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Lipidomic analysis of the extremophilic red alga *Galdieria sulphuraria* in response to changes in pH

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ABSTRACT

Analysis of polar lipids from the red micro-alga *Galdieria sulphuraria* by means of the HILIC-LC/high resolution ESI-tandem MS determined 14 classes of lipids including identification of tens of molecular species of lipids and their regioisomers. The extremophilic alga *G. sulphuraria* uses several mechanisms for adaptation to low pH and high temperature, the major change being a replacement of cellular phospholipids by betaine lipids. We also found a change in the ratio of regioisomers of the polar lipids, i.e. phospho-, glyco- and betaine lipids, in dependence on the culture medium pH. We hypothesize that biosynthesis of polar lipids via the prokaryotic and/or eukaryotic pathway is dependent on ambient pH.

1. Introduction

Organisms inhabiting extreme biotopes are mostly prokaryotic microorganisms, but also some eukaryotes. These include e.g. the eukaryotic unicellular red micro-alga *Galdieria sulphuraria*, which may even be dominant under certain conditions. This extremophile alga, living in hot (up to 56 °C) but also in cold sulfur springs, i.e. environments having a pH in the range 0–4, is able to grow photoautotrophically, photoheterotrophically, or chemoheterotrophically. This species is colored yellow-green to dark blue-green when grown heterotrophically and yellow or green during autotrophic growth. Cell size is from about 3 to 9 μm, and it has a highly proteinaceous cell wall, a single chloroplast and Floridean starch as a storage substance. Its taxonomic position has been revised by Ciniglia and colleagues [1]. *G. sulphuraria* is a highly interesting model organism for the study of adaptation to extreme environments.

Lipids and fatty acids (FA) in *G. sulphuraria* have so far received little attention. Although hundreds of articles have been published about this alga, the number of publications on fatty acids is negligible. Lang and colleagues [2] analyzed two strains of *G. sulphuraria* (SAG collection, i.e. culture collection of algae of the University of Göttingen) and found just a narrow spectrum of fatty acid methyl esters (FAME), i.e. palmitic (P), oleic (O), linoleic (L), and linolenic (Ln) acids. Depending on the type of cultivation (heterotrophic or autotrophic), Graziani and colleagues [3] identified in addition to the already mentioned FA a small amount (in units of percent of total FA) of myristic (M), palmitoleic (Po), and stearic (S) acids. Finally, Gross and colleagues [4] identified in both thermophilic and mesophilic strains also virtually the same FAs, i.e. P, S, O, L and Ln.

However, taking into account the changes in the taxonomy (cf. [5,6,7,8]), different authors identified in *G. sulphuraria*, (originally *Cyanidium caldarium* M-8 and/or *C. caldarium* [4] Allen), apart from common FA such as P, O, L and Ln, also minor odd-numbered (tridecanoic and pentadecanoic), and even-numbered minor FA (M and Po), but also polar lipids, e.g. monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfolipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) [8].

According to our current knowledge, analysis of both simple (triacylglycerols — TAG) and complex glycerolipids (phospho- and glycolipids) has not yet been published.

We analyzed the lipids of this alga cultivated at different pH (from 1 to 4). Using LC-MS analysis we identified different classes of lipids, i.e. phospho- and glycolipids, including betaine lipids, and hundreds of different molecular species, and performed tandem MS analysis of regioisomers of many of these lipids.

2. Material and methods

2.1. Chemical and standards

Acetonitrile, 2-propanol, hexane and dichloromethane were purchased from Sigma-Aldrich (Prague, CR). 1,2-Dipalmitoyl-

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diacylglyceryltrimethylhomoserine (DGTS) and 1,2-dioleoyl PI (ammonium salt) was purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA, and DGDG, MGDG, and sulfoquinovosyldiacylglycerol (SQDG) (plant leaf lipids), 1,2-dioleoyl- phosphatidic acid (PA), 1,2-dioleoyl-PC, 1,2-dioleoyl-PE, 1,2-dioleoyl-phosphatidylglycerol (PG) (Na-salt), and 1,2-dimyristoyl-phosphatidylserine (PS) (Na-salt) from Larodan (Malmö, Sweden). Because there is no commercially available diacylglyceryl hydroxymethyltrimethyl-β-alanine (DGTA), the concentration of this lipid was estimated by comparing the peak area (total ion current in the full scan data in tandem positive ESI-MS) with those of DGTS, which has a similar structure.

2.2. Cultivation

The unicellular rhodophyte *G. sulphuraria* 002 (Galdieri) Merola was obtained from Algal Collection of Dipartimento delle Scienze Biologiche, Section of Plant Biology, University "Federico II" of Naples, Italy (http://www.acuf.net/index.php?lang=en). The inoculum of *G. sulphuraria* for this experiment originated from a cultivation at pH 2.5, in standard medium and constant light regime. Laboratory cultivation units consisted of glass cultivation photobioreactors placed in a thermostatic bath and continuously illuminated by white fluorescent light tubes (Osram L 36W/830 Lumilux, Germany). The incident light intensity was 150 m^2s^{-1} of photosynthetically active radiation (PAR). The light intensity was measured using a PU 550 digital lux-meter (Metra, Czech Republic) equipped with a PAR sensor calibrated according to the LI-185 quantum sensor (LI-COR, USA). Cultures were grown in a modified Galdieria-nutrient medium [9] and aerated by a mixture of air and CO_2 (2%, v/v). Temperature was 39 °C ± 0.5 °C. The volume of the algal suspension in each cultivation photobioreactor was 150 mL. Four different pH values were used: pH 1, 2, 3 and 4 [10]. The cultures were cultivated for 10 days and were controlled by measuring the optical density. The biomass was harvested by centrifugation and lyophilized.

2.3. Isolation of lipids

Isolation and pretreatment of lipids were carried out according to Bligh and Dyer [11], with modifications as described previously [12]. For full description of the experiments see the Supplements.

2.4. FAMEs analysis

Analysis of fatty acids in the form of methyl esters (FAME) was described previously [13,14]. A complete description of all conditions of FAME analysis by GC-MS is in the Supplements.

