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Arsenolipids in the green alga *Coccomyxa* (Trebouxiophyceae, Chlorophyta)

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ABSTRACT

Lipid-like compounds containing a dimethylarsinoyl group, i.e. $Me_2As(O)$-, have been identified by liquid chromatography/inductively coupled plasma mass spectrometry (LC/ICP-MS) and non-aqueous reversed-phase high-performance liquid chromatography (positive and/or negative high-resolution tandem electrospray ionization mass spectrometry (NARP-HPLC/HR-ESI$^{+(-)}$-MS/MS) from three strains of green algae of the genus *Coccomyxa* (Trebouxiophyceae, Chlorophyta). The algae were cultivated in a medium containing 10 g arsenic/L, i.e. 133.5 mmol/L of $Na_2HAsO_4.7H_2O$. After extraction by methyl-tert-butyl ether (MTBE), total lipids were analyzed by ICP-MS or ESI-MS without any further separation or fractionation. A total of 39 molecular species of arsenic triacylglycerols (AsTAG), 15 arsenic phosphatidylcholines (AsPC), 8 arsenic phosphatidylethanolamines (AsPE), 6 arsenic phosphatidylinositols (AsPI), 2 arsenic phosphatidylglycerols (AsPG) and 5 unknown lipids (probably ceramides) were identified. The structures of all molecular species were confirmed by tandem MS. Dry matter of the individual strains contained different amounts of total arsenolipids, i.e. *C. elongata* CCALA 427 (0.32 mg/g), *C. onubensis* (1.48 mg/g), *C. elongata* S3 (2.13 mg/g). On the other hand, there were only slight differences between strains in the relative abundances of individual molecular species. Possible biosynthesis of long-chain lipids with the end group $Me_2As(O)$ has also been suggested.

1. Introduction

Coccomyxa Schmidle is a genus of coccoid green microalgae belonging to the class Trebouxiophyceae (Chlorophyta). It represents a cosmopolitan and ecologically versatile taxon found in different types of habitats, including extreme ones. For example, *Coccomyxa* is commonly encountered in acidic or neutral habitats with increased heavy metal concentrations (Barcyte and Nedbalova, 2017; Fuentes et al., 2016; Koechler et al., 2016; Malavasi et al., 2016) and it is also found as a contaminant in different chemical solutions in laboratories (Sládečková, 1959). The ability to cope with various stressful environmental conditions has evolved within different *Coccomyxa* species and tolerance to toxic substances might be a population- or strain-specific phenomenon (Barcyte and Nedbalova, 2017). Therefore, isolates from extreme habitats are particularly attractive for biotechnological research since they might have an advantage in growing under a wide range of stressful conditions, producing hitherto unknown and/or high value compounds. Increased attention has been paid to the genus *Coccomyxa* in terms of the accumulation of lipids (Bermejo et al., 2018; Ruiz-Domínguez et al., 2014). The results have shown that strains of this genus are good candidates for a biofuel or as a source of various valuable compounds, as well as for toxic metal bioremediation technologies (Leonardo et al., 2016).

The presence of arsenic-containing compounds has been demonstrated in extracts from marine fish and algae (Dembitsky and Levitsky, 2004; Duncan et al., 2015). Cultivation of unicellular algae, as well as cyanobacteria, in the presence of arsenic has been described many times (Duncan et al., 2013). However, all these cultivations, with rare exceptions, were performed at arsenic concentrations commonly found in the world's oceans, i.e. 1.5 μg/L on average, and 0.15–0.45 μg/L in freshwater (Bissen and Frimmel, 2003). The concentration of arsenic exceeding these concentrations by many orders was used only exceptionally. *Dunaliella salina* was cultured at a concentration of 2 g of Na_2HAsO_4 per 1 L (i.e. 6.41 mmol arsenic) (Yamaoka et al., 1999). At this concentration, algae still grew and accumulated arsenic in the cells. The algal extracts contained, in addition to inorganic anions - arsenite (As^{III}) and arsenate (As^{V}) - also low molecular weight arsenic-containing organic compounds, i.e. arsenobetaine (AB) or dimethylarsinoyl-propionate (DMAP). Arsenosugar compounds such as derivatives of dimethylated arsenoribosides are also common (Dembitsky and Levitsky, 2004). Besides these compounds, lipid-soluble dimethylarsinoyl compounds, especially dimethylarsinoyl hydrocarbons (AsHC) and

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dimethylarsinoyl fatty acids (AsFA) have also been identified (Wang et al., 2015).

