} PUFAs mostly in the *sn*-2 position, appears to be a likely source of these PUFAs for the 20/MLC molecular species of DGDG. The decrease in PE from 0.7% to 0.3% (of total lipids), following phosphate starvation (Table 3), is correlated with a relative decrease in the proportion of MGDG and in keeping with the suggested source–sink relationship between these lipids. Similarly, the decrease of EPA in DGTS and its concomitant increase in DGDG further supports a similar relationship between DGTS and DGDG. Treatment with the herbicide SAN 9785 resulted in a sharp decrease in DGTS and subsequently in the 20/MLC molecular species of MGDG and DGDG while the proportion of PE and consequently that of the 20/20 molecular species of MGDG, increased sharply (Khozin-Goldberg et al., 2002). PE and

Table 2
Effect of phosphate deprivation on the fatty acid composition and content of *Monodus subterraneus*

P concentration (μM)	TFA (% DW)	Fatty acid composition (mol%)											
		14:0	16:0	16:1 ω9	16:1 ω7	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	20:3 ω6	20:4 ω6	20:5 ω3
175	12.9	3.3	20.3	4.2	28.0	0.8	4.2	0.8	1.3	0.8	0.3	3.9	28.2
52.5	13.8	3.5	20.3	1.4	33.7	1.4	8.8	1.1	0.9	0.6	1.0	3.2	20.8
17.5	13.4	3.2	21.3	2.1	33.2	2.4	8.0	1.2	0.9	0.6	1.4	3.4	19.4
0	15.1	3.3	19.8	0.5	33.8	9.7	7.8	1.2	0.8	0.7	1.9	2.8	15.5

Cells were maintained at different levels of phosphate and harvested after 4 days. Biomass was transmethylated as described in Section 3. The data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 15% for minor peaks, of three independent samples, each analyzed in duplicate. The fatty acids 18:3ω3, 20:0 and 20:2 constituted less than 0.5%.

Table 3
Effect of phosphate starvation on the lipid and fatty acid composition of *Monodus subterraneus*

P concentration (μM)	Lipid	% of TFA	Fatty acid composition (mol%)												
			14:0	16:0	16:1	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3	Other
175	MGDG	35.7	4.2	12.2	22.2	0.3	2.5	0.8	1.1	0.8	0.5	0.4	4.0	49.0	2.0
17.5		19.8	5.3	10.0	19.3	0.9	2.1	0.7	0.8	0.6	0.4	0.6	5.7	51.6	2.0
0		13.3	6.7	8.4	21.2	1.2	1.5	0.8	0.3	0.6	0.4	0.6	5.0	50.6	2.7
175	DGDG	18.3	2.2	24.5	51.4	0.2	5.7	1.0	1.0	0.2	0.7	tr	0.5	11.3	1.2
17.5		17.8	2.8	21.2	48.0	0.3	4.6	0.7	1.2	0.3	0.8	0.2	0.8	18.3	0.8
0		15.1	3.1	19.1	46.5	0.9	3.9	0.6	1.3	0.3	0.8	0.3	1.1	21.5	0.6
175	SQDG	15.6	3.6	48.9	41.5	0.3	3.5	0.6	0.3	0.2	0.2	–	tr	0.4	0.5
17.5		10.6	3.0	49.0	41.4	0.6	3.7	0.9	0.2	tr	tr	tr	tr	0.3	0.5
0		8.6	4.0	47.7	41.9	0.8	3.3	1.0	tr	tr	tr	tr	tr	0.4	0.9
175	DGTS	12.2	3.4	25.8	30.0	2.2	2.4	0.8	1.3	0.8	0.9	tr	7.2	21.8	3.3
17.5		9.7	4.7	33.9	27.8	2.5	1.0	1.0	0.8	0.8	0.5	0.3	7.3	17.6	tr
0		8.6	5.4	32.8	26.7	2.9	1.8	1.2	1.4	0.7	0.4	0.3	7.7	16.4	2.3
175	PG	3.3	0.7	30.7	6.7	0.8	1.4	1.7	1.0	0.1	0.2	–	0.9	26.8	29.9[a]
17.5		1.2	2.0	23.7	13.1	4.6	2.2	3.6	0.5	tr	tr	–	1.9	15.5	32.9[a]
0		0.4	2.2	24.9	12.6	4.5	1.8	3.0	0.8	tr	tr	–	0.9	14.9	34.3[a]
175	PC	2.2	2.6	32.0	19.8	5.0	9.8	1.0	7.9	4.6	0.3	0.2	6.1	7.4	3.3
17.5		0.7	4.2	33.7	28.5	3.0	9.3	1.2	3.6	1.6	0.3	0.8	6.2	6.7	0.9
0		0.3	4.3	39.0	27.9	2.3	8.1	2.1	0.5	1.6	tr	tr	5.9	6.3	tr
175	PE	0.7	2.6	18.8	17.6	1.9	4.5	1.5	1.8	1.0	0.4	1.9	16.6	30.3	1.1
17.5		0.3	4.4	28.7	25.5	4.6	6.7	2.1	1.5	0.3	tr	1.7	11.2	11.7	1.6
0		0.3	3.8	29.4	26.5	3.5	5.3	1.3	1.6	0.4	tr	1.4	10.5	14.4	1.9
175	TAG	6.5	3.2	22.9	32.9	3.1	15.5	1.9	2.0	0.9	0.2	0.9	3.3	10.7	2.5
17.5		34.3	3.8	25.4	41.2	2.0	14.4	1.4	0.9	0.6	0.2	2.2	1.5	4.4	2.0
0		39.3	4.6	24.1	43.0	1.9	12.5	1.4	0.9	0.8	0.2	2.8	1.5	4.4	1.9

