Effect of Environmental Temperature Transition on Fatty Acid Composition of Membrane Glycerolipids in Marine Cyanobacterium *Aphanizomenon sp*.

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Abstract:

In this study the fatty acid composition and the degree of unsaturation were followed upon temperature shift from 28°C to15°C and from 15°Cto 28°C. Temperature shift from 28°C to 15°C led to changes in the overall fatty acid composition in total lipid and glycerolipid classes, with rapid and slow responses in the fatty acid content during the adaptation period. The rapid response-limited to the 12h following temperature transition-observed in PG fraction in which C18:1 increased, and almost 2.5 fold, while C16:0 and C18:3 level decreased, and a relatively rapid response was presented by decrease in C16:0 and concomitant increase in C18:3 in MGDG and DGDG. A comparison of fatty acid composition at 28°C (0h) with those at the end of the 15°C growth period (48) shows that the most significant changes were the increase of C18:3/C18:2 ratio in the total lipid, MGDG and SL. The results also show an increase in the degree of concentration in the total lipid, MGDG and SL fractions, while there is no change in DGDG and decrease in PG fraction.

When the growth temperature shift from 15°C to 28°C, the pattern of the change in fatty acid composition and the degree of unsaturation were relatively opposite to that observed upon temperature shift from 28°C to 15°C. A decrease in C16:3 and a marked increase in C16:1 was observed in total lipid, MGDG, PG and SL. In the total lipid small decrease in C18:3 was found accompanied by a doubling increase in C18:1. The amount of C16:0 and C18:0 however remained constant during temperature adaptation.

Key word : Baltic Sea; *Aphanizomenon sp*.; temperature; fatty acids; glycerolipids; Plasma membrane;

Abbreviation: C16:0 palmitic acid; C18:1 oleic acid; C18:2 linoleic acid; C18:3 linolenic acid; MGDG monogalactosyl diglyceride; DGDG digalactosyl diglyceride; PG phosphatidyl glycerol; SL sulfoquinovosyl diglyceride; TLC thin layer chromatography; FAME fatty acid methyl esters; GLC gas liquid chromatography.

Introduction:

Biological membranes are not static structure, but can have a degree of fluidity in their interior. Membrane fluidity or bilayer order has a major influence over a wide variety of membrane functions and processing (Morgan-Kiss *et al.*, 2006). The extent of fluidity of the membranes depends on the degree of unsaturation of the fatty acid estrified to the glycerol backbone of the glycerolipids of the membrane (Veatch and Keller 2005). Resent research has focused on the cellular membrane as the primary site of low temperature injury by reducing the membrane fluidity (Lee *et al.*, 2006). An organism – in particular poikilotherms – may maintain the level of molecular motion or "fluidity" of its membrane lipid by regulating the number of double bonds in the fatty acids of these lipids (Russell, 1984; Jones, 2003). When the fluidity of membrane lipids is reduced by decrease in temperature, cyanobacteria and plants respond by introducing double bond into the fatty acids of lipids, so that membrane returns to more fluid state (Vigh *et al*., 1993).

Fatty acid composition of many organisms is affected when their normal environmental growth temperature either decreases or increases (Wada and Murata, 1990; Sushchik *et al.*, 2001). These modifications in glycerolipid fatty acid composition represent adaptation of membrane fluidity that enable the organism to carry out normal physiological function at either decreased or increased environmental temperature. In fact, cellular fatty acid composition has been used as a tool for classifying bacteria at the family, genus and species levels (Vandamme *et al*., 1996). Unicellular and filamentous cyanobacteria have been grouped in five clusters depending on the number and position of double bonds counted from the carboxyl terminus (Δ) or from the methyl terminus (ω) of 16 carbon (C_{16}) and 18 carbon (C_{18}) fatty acids (Kenyon, 1972; Kenyon *et al*., 1972; Murata *et al*., 1992; Cohen *et al*., 1995). Four groups were first