The AsFA and AsHC contain the dimethylarsinoyl group instead of a terminal methyl group. Both AsFA and AsHC have been found primarily in fish oil, which contains both saturated and unsaturated dimethylarsinoyl FA. Homologues from C8 to C28, mostly with an odd number of carbon atoms, are present at low concentrations, typically ppm in fish oil (Rumpler et al., 2008). Although the positions of the double bonds in AsFA have not yet been identified, it is assumed that biosynthesis is based on the starting group dimethylarsinoyl-propionic acid (Rumpler et al., 2008). For this reason, analogues of conventional FA, e.g. palmitic (16:0), oleic (18:1ω-9) or docosahexaenoic (22:6ω-3) acids are found in fish oil (Gunstone, 2010).

Analysis of arsenical compounds is currently performed by a combination of reversed-phase liquid chromatography-mass spectrometry (RP-LC/MS) methods where a liquid chromatograph is linked to an inductively coupled plasma mass spectrometer (ICP-MS) in selective monitoring mode at *m/z* 75.0 $[As]^+$ (Amayo et al., 2011; Arroyo-Abad et al., 2013; Guttenberger et al., 2017a,b; Miyashita et al., 2009; Pereira et al., 2016; Sloth et al., 2005). In this way, arsenical compounds are sequentially eluted from the non-aqueous reversed phase column. Unfortunately, this method does not provide information on the structure of a given compound and can only ascertain whether arsenic is present or not. The structure can only be judged from retention times if standards are available. The full structure of organometallic arsenic compounds is best determined by positive electrospray ionization high-resolution tandem mass spectrometry (positive HR-ESI-MS/MS). Almost all arseno-lipids can be identified based on the fact that they contain a dimethylarsinoyl group, i.e. $Me_2As(O)$, which is characterized in tandem MS by two ions at *m/z* 104.9680 $[C_2H_6As]^+$ and at *m/z* 122.9786 $[C_2H_6OAs]^+$ (Guttenberger et al., 2017a, 2017b; Viczek et al., 2016).

Using HR-MS, polyunsaturated AsFA was identified in arsenic phosphatidylcholine (AsPC) from herring roe, and very long chain dimethylarsinoyldimethylarsoryl polyunsaturated AsFA was identified in arsenic phosphatidylethanolamine (AsPE) from salmon roe (Viczek et al., 2016). According to Taleshi et al. (2014a), a nonpolar lipid extract of oil from the blue whiting fish (*Micromesistius poutassou*) probably comprised arsenic-containing triacylglycerols (AsTAG); the presence of AsTAG was also later presumed in the Peruvian anchoveta (*Engraulis ringens*) (Pereira et al., 2016). However, few data exist on arsenolipids from photoautotrophic microorganisms that form the basis of aquatic food chains.

The objective of this work was to study the biosynthesis of lipid-soluble arsenical compounds in the green alga *Coccomyxa* when is exposed to high inorganic arsenic levels in the cultivation medium.

2. Results and discussion

2.1. Fatty acids and lipids from Coccomyxa

The fatty acids (FA) in the algal biomass of the genus *Coccomyxa* identified in the literature were in particular, C16 and C18 FA including eight FA ranging from saturated to triunsaturated ones, i.e. 16:0, 16:1, 16:2, 16:3, 18:0, 18:1, 18:2, 18:3, but not C20 or C22 FA such as arachidonic, eicosapentaenoic or docosahexaenoic FA. The taxonomic classification of the genus *Coccomyxa* to the class Trebouxiophyceae indicates the possible content of fatty acids (Lang et al., 2011), and other authors dealing with this genus (Abe et al., 2014; Bogen et al., 2013; Guschina et al., 2003; Guschina and Harwood, 2006; Msanne et al., 2012) have also presented similar data on FA identified in *Coccomyxa* algal biomass.