2.5. HILIC-LC/MS-ESI

After purification by Sep-Pak Vac Silica cartridge, the lipids were identified and quantified by LC/MS. A detailed description of both positive and negative ESI is again in the Supplements.

2.6. Statistics

All experiments were repeated at least thrice. Two-way ANOVA analyses were performed to determine significant differences ($p < 0.05$) between cultivation at different pH. In the case of significant effects, the Student–Newman–Keuls post-hoc test was applied. Three replicates ($n = 3$) of each cultivation (pH 1, 2, 3, and 4) were used for each sampling time. The software Statistica for Windows (version 12.0, Dell) was used for analyses. Data were presented as means ± SD.

3. Results and discussion

3.1. Identification and production of fatty acids

Table 1 gives the contents of total fatty acids and FA in triacylglycerols and polar lipids in the alga *G. sulphuraria* cultured autotrophically at different pH. It shows that the content of individual acids, as dependent on the pH of the culture, is not much different. Guschina and Harwood [15] assumed that algae respond to a decrease in cultivation pH by increasing the production of saturated FAs. *Euglena gracilis* [11] cultured at pH 2 and pH 5 showed basically no difference between the proportions of FA (content of saturated FA at pH 2 was 27.6%, whereas at pH 5 it was 27.9%) [16] while for *Chlamydomonas* sp. the total content of saturated FA at pH 1 was 37.2%, while at pH 6 it was 43.0% [17], i.e. it decreased with decreasing pH at variance with the theory of Guschina and Harwood [15]. Still, the saturated/unsaturated FA ratio in another *Chlamydomonas* sp. cultured at pH 2.7 and pH 7.0 was 1.38:1.00 [18], i.e. the content of saturated FA complied with the theory. The results are thus largely contradictory and satisfactory explanation for this phenomenon has not yet been proposed.

As stated above (Table 1), the theory of Guschina and Harwood [15] on the influence of pH on the unsaturation of FA appears to be only partially tenable. We therefore analyzed the lipids using two approaches, i.e. analysis of polar lipids using LC-MS (see below) and also analysis of molecular species of phospho- and glycolipids, including betaine lipids and their regioisomers, see also below.

3.2. Separation of polar lipids

The commonly used methods of analysis of lipids include TLC and HPLC and the preferred connection of HPLC with mass spectrometry (LC-MS). Separation can be carried out on both normal and reverse phases. Normal phase silica methods exploit amine or diol stationary phase, and lipids are essentially separated according to the polarity of the head groups. Reverse phase separates lipids according to the non-

Table 1
Fatty acid composition in *Galdieria sulphuraria* cultivated at different pH.

FA	pH 1	pH 2	pH 3	pH 4
Total lipids				
M	1.8 ± 0.2[a]	2.0 ± 0.3	2.1 ± 0.2	1.9 ± 0.2
P	23.1 ± 1.3	26.3 ± 1.5	27.2 ± 1.5	27.4 ± 1.4
Po	3.8 ± 0.4	3.6 ± 0.4	3.1 ± 0.3	3.7 ± 0.5
S	4.2 ± 0.4	4.4 ± 0.5	4.1 ± 0.4	4.6 ± 0.3
O	17.3 ± 0.8	16.8 ± 1.2	17.5 ± 1.5	17.1 ± 1.2
L	36.2 ± 1.2	34.8 ± 1.8	35.1 ± 2.2	33.8 ± 2.3
Ln	9.6 ± 0.7	9.0 ± 0.8	8.9 ± 0.7	9.3 ± 0.9
Unkn + tr[b]	4.0 ± 0.6	3.1 ± 0.3	2.0 ± 0.3	2.2 ± 0.3
Triacylglycerols				
M	3.4 ± 0.3	4.2 ± 0.4	4.4 ± 0.4	4.3 ± 0.4
P	25.2 ± 1.8	25.9 ± 2.3	26.4 ± 1.5	26.0 ± 2.4
Po	4.9 ± 0.4	5.7 ± 0.4	5.4 ± 0.4	5.1 ± 0.5
S	6.3 ± 0.5	7.1 ± 0.6	7.7 ± 0.6	6.9 ± 0.7
O	21.4 ± 1.9	19.8 ± 1.1	19.7 ± 1.2	22.5 ± 1.9
L	29.8 ± 2.1	31.5 ± 1.5	32.2 ± 2.3	30.6 ± 2.5
Ln	5.4 ± 0.5	2.8 ± 0.3	2.2 ± 0.3	1.3 ± 0.2
Unkn + tr	3.6 ± 0.4	3.0 ± 0.3	2.0 ± 0.2	3.3 ± 0.3
Polar lipids				
M	2.1 ± 0.2	2.3 ± 0.3	2.0 ± 0.2	2.4 ± 0.2
P	26.1 ± 2.4	27.6 ± 2.5	30.2 ± 2.6	29.9 ± 2.5
Po	4.2 ± 0.4	4.7 ± 0.4	5.1 ± 0.4	4.8 ± 0.4
S	3.7 ± 0.3	3.9 ± 0.3	4.0 ± 0.4	3.8 ± 0.3
O	19.4 ± 1.4	22.3 ± 2.3	21.4 ± 2.1	21.0 ± 1.3
L	29.1 ± 2.3	23.2 ± 2.1	22.5 ± 2.3	21.8 ± 1.5
Ln	13.6 ± 1.0	14.2 ± 0.8	13.1 ± 1.0	13.7 ± 0.9
Unkn + tr	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	2.6 ± 0.3

[a] Arithmetic means from three analyses ± standard deviations.
[b] Unknown fatty acids and those present in traces.

polar part of the molecule, i.e. according to the chain length, position and number of double bond(s).

The mobile phase is represented by different mixtures of solvents, with preferred gradient elution using two mixtures, e.g. apolar phase (hexane, $CHCl_3$) and polar (water, methanol or acetonitrile) with the buffer containing ammonium ions, acetic or formic acid. The mobile phase used for the separation is often a binary mixture of two quaternary mixtures. Popendorf [19] used a gradient from elution mixture A (n-hexane:isopropanol:formic acid:25% aqueous ammonium hydroxide) to mixture B (isopropanol:water:formic acid:25% aqueous ammonium hydroxide). The presence of water in an amount of about 5% is essential for good separation [20]. We took the optimized solvent mixture from the paper of Anesi et al. [20] using a column with the same stationary phase but from another manufacturer. On the basis of published HPLC data on total lipids from plants or algae [19–22] and our previous experience [23], we selected a diol column as the most appropriate. The chromatograms in Fig. 1 demonstrate a base-line separation of all major lipid classes, except for DGTA and DGTS.