found by Kenyon *et al*. (1972) and confirmed by Murata *et al*. (1992). The fifth group described by Cohen *et al*. (1995) was positioned according to the Kenyon-Murata classification system between groups 1 and 2. The strains of group 1 are devoid of polyunsaturated fatty acid (PUFA) and contain only saturated and monounsaturated fatty acids. The strains of the group defined by Cohen *et al*. (1995) contain 18:2Δ9,12 (18:2ω6, linoleic acid) as the only C_{18} PUFA. Group 2 consists of cyanobacterial strains containing 18:3 Δ9,12,15 (18:3ω3, *a*-linolenic acid) as the only C_{18} PUFA. The strains of group 3 have 18:3Δ6,9,12 (18:3ω6, *γ*-linolenic acid) as the major C_{18} PUFA, but no or only traces of 18:3Δ9,12,15. Strains of group 4 contain either 18:3Δ9,12,15 or 18:3Δ6,9,12 or both, but also produce 18:4Δ6,9,12,15 (18:4ω3, octadecatetraenoic acid). The double bonds in the hydrocarbon chains of PUFA are introduced by fatty acid desaturases, which play an important role in the acclimation of the various organisms to changes in environmental temperatures (Murata and Wada, 1995; Nishida and Murata, 1996).

In the Baltic Sea, high abundances of planktonic cyanobacteria are common during the summer months (Stal *et al.*, 2003). The dominating genera are two photoautotrophic filamentous diazotrophs (*Nodularia* and *Aphanizomenon*) that use heterocysts in the process of assimilating nitrogen from the environment through N_2 fixation (Gallon *et al.*, 2002; Janson & Granéli, 2002). Early studies showed that they occur at the most nutrient-deplete time of the year, during an approximately two month long time-window from the end of June to the end of August when surface-layer temperatures are high (Wasmund, 1997). These cyanobacteria form colonies or larger aggregates that float to the water surface where they accumulate as dense scums, because they contain gas vesicles, (Walsby *et al*., 1995, 1997). Despite the fact that these cyanobacterial blooms occur locally and for relatively short periods (Evans *et al.*, 2000), *Aphanizomenon* sp. is abundant in the water mass during the whole year, thus showing the ability to grow in low or high temperatures (Niemistö *et al.*, 1989). *Aphanizomenon* exhibit maximum growth at temperatures of 12–25°C at photon irradiance levels of 25–45 μmol m^{-2} s^{-1}, and are more homogeneously distributed through the mixed layers and forms scums

onto the water surface(Evans *et al*., 1996, 2000; HELCOM, 2002; Laanemets *et al*., 2004).

In summer when the water column is thermally stratified, wind-driven coastal upwelling is an important mesoscale phenomenon that dramatically changes the euphotic layer temperature and nutrient conditions in the Baltic Sea. The surface-layer water temperature may drop more than ten to fifteen degrees within hours, while nutrient concentrations increase markedly (Vahtera *et al.*, 2005). The solar heating and wind mixing form a sharp seasonal thermocline at a depth of about 10–15 m starting from May. The seasonal thermocline is the strongest in July/August, when the temperature difference between the warm upper mixed layer and the cold intermediate layer varies within 12–20°C (Laanemets *et al.*, 2004). In the Baltic, *Aphanizomenon sp*. and other cyanobacteria are exposed during the summer to a wide range of temperature from a maximum of 30^{o}C whilst floating at the surface under calm condition to a minimum of 10^{o}C when they sink to the thermocline following a wind induced mixing event (Helsinki Commission, 2006). The aim of this study is to determine change in fatty acid composition in the total lipid fraction and major glycerolipid classes of *Aphanizomenon sp*. following (i) a downward temperature transition from 28^{o}C to 15^{o}C, and (ii) an upward temperature shift from 15^{o}C to 28^{o}C.

Materials and Methods :

Growth and maintenance of laboratory cultures

A culture from a strain of *Aphanizomenon* originally isolated from the Gulf of Finland by Dr Kaarina Sivonen, University of Helsinki, Finland, was obtained from the Biochemistry Research Group, University of Wales Swansea , henceforth referred to as *Aphanizomenon sp.*, and was grown photoautotrophically in modified ASM-1 liquid medium lacking a fixed nitrogen source (Gallon et al., 1978). The medium ingredients were mixed thoroughly and the pH value of the medium was adjusted to 7.6 by adding of small volume of 0.1 M HCI. Volumes of the medium were transferred into conical flasks , typically to 1\2 – 3\4 of the flask volume, which were then loosely plugged with non-absorbent cotton wool and sterilized in an autoclave at 121^{o}C at pressure of 1.5 kg-force/cm^{2}

for 15 minutes. When the flask attained room temperature, it was inoculated. Normally, 10% by volume of inoculum was used from a culture which had previously been grown for 10 – 12 days. Inoculations were carried out by sterile transfer in a console safety cabinet. Inoculated flasks were placed in an illuminated refrigerated orbital incubator on a 12 h light (90 μE $m^{-2}s^{-1}$) /12 h dark cycle , the speed of shaking was 12 rpm and normally culture were maintained at a growth temperature of 28°C.