Furthermore, common classes of individual lipids were also identified in the biomass of *Coccomyxa* strains by different authors (see below), Similarly to other representatives of the genus, these included neutral lipids (triacylglycerols (TAG), diacylglycerols (DAG), and sterol esters (SE)) (Guschina et al., 2003) or polar lipids, i.e. phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), phosphatidylserines (PS) (Kiseleva and Kotlova, 2007), phosphatidylinositols (PI) (Guschina et al., 2003), glycolipids - monogalactosyldiacylglycerols (DGGG), diacylglyceryltrimethylhomoserine DGTS) (Kiseleva and Kotlova, 2007), and also sulphoquinovosyl diacylglycerols (SQDG) (Guschina et al., 2003) or trigalactosyldiacylglycerols (TGDG) (Allen et al., 2017).

2.2. Cultivation of algae and cyanobacteria in arsenic-containing medium

Many green algae are able to grow in a medium with a high arsenic salt concentration. An example is *Dunaliella salina*, which grew in a medium containing 2 g of arsenate per 1 L (Yamaoka et al., 1999). Green alga *Dunaliella* sp. was cultivated in a medium containing up to 100 mg/L sodium arsenate (Na_2HAsO_4 . $7H_2O$) and sodium arsenite ($NaAsO_2$) (Takimura et al., 1996). *Chlorella vulgaris* was successfully cultivated in a solution containing 1 g of arsenate per liter of medium (Murray et al., 2003).

Miyashita et al. (2009) analyzed water-soluble arsenicals extracted from algae growing in an arsenic-rich river. The green alga *Cladophora glomerata* was found to contain a total arsenic content of 18 μg/g of dry matter but less than 50% of this total was extracted using a methanol-water mixture. This may indicate that a substantial proportion of arsenical compounds are retained in the algae and may thus be lipid-like compounds. Similarly, *Chlamydomonas reinhardtii* contained a total arsenic level of 11 μg/g of dry matter, but only about 1/3 was extractable using a methanol/water mixture.

The cyanobacterium *Nostoc*, cultivated in a nutrient solution containing up to 100 μmol/L of As(III), contained more than 1.5 mg As/g of dry matter. In an extract obtained using a mixture of CH_2Cl_2-MeOH, the arsenic content was over 5 μg/g (dry mass), and about 25 μg/g in an aqueous extract obtained from dry mass (Xue et al., 2017). The alga *Coccomyxa* sp. was able to grow without difficulty in arsenic or arsenate medium containing a concentration of tens of mmol As/L (Koechler et al., 2016). It can thus be assumed that algae and cyanobacteria are able to accumulate arsenicals in tenths of a percent of dry matter and that most of the arsenical compounds, designated as total arsenic, have not been identified.

In the *Coccomyxa* strains used in our study, the content of arsenolipids, i.e. relative abundance of the individual molecular species, was similar, but not the absolute content (weight) of arsenolipids. While in *C. elongata* CCALA 427 isolated from a conventional habitat (freshwater pool), total arsenolipids obtained by extracting the cells with methyl-tert-butyl ether (MTBE) amounted to 0.32 mg/g DM, in two strains from an extreme environment, the content of total arsenolipids was 1.48 mg/g (*C. onubensis*) and 2.13 mg/g (*C. elongata* S3). The strain *C. onubensis* was isolated from an unusual habitat, a burning coal spoil heap that was recently shown to host extremophilic algal communities (Barcyte et al., 2018). These residues of mining activities from the Silesian region (Czech Republic/Poland contained a wide range of trace elements, including arsenic, that reached an average concentration in the order of ppm (Smolinski et al., 2016)). *C. elongata* S3 was found growing as a contaminant in a laboratory solution of arsenic salt. Based on differences in the content of arsenolipids among strains, we assume that the environmental pressure on organisms for the biosynthesis of arsenolipids could be high and it could mirror arsenic availability in the original habitats of each strain. However, more data are needed to unravel such possible patterns.

2.3. Analysis of arsenolipids

Table 1 and Fig. 1 present the overall results of our MS analysis of arsenic molecules. The methodological approach applied e.g. by Viczek et al. (2016) was followed, i.e. complex isolation and fractionation of total lipids was not performed. Based on previous experience with

Table 1
HPLC/ESI-MS of arsenolipids from three strains of the green alga *Coccomyxa*, retention time (tR, min), lipid class, fatty acyls, including the ion according to which the lipid class was identified and the relative abundance of individual molecular species ("s" small, "m" medium, and "l" large abundance).