As a eukaryotic organism, algae of genus *Galdieria* contain in their cells besides neutral lipids (mainly TAG) also polar lipids such as phospholipids and glycolipids. The major phospholipids found here were PC, PE, PG, PI, PS, and PA, among glycolipids it was particularly MGDG, DGDG, and SQDG, but also less common glycolipids such as DGCC, DGTA or DGTS. We therefore selected appropriate commercially available standards (see Fig. 1) that show base-line separation of all the classes of polar lipids using gradient elution, except DGTS and DGTA, which were separated on the same column but with a different mobile phase. We used previously published gradient elution [19,24] for the separation of these two lipid classes.

Identification was performed using total ion current in positive mode, where individual lipid classes were identified based on precursor and neutral loss scanning utilizing the characteristic fragments generated by positive ESI, see Table 1S (Supplements).

Separation of both commercially obtained lipid standards and algal lipids, see Experimental, was performed on a diol column by gradient elution. Base-line separation of all classes of lipids (Fig. 1) was carried out for 60 min in positive mode (except SQDG) and the lipids were checked as $[M + H]^+$ and $[M + NH_4]^+$ or $[M - H]^-$ ions (Table 1S). TAG residues were poorly retained on HILIC column and were eluted at the start of the chromatogram (tR = 11.3 min). As is evident from Fig. 1, diol phase provides partial intra-class separation leading to individual molecular species. This effect is particularly noticeable in those molecular species that differ significantly either in chain length or in the number of double bonds. However, intra-class separation into individual molecular species is insufficient in contrast to inter-class separation. Nevertheless, unless a given lipid class consists of many molecular species (see e.g. Fig. 2, which is basically a cutout of Fig. 1 from 18.3 to 19.5 min) then the first eluting and therefore the least polar molecular species are 36:2, then 36:3 and finally 36:4 but the separation is not complete. To further pinpoint not only a molecular species, but also regioisomers of individual lipid classes, we used tandem MS (MS^2) (Fig. 3).

3.3. Structure of polar lipids

Glycerolipids located in the cells of *G. sulphuraria* were identified on the basis of the fragmentation mechanism obtained using mass spectrometry of commercially available standards, see Material and methods. In positive-ion tandem ESI spectrum, MGDG exhibits ion $[M + NH_4]^+$ at 772.5939 Da, which produces two intense fragments $[M + NH_4–R_iCOOH]^+$ resulting from the loss of neutral particles (free acids) either from the *sn*-1 (i = 1) or the *sn*-2 position (i = 2) of glycerol.

Ion of the type $[M + NH_4–Gal]^+$ at *m/z* 609.5332 arises by elimination of a monosaccharide (galactose) from the ion $[M + NH_4]^+$. The very intense peak in the spectrum of MGDG is an ion having a value of 238.1291 Da ($[C_9H_{16}O_6 + NH_4]^+$), which arises from the galactosyl group head and is characteristic for MGDG.

Cleavage of another glycolipid, i.e. DGDG, is quite analogous to MGDG. Two ions, i.e. $[M + NH_4–R_iCOOH]^+$ and $[M + NH_4–Gal–R_iCOOH]^+$ (i = 1,2) generated from the ion at 934.6467 Da ($[M + NH_4]^+$) are formed by gradual loss of both a fatty acid, and one galactosyl moiety. Ion at 400.1819 Da ($[C_{15}H_{26}O_{11} + NH_4]^+$) is generated from the head digalactosyl group due to further loss of galactose to produce an ion of 238.1291 Da value ($[C_9H_{16}O_6 + NH_4]^+$) (Fig. 4). Both MGDG and DGDG may give rise to an ion having a value of 238.1291 Da, which can be used to identify the two glycolipids in positive ESI. It should be noted, however, that in the case of DGDG the ion at *m/z* 400.1819 has a higher intensity than the ion at *m/z* 238.1291; this makes it possible to identify DGDG on the basis of the presence of the ion at 400.1819 Da.

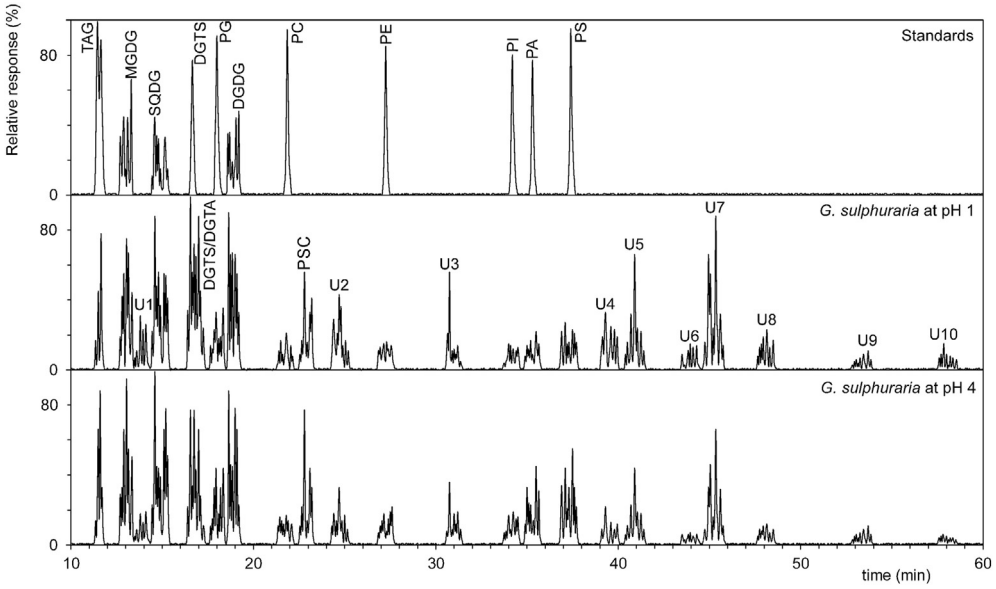

Fig. 1. HPLC/ESI-MS separation of standards and major lipid classes of alga of *Galdieria sulphuraria*, cultivated at pH 1 and pH 4. For experimental conditions, see "Material and methods". Abbreviations are explained in the text.