Aphanizomenon sp. culture was grown in 1500 ml of modified ASM-1 medium (Rapala *et al*., 1997) contained in 2L conical flask for 10 days at 28°C as described above. The incubator temperature was then reduced to 15°C. Under these conditions the temperature of culture in the incubator were found to have fallen to 15°C within 1.5 h of the incubator temperature being lowered. Culture was maintained at 15°C for 48h, during which time samples of culture were collected at 0h (immediately before lowering the temperature), then at 12h, 24h, 36h and 48h for the analysis of fatty acid composition. In another experiment, the organism was grown at 15°C for 10 days, then the temperature of the incubator was raised to 28°C and growth continued for 48h. Samples of culture were collected at the time the temperature was raised (0h) to28°C and after further periods of 12h, 24h, 36h and 48h for fatty acid composition analysis.

Analysis:

Total lipid was extracted from *Aphanizomenon sp*. cells which had been harvested by centrifugation, normally at 3,000xg essentially according to the method of Bligh and Dyer (1959) as modified by Murata and Sato (1981).

For separation of glycerolipid classes were done by the method of Harwood *et al*. (1988), in which the total lipid extracts was applied as a short band to silica gel-G TLC plates together with authentic samples of MGDG, DGDG and PG. The chromatogram was developed in a solvent of chloroform: methanol: acetic acid: water (170: 30: 20: 7, v/v/v/v). Developed plates were sprayed with 0.05% (w/v) primulin in acetone/

H_2O (4: 1, v/v), and visualized under UV light, or visualized by iodine as described by Christie (2003).

Fatty acid methyl esters (FAME) of total lipid fractions and isolated glycerol lipid fractions were prepared by transesterification. Samples (100 – 200 µl) of total lipid extracts or isolated glycerolipid fractions were evaporated to dryness under oxygen free nitrogen (OFN), and then heated at 55^{o}C for 15h with 2ml 2% H_2SO_4/ absolute methanol (Christie,2003) and extracted by petroleum ether.

The methyl ester samples prepared by transesterification of the total lipids fractions and glycerolipid classes were purified by preparative TLC on Whatman 60A silica gel-G coated plates. An authentic solution of fatty acid methyl ester standard (5mg/ml) was used for identification of the sample methyl esters. A solvent system of diethylether: petroleum ether (1: 9, v/v) was used to develop the chromatogram. Developed plates were sprayed with 0.05% (w/v) primulin in acetone/water (4: 1, v/v), visualized under UV light (340nm), and detected fatty acid methyl esters were removed from the plates by extraction in petroleum ether. The recovery of FAME from transesterification and preparative TLC was estimated by adding a known amount of internal standard heptadecanoic acid to representative total lipid extract before transesterification, then processing as described above. An apparent recovery of 73% was routinely obtained. Preparation of fatty acid methyl esters for gas chromatographic analysis of marine lipids: insight studies.

FAME recovered from TLC plates were identified and quantified by GLC (Perkin Elmer-8700 gas liquid chromatography, Boston, MA,USA) equipped with a flam ionization detector using a highly "polar" capillary column (BPX70, 0.25µm film, 25m x 0.22mm id). Oven temperature was programmed from 130^{o}C to 210^{o}C at a rate of 20^{o}C min^{-1}. Injections were made in the split injection mode (10: 1) at an injection temperature of 160^{o}C using helium as carrier gas at a flow rate of 10ml min^{-1}. Individual components were identified by comparison their retention times with authentic FAME standards and by comparison with a GLC-MS total ion chromatogram determined under similar conditions. The relative % composition of fatty acid was calculated from area of the individual peaks

of interest in the chromatogram. Absolute quantification of FAME was obtained by injection of known amounts of an appropriate authentic FAME standard and determination of peak area. The amount of unknown FAME was then calculated by proportionation.

The degrees of saturation were calculated from the relative amounts of fatty acids in each lipid class as the values of the average number of double bonds per lipid molecule. For example value of zero with only saturated fatty acid and a value of 2 for C16:1, and C18:1, and a value of 4 for C16:2, and C18:2, and a value of 6 for C16:3, and C18:3(Havaux *et al*.,1984).