tR (min)	Lipid class	Fatty acyls	*m/z*	Identified based on	*C. elongata* 427	*C. elongata* S3	*C. onubensis*
15.14	AsLPC 16:1		600.264[a]	$[M+H]^+$	s	s	s
15.29	AsLPC 18:2		626.280	$[M+H]^+$	s	s	s
15.44	AsFA 18:3		383.157	$[M-H]^-$	m	m	m
16.02	AsFA 14:0		333.141	$[M-H]^-$	s	s	s
16.46	AsFA 16:1		359.156	$[M-H]^-$	m	m	m
17.05	AsLPC 16:0		602.280	$[M+H]^+$	s	s	s
17.20	AsFA 18:2		385.173	$[M-H]^-$	m	m	m
17.35	AsLPC 18:1		628.295	$[M+H]^+$	s	s	s
17.79	AsLPE 18:2		584.233	$[M+H]^+$	s	s	s
18.38	AsLPE 16:0		560.233	$[M+H]^+$	s	s	s
19.26	AsLPE 18:1		586.249	$[M+H]^+$	s	s	s
19.70	AsFA 16:0		361.172	$[M-H]^-$	m	m	m
19.85	AsLPE 18:0		588.264	$[M+H]^+$	s	s	s
20.87	AsFA 18:1		387.188	$[M-H]^-$	l	m	m
21.02	AsLPC 18:0		630.311	$[M+H]^+$	s	s	s
25.28	AsFA 18:0		389.203	$[M-H]^-$	s	s	s
45.72	AsPI 32:1	PI $Me_2As(O)$-15:0/16:1[b]	913.443	$[M-H]^-$	m	m	m
47.92	AsPI 34:2	PI $Me_2As(O)$-15:0/18:2	939.459	$[M-H]^-$	l	m	m
48.36	AsPI 36:3	PI $Me_2As(O)$-17:1/18:2	965.474	$[M-H]^-$	s	s	s
53.80	AsPE 36:5	PE $Me_2As(O)$-17:2/18:3	842.431	$[M-H]^-$	s	s	s
57.04	AsPC 32:2	PC $Me_2As(O)$-13:0/18:2	836.478	$[M+H]^+$	s	s	s
57.62	AsPC 34:3	PC $Me_2As(O)$-15:1/18:2	862.493	$[M+H]^+$	l	l	m
59.09	AsPI 34:1	PI $Me_2As(O)$-15:0/18:1	941.474	$[M-H]^-$	m	m	m
59.98	AsPG 34:1	PG $Me_2As(O)$-15:0/18:1	853.457	$[M-H]^-$	m	m	s
60.27	AsPC 36:4	PC $Me_2As(O)$-17:2/18:2	888.508	$[M+H]^+$	m	m	l
60.56	AsPC 34:3	PC $Me_2As(O)$-15:0/18:3	862.493	$[M+H]^+$	l	l	m
62.03	AsPI 36:2	PI $Me_2As(O)$-17:0/18:2	967.490	$[M-H]^-$	s	m	s
70.71	AsPC 34:2	PC $Me_2As(O)$-15:1/18:1	864.508	$[M+H]^+$	m	s	m
71.59	AsPC 32:1	PC $Me_2As(O)$-13:0/18:1	838.494	$[M+H]^+$	s	s	s
73.06	AsPC 36:3	PC $Me_2As(O)$-17:1/18:2	890.524	$[M+H]^+$	m	m	m
73.65	AsPC 34:2	PC $Me_2As(O)$-15:0/18:2	864.508	$[M+H]^+$	l	l	l
74.38	AsPE 36:4	PE $Me_2As(O)$-17:1/18:3	844.447	$[M-H]^-$	m	m	s
74.82	AsPI 36:1	PI $Me_2As(O)$-17:0/18:1	969.505	$[M-H]^-$	m	m	s
75.71	AsPG 36:1	PG $Me_2As(O)$-17:0/18:1	881.488	$[M-H]^-$	m	m	m
77.91	AsPE 34:2	PE $Me_2As(O)$-15:0/18:2	820.447	$[M-H]^-$	l	l	l
78.06	AsPE 36:3	PE $Me_2As(O)$-17:1/18:2	846.463	$[M-H]^-$	s	m	m
86.44	AsPC 36:2	PC $Me_2As(O)$-17:1/18:1	892.540	$[M+H]^+$	l	l	l
87.91	AsCer 34:1	Cer d18:1/$Me_2As(O)$-15:0	644.459	$[M+H]^+$	s	s	s
88.49	AsPC 32:0	PC $Me_2As(O)$-15:0/16:0	840.509	$[M+H]^+$	s	s	s