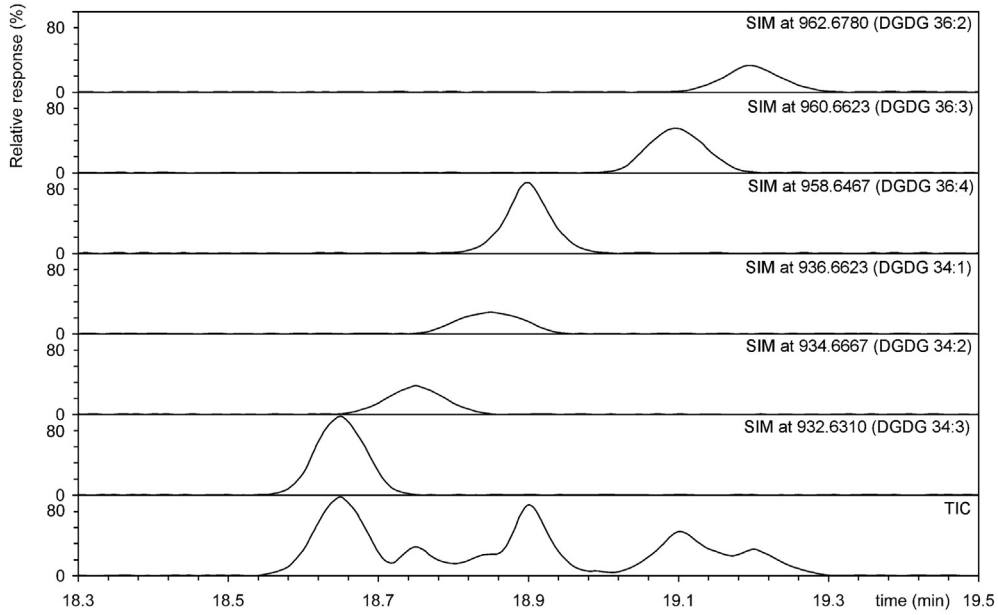

Fig. 2. A chromatogram cutoff from Fig. 1, expanded between 18.3 to 19.5 min.

Another glycolipid, SQDG, was identified in negative ESI since it contains a sulfo group. The mass spectrum was shown to feature $[M - H]^-$ ion at *m/z* 817.5141. This ion is cleaved with a simultaneous loss of two carboxylate anions $[R_iCOO]^-$ at *m/z* 255.2324 (*sn*-1, palmitic acid) and at *m/z* 279.2330 (*sn*-2, linoleic acid). Relevant negatively charged ions, i.e. $[M–R_iCOOH–H]^-$) at *m/z* 561.2739 and 537.2739 arise by a loss of neutral particles (fatty acids) from the precursor ion. Like with MGDG and DGDG, the spectrum shows an intense ion having a value of 225.0075 Da ($[C_6H_9O_7S]^-$) (Fig. 5), which is essentially the sugar moiety and can be used for identification of SQDG. To determine which acid is esterified in the *sn*-1 or *sn*-2 position we can use with advantage the knowledge that the *sn*-2 lyso lipid is more stable and therefore that palmitic acid is esterified in *sn-1* position. Therefore, the resulting structure of SQDG was designated as *sn*-1-16:0-*sn*-2-18:2 SQDG.

Betaine lipids are a class of acylglycerolipids having quaternary amino alcohol bound via an ether linkage to diacylglycerol moiety. These glycerolipids do not contain phosphoric acid in their molecule, although they may, e.g. in red algae, replace conventional phospholipids such as PE, and PC in membranes [25]. Based on chromatographic separation we decided to characterize betaine lipids (a different mobile phase was used for separation of DGTS and DGTA). DGTS and DGTA cannot be distinguished by mass spectrometry alone since both compounds have the same elemental composition. Some studies described identification of DGTS and DGTA based on the elimination of $CH_2–CH_2–N^+(CH_3)_3$ [26] but Armada et al. [27] question these data (see the spectra of DGTS and DGTA in their Figs. 1 and 2).

In the tandem mass spectrum, an ion at *m/z* 753.6357 and two pairs of ions are formed due to the characteristic loss of R_1COOH, R_2COOH, $R_1'–CH{=}C{=}O$, and $R_2'–CH{=}C{=}O$ at *m/z* 480.3684, 456.3684, 498.3789, and 474.3789, respectively and at *m/z* 649.5049 (loss of $CH_2–CH_2–N^+(CH_3)_3$). The spectrum features also an abundant ion at *m/z* 236.1492 ($C_{10}H_{22}NO_5$), which supports the structure determination