Result and Discussion :

Effect of Reduction in Growth Temperature From 28^{o}C to15^{o}C on Fatty Acid Composition

Figure 1 shows the time dependant changes in fatty acid composition in total lipid and glycerolipid classes when the temperature shifts from 28^{o}C to15^{o}C. At 28^{o}C (0h) MGDG and DGDG had almost a similar fatty acid composition and contained polyunsaturated C-16 and C-18 fatty acids, whilst in the acidic glycerolipid PG and SL, there was no polyunsaturated C-16 fatty acids, and the level of C18:3 in SL was generally lower than in galactolipid fractions. Temperature shift from 28^{o}C to15^{o}C led to changes in the overall fatty acid composition in total lipid and glycerolipid classes. The changes observed in the fatty acid composition of the total lipid after lowering the temperature were broadly similar to those observed in the MGDG and DGDG fractions, where the reduction of growth temperature led to decrease in C16:0 and C18:1 while C16:3 and C18:3 increased. In contrast PG fraction the level of C18:1 increased whilst the level of C18:3 decreased. In SL fraction C16:0 was the dominant fatty acid and its level decreased slightly, whilst C16:1 increased, and a fall in C18:1 level was accompanied by an increase of C18:3.

The results in Fig.1 demonstrate the presence of relatively rapid and slow responses in the mechanisms by which the fatty acid content is modulated temperature acclimation. The most significant change upon shifting the temperature from 28^{o}C to 15^{o}C was observed in the shooting

of C18:1 (increased almost 205 fold) in PG during the first 12h and drop in C18:3 during the first 24h. This observation appears to indicate that de novo synthesis of C18:1 may be an important adaptive mechanism in retailoring this lipid class.

Table 1 present a comparison of fatty acid composition at 28^{o}C (0h) with those at the end of the 15^{o}C growth period (48h). The most significant changes were the increased linolenic (C18:3) to linoleic (C18:2) acid ratio in total lipid (from 3.054 to 6.68), MGDG (from 5.87 to 21.76) and SL (from 1.98 to 3.91). This increased of unsaturation of fatty acids at low temperature can be attributed to a direct (Mazliak, 1981) or indirect (Kiseleva *et al*. 1999) effect of temperature on fatty acid saturation activities.

Table 1 also shows the effects of temperature change from 28^{o}C to 15^{o}C on the degree of unsaturation of total lipid and glycerolipid classes. We observed an increase in the average number of double bond per lipid molecule in the total lipid and MGDG and SL fractions, while there is little change in DGDG and decrease in PG fractions. In general, in microorganisms, the fatty acid composition of membrane glycerolipid is dependent on growth temperature (Lynch and Thompson, 1982). Increase in the level of degree of unsaturation in fatty acids is considered to provide a mechanism for the thermoadaptive regulation of membrane lipid fluidity (Mortensen *et al*., 1988). Previous reports have suggested that decreasing temperature cause a general increase in the degree of unsaturation of fatty acids in marine phytoplankton (Pohl and Zurheide, 1979; Harwood and Russel, 1984). We suggest that in *Aphanizomenon sp*. that C18:1 serve partially as modulator of low temperature adaptation.

Effect of Increasing in Growth Temperature From 15^{o}C to 28^{o}C on Fatty Acid Composition

Fig. 2 shows the pattern of changes in fatty acid composition in the total lipid classes upon temperature shift from 15^{o}C to 28^{o}C. The most significant change was observed in the shooting 0f C16:1 in PG and drop in C16:0 during the first 12h whilst over the same period C16:3 was decreased to 53.4% of its original value. These observations may suggest

that saturation during initial adaptation to higher temperature could induce partially by conversion of C16:3 to C16:1.

After 48h the temperature shift from 15^{o}C to 28^{o}C (Table2) caused an increase in the level of saturation in the fatty acyl chains of the membrane glycerolipid, as the average number of double bond per fatty acid molecule in the total lipid decreased by 14.563% (decreased from 3.296 to 2.816). Among C-16 acids, there is no evidence for an increase in the proportion of C16:0, but a decrease in C16:3 and a marked increase in C16:1 were observed in total lipid, MGDG, PG and SL. Also, changes in C-18 fatty acids were observed. In the total lipid small decrease (17.08%) in C18:3 was found, accompanied by an approximate doubling in the proportion of C18:1 within 48h of the upward temperature shift, and the level of C16:3 also decreased by 25% whilst C16:1 increased by 96.2%. The amount of saturated C-16 and C-18 fatty acids however, remained constant during temperature adaptation. These finding suggested that the observed changes in fatty acid composition are most probably due to the accelerated syntheses of C16:1 and C18:1 and substitution of the preexisting polyunsaturated fatty acid by newly synthesized fatty acids.