Cells were maintained at different levels of phosphate and harvested after 4 days. Lipids were extracted and separated by 2D-TLC and transmethylated as described in Section 3. Repetitions and accuracy as in Table 2.

[a] Mostly 16:1Δ3t.

Table 4
Effect of phosphate starvation (0 μM) on the molecular species composition of DGDG in *Monodus subterraneus*

Molecular species *sn*-1/*sn*-2	% of total	
	Control	–P
20:5/16:1	26.1	43.4
16:1/16:1	19.0	20.5
20:5/16:0	2.7	7.3
16:1/16:0[a]	52.2	28.8

[a] Major combination.

DGTS of *Nannochloropsis*, a marine eustigmatophyte, are also rich in EPA (Schneider et al., 1995; Haigh et al., 1996). Although Schneider and Roessler (1994) and Schneider et al. (1995) suggested that PE is the substrate to the Δ5 desaturase, their labelling data are in keeping with DGTS being also a substrate for this desaturation. Possibly, the role of DGTS in this alga could be similar to that suggested for *M. subterraneus*. In DGTS, EPA appears mostly in the *sn*-2 position, while in DGDG it is restricted to the *sn*-1 position. It thus follows that in contrast to the 20/20, eukaryotic-like, molecular species of MGDG that are produced by import of DAG, the 20/MLC, prokaryotic-like molecular species of DGDG are apparently constructed by import of EPA, rather than EPA-containing DAG, possibly using 20/MLC MGDG as an intermediate (Khozin-Goldberg et al., 2002). Similarly, true prokaryotic molecular species of higher plants and algae are not made of imported DAG, but rather by stepwise incorporation of 18:1 and 16:0 into the glycerol backbone (Browse and Somerville, 1994).

During phosphate deprivation of *Arabidopsis*, the UDP-galactose dependent DGDG synthase, DGDG2, that is localized to the outer side of chloroplastic envelope membranes, is responsible for the synthesis of DGDG in extraplastidial membranes having a non-chloroplastic fatty acid composition with molecular species rich in C_{16} at the *sn*-1 position (Kelly and Dörmann, 2002). This DGDG resembles PC in its fatty acid composition and can partially replace phospholipids in extraplastidial membranes under phosphate limitation. In contrast, DGDG accumulated under P-deprivation in *M. subterraneus* cells, did not resemble PC and the positional distribution of its fatty acids was not altered, preserving the C20/C16 structure of its molecular species. In contrast to higher plants, chloroplasts of Eustigmatophyceae are enclosed by the endoplasmic reticulum (Dodge, 1973). This proximity may enable translocation of lipid intermediates, supporting the hypothetical DGTS–DGDG pathway.

We suggest that in *M. subterraneus*, the PE-MGDG pathway predominates under P-sufficient conditions, while the suggested DGTS–DGDG pathway, which is minor under these conditions, can be augmented when P is limiting and utilized as a survival strategy that enables the alga to conserve phosphate without compromising membrane structure. Whereas DGTS can partially compensate for the decrease in PE, no lipid can replace PC, which appears to be the only lipid capable of C_{18} desaturation. Indeed, when kept in phosphate-free medium, non-esterified fatty acids, mostly 18:0, accumulated.