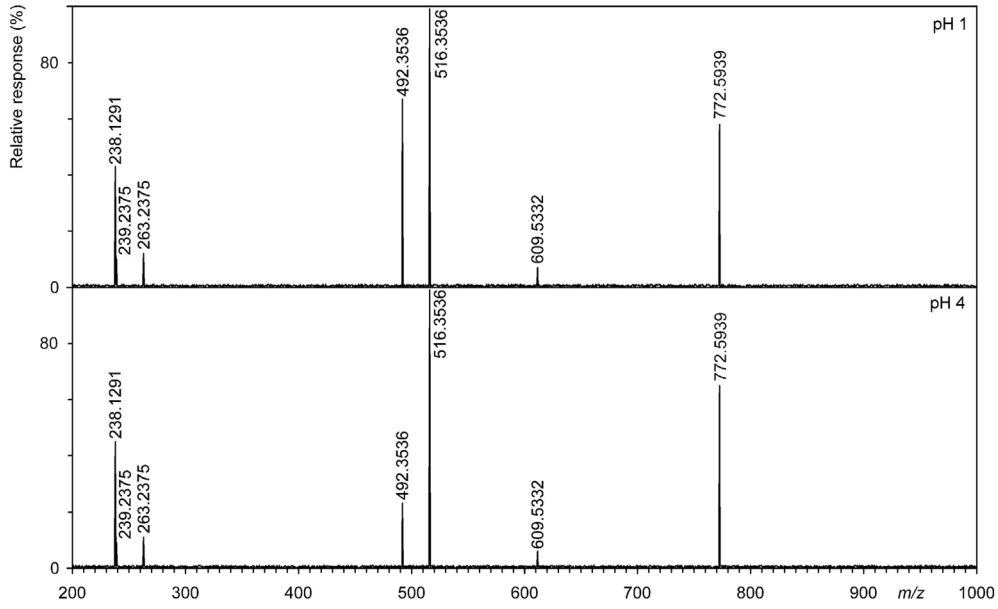

Fig. 3. Tandem mass spectrum of natural MGDG, molecular species 1-palmitoyl-2-linoleyl-MGDG.

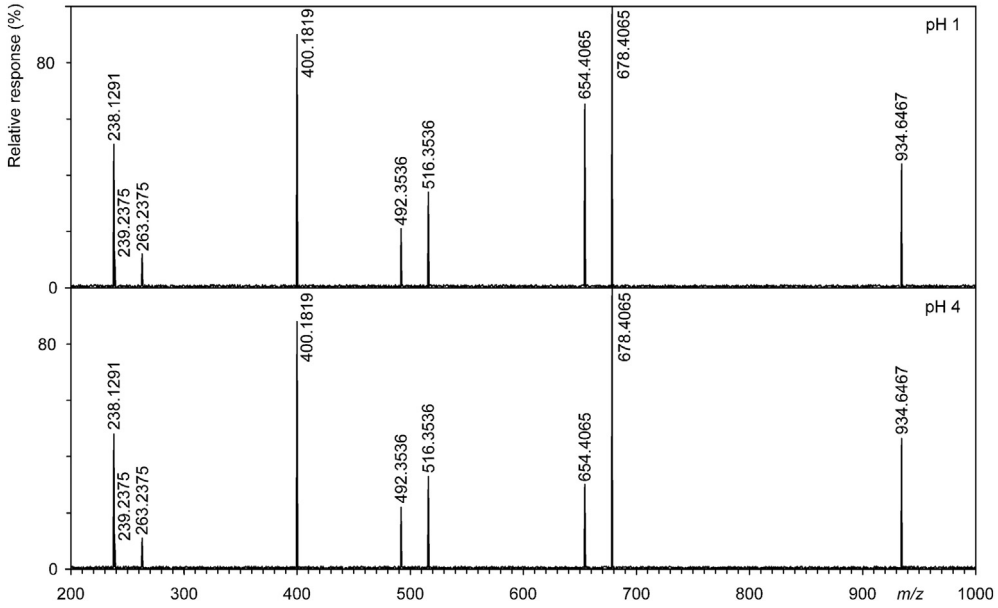

Fig. 4. Tandem mass spectrum of natural DGDG, molecular species 1-palmitoyl-2-linoleyl-DGDG.

as DGTS [24,26]. A dependence of the intensities of ions of type $[M + H–R_iCOOH]^+$ on *sn*-1 or *sn*-2 has already been demonstrated, e.g. for phospholipids [28] or for MGDG and DGDG [29]. Abida et al. [30] assume, based on the chemical similarities, that the situation in DGTA is the same as in phospholipids, i.e. the intensity of the ion $[M + H–R_2COOH]^+$ is higher than that of $[M + H–R_1COOH]^+$.

An isomorph compound with a pseudomolecular ion at *m/z* 753.6357 exhibits, in mass spectrum, ions at the same *m/z* value 753.6357, but of different abundance. The most abundant is the ion at *m/z* 480.3684 (loss of FA at the *sn*-1) and an ion at *m/z* 456.3684 (loss of FA at the *sn*-2). The much lower abundance of the ion at 649.5049 (loss of $CH_2–CH_2–N^+(CH_3)_3$) and the same abundance of an ion at 677.5356 (loss of $N^+(CH_3)_3$) as in DGTS supports the proposed structure of DGTA (Fig. 6), which was also supported by the different elution time from HILIC column.

To our current knowledge, mass spectra of phosphatidylsulfocholines (PSC) have been measured only by desorption chemical ionization MS [31] and field desorption (FD) MS [32]. Nevertheless, mainly due to the fact that the FD-MS is one of the soft ionization techniques (although currently hardly in use), major ions could be expected in the positive ESI spectrum on its basis.

Another group of peaks at retention time 22.8 min gives a majority pseudomolecular $[M + H]^+$ ion at *m/z* 761.5155 ion and an ion at *m/z* 728.5356 $[M + H\text{-}60]^+$, i.e. $[M + H–S(CH_3)_2]^+$, wherein this ion is a base peak. Here however the similarity of ESI spectra and FD spectra ended. Further, the spectrum contains two further pairs of the most abundant ion at *m/z* 506.2825, 482.2825, 488.2719, and 464.2719. These ions are formed by R_iCOOH loss, or loss of the corresponding ketene ($R_i'–CH{=}C{=}O$) from $[M + H]^+$. We assume that in this case the rule is that the loss of the carboxylic acid linked to the *sn-1* glycerol