Similar changes were obtained in MGDG following the temperature shift from 15^{o}C to 28^{o}C. The saturated fatty acids remain unchanged whilst C18:3 and C16:3 decreased by 11.1% and 27.7% respectively, and C16:1 increased by 91.76% and C18:1 by 42.1%. The similarity in the fatty acid patterns and changes observed during temperature adaptation in the two galactolipid fraction was consisted with the conversion of MGDG to DGDG by galactosylation during the acclimation to higher temperature, as has been suggested previously (Sato and Murata,1980). The acidic lipid SL contained a substantial proportion of C16:0 which remained unchanged following the upward temperature shift. Moreover, the trend of the fatty acids present in PG was similar to that in SL. The responses seen in the glycoglycerolipid C-16 fatty acids of *Aphanizomenon sp*. are different from those reported by Sato and Murata (1980), who found that in the first 5h after temperature shift from 22^{o}C to 38^{o}C in *A. variabilis*, the relative content of C16:0 increased whilst C16:1 decreased specifically in MGDG and DGDG. Also in *A.variabis*, a decrease in the proportion of

C18:3 was observed in all of the lipid classes which was similar to that found in *Aphanizomenon sp*. The results observed during the temperature shift from 15^{o}C to 28^{o}C concluded that there is a decrease in the level of unsaturation similar to that found in other cyanobacteria species. But that the increase in saturation level may be brought about by different mechanisms from those observed previously.