The MGDG/DGDG ratio is crucial for the physical state of chloroplast membranes, for stabilizing the protein–pigment complexes of the photosynthetic apparatus and may be regulated by phosphate availability (Dörmann and Benning, 2002). These galactolipids were found to be critical for the functional intactness of the complexes of photosystems I and II (Dörmann and Benning, 2002). While DGDG supports bilayer structures, MGDG does not (Williams, 1998). In control cultures of *M. subterraneus*, the MGDG/DGDG ratio is 2:1, decreasing to about 1:1 under P-deprivation. Distinctly from higher plants where MGDG substantially dominates over DGDG (Joyard et al., 1998), in microalgae the MGDG/DGDG ratio varies significantly between different species from 2 to 4 to close to 1 and is affected by various environmental factors and nutrient availability (Thompson, 1996; Harwood, 1998).

3. Experimental

3.1. Growth conditions

Cultures of *M. subterraneus* were cultivated on BG11 medium (Stanier et al., 1971), in 1 l tubes under an air-CO_2 (99:1, v/v) atmosphere. Tubes were placed in a temperature regulated water bath and illuminated from aside at a light intensity of 180 μmol quanta m^{-2} s^{-1}. Cultures were diluted every other day to reach a chlorophyll concentration of 12–15 mg ml^{-1} for at least 4 days prior to the onset of the experiment. The cultures were then centrifuged, washed three times and resuspended in 1 l of the appropriate medium (full medium – 175 μM K_2HPO_4, phosphate limited – 52.5 or 17.5 μM, or phosphate-free medium). Cultivation was continued in batch for another 4 days. Experiments were repeated at least twice, each one in two columns, analyzed separately. Growth parameters: chlorophyll volumetric concentration, dry weight measurements and cell number, were assayed as previously described (Zhekisheva et al., 2002).

3.2. Lipid and fatty acid analysis

Lipid extractions were performed as previously described (Khozin-Goldberg et al., 2002). Briefly, samples of freeze-dried biomass (50–100 mg) were treated with 0.2 ml DMSO at 70 °C for 10 min and extracted with methanol at 4 °C for 1 h. Following centrifugation, water, hexane and diethyl ether were added to the methanol extract to form a final ratio of 1:1:1:1 (v/v/v/v), and lipids were recovered into the diethyl ether:hexane phase. The aqueous phase was acidified with 2 N HCl to pH 4 and re-extracted with a mixture of diethyl ether:hexane (1:1, v/v). The lipid-containing upper phases were combined and evaporated to dryness.

Lipids were fractionated into neutral and polar lipid fractions on Bond-Elute Silica JR-Si cartridges (Varian, Middelburg, The Netherlands) by sequential washings with chloroform and methanol. Polar lipids were further separated by two-dimensional TLC on silica gel plates (Merck, Darmstadt, Germany) using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction and of chloroform:methanol:1-ethylpropylamine:conc. ammonia (65:35:0.5:5, v/v/v/v) for the second direction. Neutral lipids were resolved with petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). Methyl esters of fatty acids were prepared by transmethylation of either the algal biomass or the lipid with 2% sulfuric acid in absolute methanol as previously detailed (Khozin et al., 1997). Fatty acids analysis was carried out as previously described (Bigogno et al., 2002b).

The data presented in the tables and figures represent mean values of at least three independent experiments with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks. Each sample was analyzed in duplicate.

3.3. Molecular species analysis

Molecular species analysis of DGDG, eluted from silica gel TLC plates, was performed by reversed phase HPLC (RP 18 column, 5 μm, 250 mm, Lichrospher 100, Merck, Darmstadt, Germany) on a Hitachi L7100 chromatograph (Merck) equipped with a UV detector (Lambda-Max 481, Millipore, Waters) and an evaporative light scattering detector (drift temperature tube 125 °C, nebulizer gas flow 2.5 l min^{-1}, Varex, Burtonsville, Md). Molecular species of DGDG were resolved by methanol:water (93:7, v/v), identified by GC analysis of their fatty acids in the presence of an internal standard (17:0). Molecular species ratio was calculated on the basis of the uncalibrated light scattering detector responses. Positional analysis of DGDG was performed as previously described using *Rhizopus* lipase (Khozin-Goldberg et al., 2002).

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