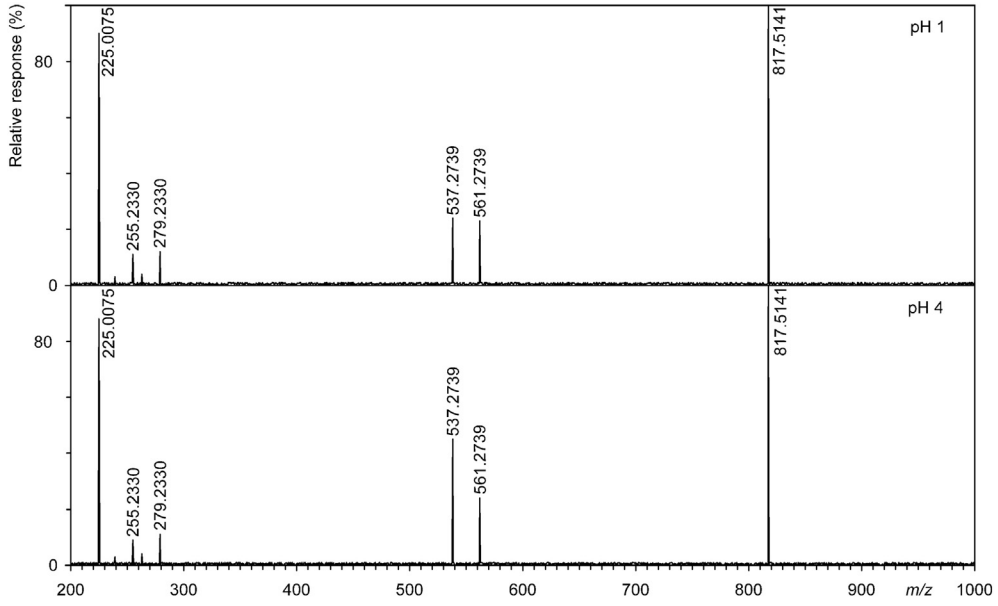

Fig. 5. Tandem mass spectrum of natural SQDG, molecular species 1-palmitoyl-2-linoleyl-SQDG.

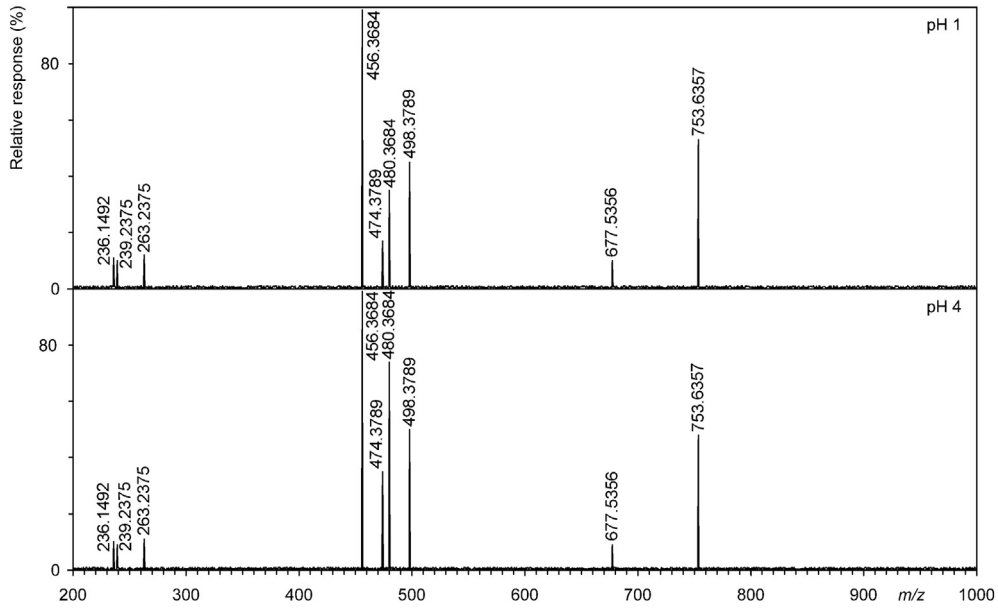

Fig. 6. Tandem mass spectrum of natural DGTA, molecular species 1-palmitoyl-2-linoleyl-DGTA.

position always produces a more intense peak than that derived from the loss of the *sn*-2 acyl chain. It thus follows that this PSC is *sn*-1-16:0–18:2-PSC.

Based on our previous papers [33–35] and a review by Hsu and Turk (2009) [28] all the above mentioned phospholipids form an abundant ion of the type $[M + H]^+$ or $[M + NH_4]^+$, see Table 1S. Tandem MS was used to characterize the structure of each class of phospholipids, as well as the structure of their molecular species, including the position of fatty acids in the *sn*-1 and *sn*-2, respectively. A rule appears to hold that preferential loss of R_1COOH using the α-hydrogens of the fatty acyl group at the *sn*-2 gives rise to more prominent $[M + H–R_1COOH]^+$ ion than the $[M + H–R_2COOH]^+$ ion, see also the paper by Abida et al. [36].

3.4. Effect of pH change on proportions of polar lipids

The composition of fatty acids in individual classes of polar lipids and TAG for *Chlamydomonas* sp. as dependent on the pH of the culture medium was published by Tatsuzawa et al. [17]. The content of palmitic acid (major FA) in TAG increases with decreasing cultivation pH (31% at pH 1, 32% at pH 3, 26% at pH 6), while that of MGDG decreases (18% at pH 1, 41% at pH 3, 46% at pH 6). This contradicts the above hypothesis since MGDG as structural membrane lipid should behave according to it. These data point to the understandable scarcity of studies in this field because few algae are capable of growing in the pH range of 1–7, and document the unsatisfactory information on the FA saturation/unsaturation ratio in dependence on cultivation pH.

In terms of the content of individual lipid classes, our results can be compared with only two already published articles [17,18]. Seven classes of lipids (TAG, MGDG, DGDG, SQDG, PG, PI and DGTS, for abbreviations see above) were identified by TLC in *Chlamydomonas* sp. cultivated at pH 1, pH 3 and pH 6. The influence of pH on the content of TAG and polar lipids showed no clear trend and cannot be traced back to simple pH dependence. For example, the amount of TAG at pH 1 was 63% of the total lipid, at pH 3 it dropped to 46% and at pH 6 it again rose to 51%. In the study on *E. gracilis* by Hayashi et al. [16], the content of both polar lipids and TAG in total lipids was the lowest in cultivation carried out at pH 5.

Betaine lipids, e.g. DGTS and DGTA, were found in extra-chloroplast and thylakoid membranes of eukaryotic algae, predominantly in green algae, and have been suggested to act as substitutes for PC [37]. It is believed that betaine lipids replace phospholipids under phosphate limitation of growth. Freshwater algal species have usually mainly PC and little DGTS, while marine species have much more DGTS, which can be caused by low levels of phosphate in sea water.