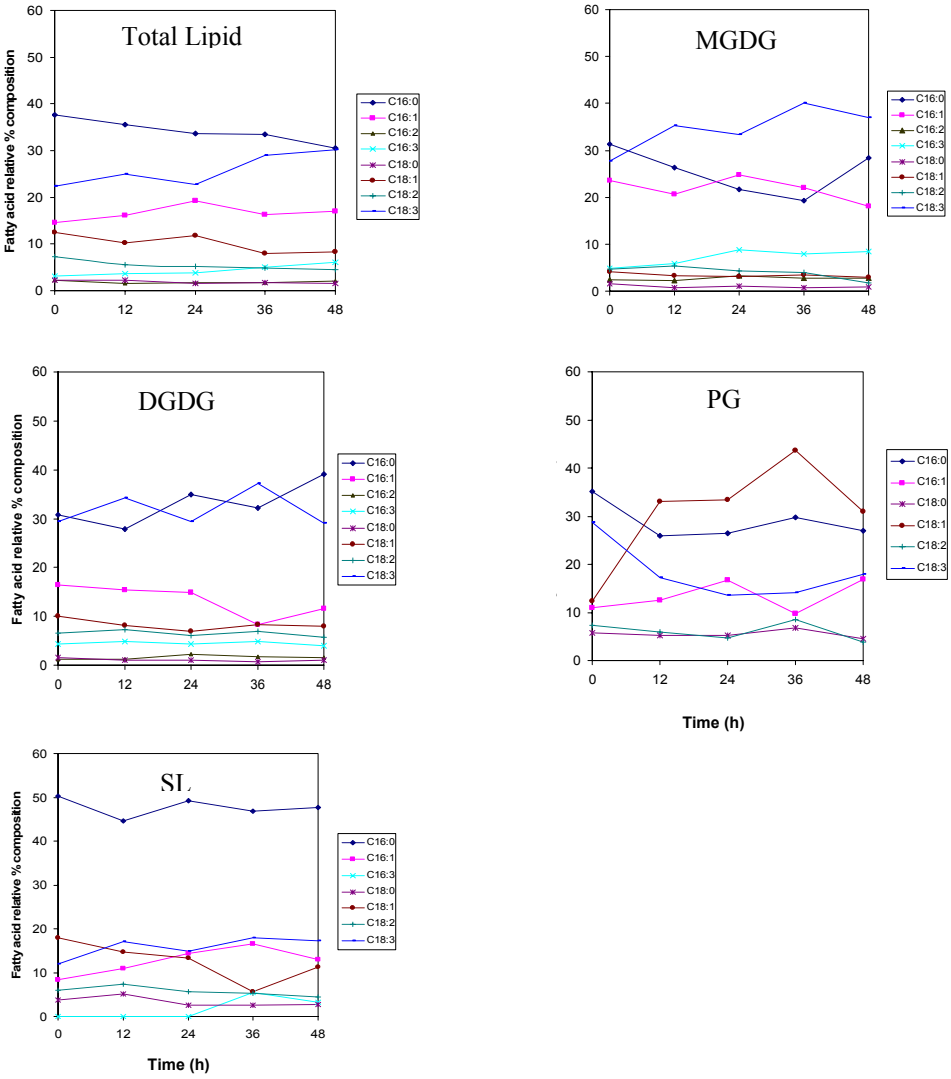

Fig. (1) : Relative percentage composition of major fatty acids in Total Lipid, MGDG, DGDG, PG and SL fractions from *Aphanizomenon sp.* Culture following a temperature shift from 28°C to 15°C.

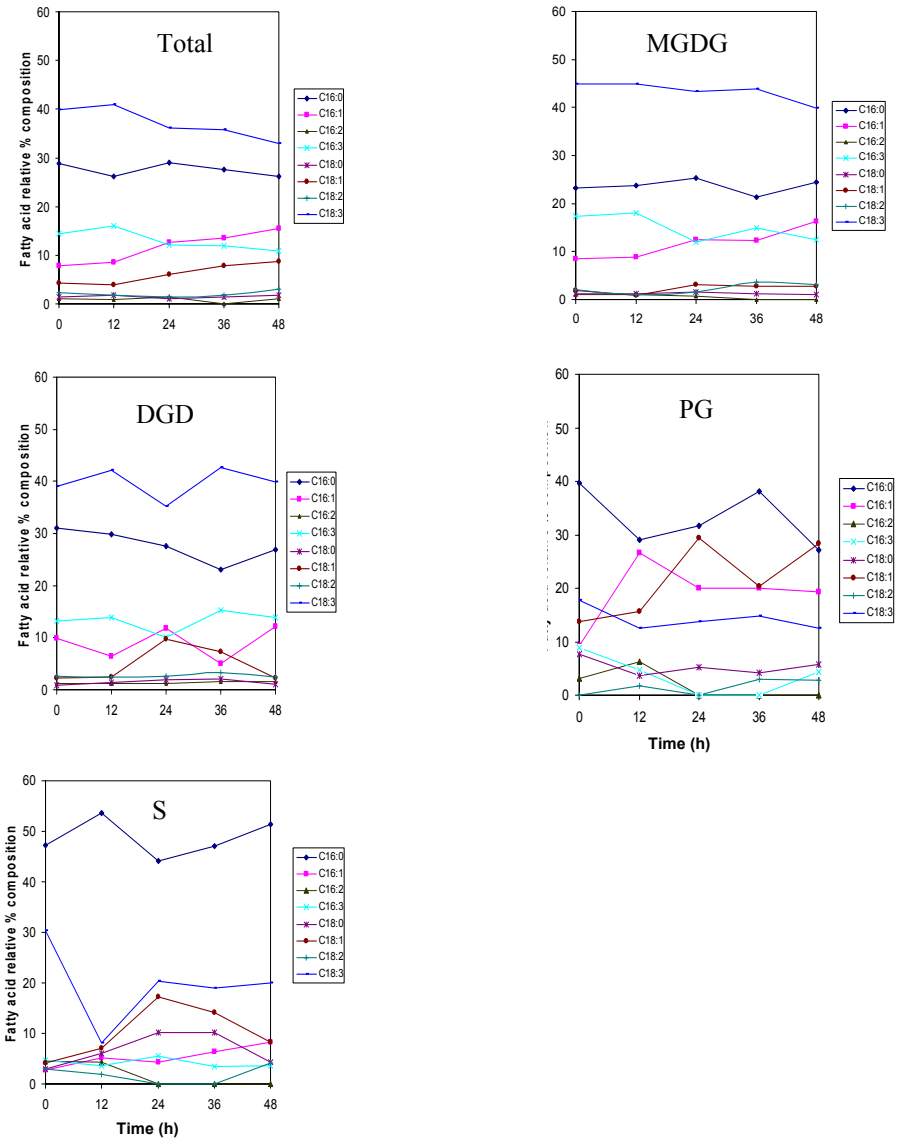

Fig. (2) : Relative percentage composition of major fatty acids in Total Lipid, MGDG, DGDG, PG and SL fractions from *Aphanizomenon sp.* Culture following a temperature shift from 15°C to 28°C.