88.64	AsPC 34:1	PC $Me_2As(O)$-15:0/18:1	866.524	$[M+H]^+$	m	l	l
88.94	AsCer 36:2	Cer d18:1/$Me_2As(O)$-17:1	670.475	$[M+H]^+$	s	s	s
90.41	AsHexCer 36:2	HexCer d18:1/$Me_2As(O)$-17:1	832.528	$[M+H]^+$	s	s	s
91.88	AsPC 36:2	PC $Me_2As(O)$-17:0/18:2	892.540	$[M+H]^+$	l	l	m
93.20	AsPE 34:1	PE $Me_2As(O)$-15:0/18:1	822.563	$[M-H]^-$	m	m	m
96.43	AsPE 36:2	PE $Me_2As(O)$-17:0/18:2	848.478	$[M-H]^-$	m	m	s
99.08	AsDAG 34:2		716.481	$[M + NH_4]^+$	s	s	s
99.23	AsDAG 36:3		742.497	$[M + NH_4]^+$	s	s	s
100.11	AsHexCer 36:1	HexCer d18:1/$Me_2As(O)$-17:1	834.544	$[M+H]^+$	s	s	s
107.60	AsPC 36:1	PC $Me_2As(O)$-17:0/18:1	894.555	$[M+H]^+$	m	m	m
107.75	AsPC 34:0	PC $Me_2As(O)$-15:0/18:0	868.540	$[M+H]^+$	m	m	m
108.05	AsCer 36:1	Cer d18:1/$Me_2As(O)$-17:0	672.491	$[M+H]^+$	s	s	s
112.16	AsPE 36:1	PE $Me_2As(O)$-17:0/18:1	850.494	$[M-H]^-$	m	m	m
112.90	AsPE 34:0	PE $Me_2As(O)$-15:0/18:0	824.478	$[M-H]^-$	s	m	s
114.51	AsDAG 36:2		744.512	$[M + NH_4]^+$	s	s	s
115.10	AsDAG 34:1		718.497	$[M + NH_4]^+$	s	s	s
115.40	AsDAG 32:0		692.481	$[M + NH_4]^+$	s	s	s
126.98	AsPC 36:0	PC $Me_2As(O)$-17:0/18:0	896.572	$[M+H]^+$	s	s	s
133.48	AsDAG 36:1		746.528	$[M + NH_4]^+$	s	s	s
135.09	AsDAG 34:0		720.512	$[M + NH_4]^+$	s	s	s
177.58	AsTAG 50:4	$Me_2As(O)$-15:1/18:2/16:1	950.679	$[M + NH_4]^+$	m	m	m
178.16	AsTAG 52:5	$Me_2As(O)$-15:1/18:2/18:2	976.695	$[M + NH_4]^+$	m	m	m
178.90	AsTAG 50:4	$Me_2As(O)$-13:0/18:2/18:2	950.679	$[M + NH_4]^+$	s	s	s
179.05	AsTAG 54:6	$Me_2As(O)$-17:1/18:3/18:2	1002.710	$[M + NH_4]^+$	m	l	m
179.93	AsTAG 44:1	$Me_2As(O)$-13:0/14:0/16:1	872.632	$[M + NH_4]^+$	s	s	s
180.52	AsTAG 50:4	$Me_2As(O)$-15:0/18:3/16:1	950.679	$[M + NH_4]^+$	m	m	m
181.69	AsTAG 48:3	$Me_2As(O)$-15:0/14:0/18:3	924.663	$[M + NH_4]^+$	s	s	s
183.16	AsTAG 52:5	$Me_2As(O)$-15:0/18:3/18:2	976.695	$[M + NH_4]^+$	m	m	m
188.45	AsTAG 50:3	$Me_2As(O)$-15:1/18:1/16:1	952.695	$[M + NH_4]^+$	m	m	m
189.04	AsTAG 52:4	$Me_2As(O)$-15:1/18:1/18:2	978.710	$[M + NH_4]^+$	m	m	m
189.34	AsTAG 48:2	$Me_2As(O)$-13:0/18:1/16:1	926.679	$[M + NH_4]^+$	s	s	s
189.78	AsTAG 54:5	$Me_2As(O)$-17:1/18:2/18:2	1004.726	$[M + NH_4]^+$	l	l	l
190.07	AsTAG 50:3	$Me_2As(O)$-15:0/16:1/18:2	952.695	$[M + NH_4]^+$	m	m	m
190.81	AsTAG 52:4	$Me_2As(O)$-15:0/18:2/18:2	978.710	$[M + NH_4]^+$	m	m	l