Six algae of the class Rhodophyceae, among others *Porphyridium purpureum* and *Cyanidium caldarium* [38], completely lacked conventional betaine lipids, i.e. DGTS or DGTA. The same conclusion was reached by Kostetsky et al. [39] in the red alga *Ahnfeltia tobuchiensis*.

Conversely, low concentrations of DGTS were identified in five out of 22 species of red algae, whereas DGTA was detected in a low concentration in one species only (cited according to [37]). DGTS was detected also in the rodophytes *Phyllophora pseudoceranoides*, *Lomentaria articulata*, *Mastocarpus steliatus*, *Membranoptera alata* and *Phycodrys rubens* [40]. Other two red algae, i.e. *Pterocladiella capillacea* and *Polysiphonia urceolata*, contained betaine in the range of 0.2 to 0.45% of dry weight [41].

Phosphatidylsulphocholine (PSC), a rather unusual lipid and a sulfur analog of phosphatidylcholine, was identified in two marine red algae, *Chondrus crispus* and *Polysiphonia lanosa* [42]. Dimethylsulfoniopropionate (DMSP) was found in many Rhodophyta algae; unfortunately, very little is known about its effect on the biosynthesis of PSC, yet it is assumed that DMSP acts as precursor of the biosynthesis of PSC [12] that has been identified, e.g., in the red algae *Polysiphonia paniculata* [43] or *Gelidium arbusculum* [44].

DMSP biosynthesis is important not only in relation to osmoprotection but also because the DMSP produced by marine algae is the precursor of atmospheric dimethyl sulfide gas [45,46].

As seen in Fig. 7, the content of phospholipids (PG, PC, PE, PI, PS, and PA) and glycolipids (MGDG, DGDG and SQDG) increases with increasing pH while the content of betaine lipids (DGTA and DGTS) decreases. The content of the phosphatidylcholine analog PSC copies the content of PC, which could mean that sufficient sulfur is present in the solution.

This phenomenon has not yet been observed but one should be aware that the effect of change of culture pH on the proportion of phospho-, glyco- and betaine lipids has not yet been explored. We believe that the reason for the change in the content of phospholipids and their replacement by betaine lipids is the lack of available phosphate in the culture medium. The culture medium contains metal ions which, at a given pH, form insoluble phosphates with $H_2PO_4^-$. Moreover, the major compound present in solution at pH 1 is only the undissociated H_3PO_4 ($pK_{a1} = 2.12$). The difference in the representation of the

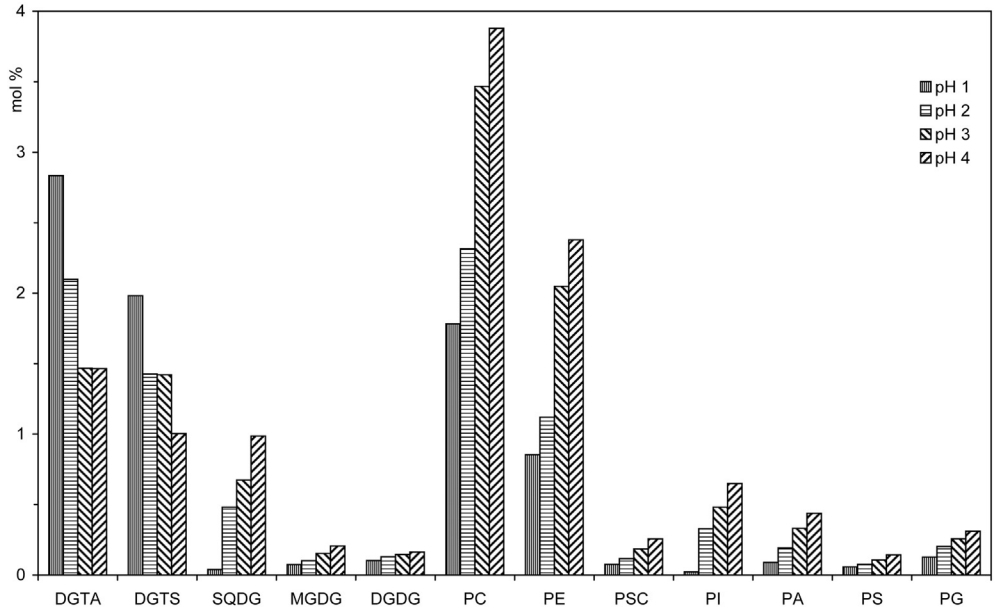

Fig. 7. The content of phospholipids, glycolipids and betaine lipids in *Galdieria sulphuraria* as dependent on cultivation pH.

various classes of lipids is best seen on one of the most abundant molecular species, 16:0–18:2 phospholipids/betaine lipids and glycolipids (see Fig. 8).

The phosphorus starvation hypothesis is supported by several facts. Firstly: at pH 1, to which the nutrient solution was acidified with sulfuric acid, according to the Henderson–Hasselbalch equation only ~7% of the total phosphorus is present as $H_2PO_4^-$ ion, most of it being present as undissociated phosphoric acid (H_3PO_4). Out of 300 mg KH_2PO_4 (m.w. 136) in 1 L of nutrient solution only about 21 mg is thus present in dissociated form, which is a 0.154 mM solution. Riekhof et al. [47] reported that phosphate starvation in the yeast *Kluyveromyces lactis* induces the replacement of PC by DGTS in a concentration range of 1 mM–0.15 mM, see their Fig. 2 (TLC of lipids). The TLC clearly shows the stimulatory effect of phosphorus deficiency on increased production of DGTS, with a clear break just between these two concentrations. Based on these findings we believe that, in our case, despite phosphate being present in solution in sufficient quantity (considering its total amount, not only dissociated form), i.e. a concentration of 0.15 mM, which holds for both our own and literature data, the alga is subject to phosphorus starvation. With increasing pH the amount of ionic $H_2PO_4^-$ (and gradually also HPO_4^{2-}) increases and the effect of phosphorus starvation decreases, see Figs. 7 and 8.