Table (1)

Effects of growth temperature shift from 28°C (0hr.) to 15°C (48hr.) on the degree of S saturation/unsaturation as determined by the average number of double bond per lipid molecule.

Fatty acid composition (mole %)										
		C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3	Average No. of double bond per
Total lipid	28° (0hr.)	37.60	13.55	2.60	4.10	3.15	11.45	6.50	22.00	2.43
	15° (48hr.)	31.20	15.25	2.05	7.15	1.65	7.60	4.10	31.15	3.001
MGDG	28o (0hr.)	30.25	20.80	3.70	7.20	2.09	4.85	5.20	25.20	2.813
	15° (48hr.)	26.45	15.95	2.50	10.30	1.05	2.75	2.35	38.80	3.514
DGDG	28° (0hr.)	33.35	12.10	1.40	6.15	3.55	10.45	5.50	27.65	2.755
	15° (48hr.)	38.75	11.20	2.10	4.10	2.50	5.95	5.59	30.15	2.72
PG	28° (0hr.)	35.45	11.15	0.00	0.00	4.00	10.05	6.00	27.55	2.317
	15° (48hr.)	32.05	13.75	0.00	0.00	5.25	23.40	1.90	19.90	2.013
SL	28° (0hr.)	53.60	6.15	0.00	0.00	6.40	15.95	7.00	9.95	1.319
	15° (48hr.)	49.00	9.40	0.00	4.05	4.45	9.35	5.10	18.45	1.929

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تأثير تغير درجة الحرارة وسط النمو على تركيب جلايكوليبيدات الغشاء من الأحماض الدهنية في السيانوبكتيريا Aphanizomenon sp. من نوع

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الملخص :

في هذة الدراسة تم تتبع تركيب الاحماض الدهنية و درجة عدم التشبع عند تحول درجة حرارة النمو من ٢٨°م الى ١٥°م و من ١٥°م الى ٢٨°م في السيانوبكتيريا من نوع *Aphanizomenon sp.* . إن تحول درجة حرارة من ٢٨°م الى ١٥°م قد أدى إلى تغير كلي في تركيب الاحماض الدهنية في الليبيدات الكلية و الجليسروليبيدات مع إستجابة سريعة و بطيئة في محتوى الاحماض الدهنية أثناء فترة الاقلمة. إن الاستجابة السريعة كانت محصورة على ١٢ ساعة التي تلت تحول الحرارة قد شوهدت في جزء PG حيث وجد أن C18:1 قد زاد بحوالي إثنان و نصف مرة بينما مستوى C16:0 و C18:3 قد إنخفض و الأستجابة السريعة نسبيا قد وجدت في إنخفاض C16:0 مع زيادة متلازمة في C18:3 في MGDG و DGDG . و مقارنة تركيب الاحماض الدهنية عند ٢٨°م (الزمن صفر) مع تلك عند نهاية فترة النمو على ١٥°م (٤٨ ساعة) أوضحت أن معظم المعنوية هي زيادة نسبة C18:3/C18:2 في الليبيدات الكلية و MGDG و SL . و أوضحت النتائج أيضا زيادة في درجة عدم التشبع في أجزاء الليبيدات الكلية و MGDG و SL بينما لا يوجد تغيير في DGDG و إنخفاض في جزء PG .

عند تحولت درجة حرارة النمو من ١٥°م الى ٢٨°م فإن نمط التغيير في تركيب الاحماض الدهنية و درجة عدم التشبع مخالفة للتي شوهدت عند تحول درجة حرارة النمو من ٢٨°م الى ١٥°م . فقد حدث إنخفاض C16:3 و زيادة محسوسة في C16:1 في الليبيدات الكلية و MGDG و PG و SL . في الليبيدات الكلية لوحظ إنخفاض صغير في C18:3 الذي صاحبه زيادة مضاعفة في C18:1 . مع ذلك فإن كمية C16:0 و C18:0 ظلت ثابتة أثناء الأقلمة لدرجة الحرارة .