(*continued on next page*)

Table 1 (*continued*)

tR (min)	Lipid class	Fatty acyls	*m/z*	Identified based on	*C. elongata* 427	*C. elongata* S3	*C. onubensis*
191.10	AsTAG 54:5	$Me_2As(O)$-17:1/18:3/18:1	1004.726	$[M + NH_4]^+$	l	l	l
191.39	AsTAG 48:2	$Me_2As(O)$-15:0/14:0/18:2	926.679	$[M + NH_4]^+$	s	s	s
191.69	AsTAG 44:0	$Me_2As(O)$-15:0/14:0/14:0	874.648	$[M + NH_4]^+$	s	s	s
191.98	AsTAG 52:4	$Me_2As(O)$-15:0/18:1/18:3	978.710	$[M + NH_4]^+$	m	l	m
192.57	AsTAG 46:1	$Me_2As(O)$-15:0/16:1/14:0	900.663	$[M + NH_4]^+$	s	s	s
196.54	AsTAG 50:3	$Me_2As(O)$-15:0/16:0/18:3	952.695	$[M + NH_4]^+$	m	m	m
199.19	AsTAG 54:4	$Me_2As(O)$-17:1/18:2/18:1	1006.742	$[M + NH_4]^+$	l	l	l
199.33	AsTAG 52:3	$Me_2As(O)$-15:1/18:1/18:1	980.726	$[M + NH_4]^+$	l	m	l
199.48	AsTAG 48:1	$Me_2As(O)$-15:0/18:1/14:0	928.695	$[M + NH_4]^+$	s	s	s
200.36	AsTAG 50:2	$Me_2As(O)$-15:0/18:1/16:1	954.710	$[M + NH_4]^+$	m	m	m
200.95	AsTAG 52:3	$Me_2As(O)$-15:0/18:1/18:2	980.726	$[M + NH_4]^+$	l	l	l
201.39	AsTAG 54:4	$Me_2As(O)$-17:0/18:2/18:2	1006.742	$[M + NH_4]^+$	s	m	s
202.13	AsTAG 50:2	$Me_2As(O)$-15:0/18:2/16:0	954.710	$[M + NH_4]^+$	l	l	l
202.86	AsTAG 48:1	$Me_2As(O)$-15:0/16:0/16:1	928.695	$[M + NH_4]^+$	m	m	m
203.15	AsTAG 46:0	$Me_2As(O)$-15:0/16:0/14:0	902.679	$[M + NH_4]^+$	s	s	s
208.45	AsTAG 52:2	$Me_2As(O)$-15:0/18:1/18:1	982.742	$[M + NH_4]^+$	l	l	l
208.59	AsTAG 54:3	$Me_2As(O)$-17:1/18:1/18:1	1008.757	$[M + NH_4]^+$	l	l	l
210.80	AsTAG 54:3	$Me_2As(O)$-17:0/18:1/18:2	1008.757	$[M + NH_4]^+$	m	s	s
213.00	AsTAG 48:0	$Me_2As(O)$-15:0/14:0/18:0	930.710	$[M + NH_4]^+$	s	s	s
213.44	AsTAG 50:1	$Me_2As(O)$-15:0/16:0/18:1	956.726	$[M + NH_4]^+$	l	m	l
219.32	AsTAG 54:2	$Me_2As(O)$-17:0/18:1/18:1	1010.773	$[M + NH_4]^+$	m	s	m
220.79	AsTAG 52:1	$Me_2As(O)$-15:0/18:0/18:1	984.757	$[M + NH_4]^+$	s	s	m
222.12	AsTAG 50:0	$Me_2As(O)$-15:0/16:0/18:0	958.742	$[M + NH_4]^+$	s	s	s
228.88	AsTAG 54:1	$Me_2As(O)$-17:0/18:0/18:1	1012.788	$[M + NH_4]^+$	s	s	s
230.35	AsTAG 52:0	$Me_2As(O)$-15:0/18:0/18:0	986.773	$[M + NH_4]^+$	s	s	s