A possible reason why the alga *Galdieria* can cope with the higher temperatures in thermal springs could be the effect of pH on the transition temperature of different phospholipids. An example can again be PC for which, e.g. for the molecular species 16:0/18:0-PC, the transition temperature of the lamellar gel-liquid crystalline phase increases from 42.0 °C at pH 2.7 up to 50.0 °C at pH 1 [48]. Thus, a change in ambient pH enables the alga to accommodate higher temperatures without being forced to change the structure of fatty acids in the cell

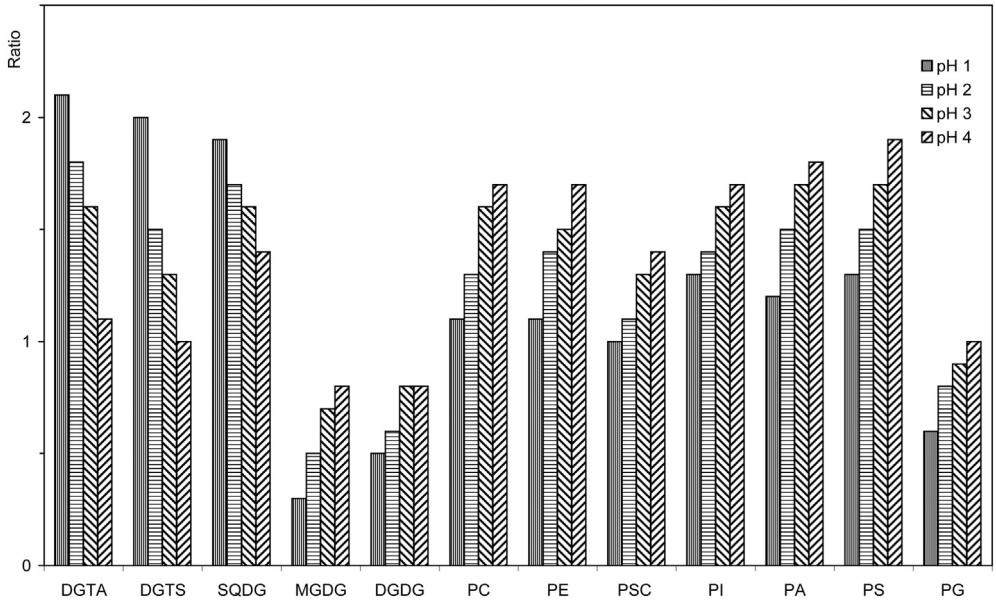

Fig. 8. The content of molecular species of 1-palmitoyl-2-linoleyl-lipids in *Galdieria sulphuraria* as dependent on cultivation pH.

membranes. Other phospholipids such as PA and PS, which were present in minor amounts (see Fig. 1), behave differently, or these changes occur at a much higher pH, see [49].

A similar effect can be found when PC is replaced by DGTS [50]. The phase transition temperatures (Tm) of 1-palmitoyl-2-stearoyl DGTS and of 1,2-distearoyl DGTS are 53 and 59 °C, respectively, each of these Tm values being significantly higher than the Tm values (47 °C and 54 °C) of the PC with an identical combination of fatty acids.

3.5. Effect of pH on the proportion of molecular species of polar lipids differing in fatty acids at sn-1 and sn-2, respectively

Another phenomenon that we observed when analyzing samples of algae cultured at different pH was a change in the representation of the various molecular species of polar lipids, see Fig. 8. This figure and also mass spectra (Figs. 1S–3S, Supplements) clearly show that a pH change brings about a change in the proportion of all regioisomers of polar lipids, as shown on one of the most abundant molecular species, the 16:0–18:2, and 18:2–16:0. Based on previously published results [51] on the biosynthesis of 18–16 diacylglycerols in the prokaryotic pathway and 16–18 diacylglycerols in the eukaryotic pathway, we expected a change in the representation of molecular species of polar lipids, which was also confirmed. In previously published studies, which describe the analysis of molecular species depending e.g. on the cultivation temperature, there is no mention of a change in representation of molecular species — regioisomers. Han et al. and Khozin-Goldberg and Cohen [41,52] described in their publications on the fresh water eustigmatophyte *Monodus subterraneus* an effect of phosphate starvation on the molecular species composition of DGDG and discovered that in starving algae the content of saturated 16:0–16:0-DGDG is reduced while the content of more unsaturated 20:5–16:1-DGDG grows. Flaim [53] showed an increased content of 18:4–18:4-MGDG in the dinoflagellate *Peridinium aciculiferum* with increasing cultivation temperature, while the content of 18:3–18:3-PC remained practically unchanged. Leblond [54] studied the effect of temperature on the relative abundance of *Symbiodinium microadriaticum* galactolipids. The content of, e.g., 18:4–18:5-MGDG and DGDG decreased with increasing temperature while the abundance of 18:4–18:4-MGDG and DGDG increased. The structure of betaine lipids, i.e. the position of fatty acids in the *sn*-1 and *sn*-2, was not determined. Table 1 shows that both total FA, and FA in the polar lipids do not change much; we therefore think that algae respond to a change in the pH of the nutrient solution by changing the ratio of FAs in the *sn*-1 and/or *sn*-2 position, giving rise to a different ratio of biosynthesized regioisomers.

4. Conclusion

Polar lipids in the alga *G. sulphuraria* were separated and their structures were determined using high resolution HILIC-LC/ESI-tandem MS. The method allowed us to identify 14 classes of lipids, each of them containing tens of molecular species including regioisomers. The alga was found to use several mechanisms to cope with the extreme low-pH environment in which it grows. Due to very low pH, the culture medium contains only very little phosphate ions and a strong prevalence of undissociated phosphoric acid. This leads to phosphorus starvation, which the alga counters by replacing cellular phospholipids with betaine lipids. Another mechanism involves a change in the ratio of regioisomers in dependence on the culture medium pH, i.e. biosynthesis of polar lipids via the prokaryotic and/or eukaryotic pathway depending on the pH. Neither of these two phenomena has so far been described.

The comprehensive analysis of polar lipids by means of the high resolution HILIC-LC/ESI-tandem MS used with G. *sulphuraria* can be applied to other photosynthetic and non-photosynthetic organisms as well.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.12.005.

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