[a] Differences between calculated (expected) and measured m/z values ranged around ± 0.0035 Da.
[b] In this Table $Me_2As(O)$ represents the (dimethylarsinoyl) group, e.g. $Me_2As(O)$-15:0.

lipidomic analyses (Duncan et al., 2013; Rezanka et al., 2018; Rezanka and Sigler, 2014), total lipids were directly analyzed. As the algae were grown in a medium containing 10 g As/L (Na_2HAsO_4 $7H_2O$, 41.64 g/L, 133.4 mmol), only the peaks (i.e., the arsenical compounds) having a retention time (tR) greater than 10 min were analyzed (Table 1, Fig. 1) to eliminate the interference of anions and water-soluble arsenic-containing organic compounds. Furthermore, MTBE instead of chlorinated hydrocarbons was used for extraction of total lipids, as reported by (Matyash et al., 2008). This eliminates the interference of ^{75}As and $^{40}Ar^{35}Cl$ ions. The use of two columns connected in series for NARP-HPLC allowed the elution of all water-soluble arsenic compounds within 10 min, using the mobile phase commonly applied in lipidomic analyses.

Comparison of retention times (tR) of arsenical and non-arsenical lipids presents a problem. Guttenberger et al. (2017a) published the order of elution of As-lysoPC (tR = 7.7 min), free AsFA (tR = 10.4 min) and AsPC with tR 17.0 min. The elution order of these three compounds was determined without any comparison to non-arsenical lipids.

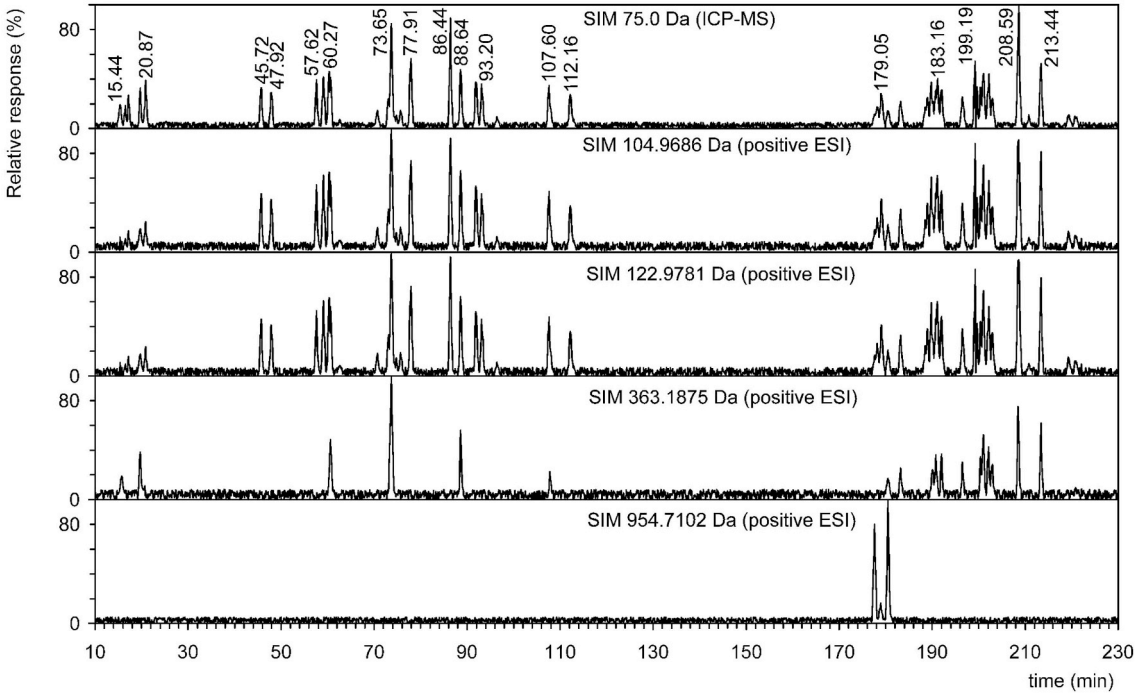

Fig. 1. LC/ICP-MS and NARP-HPLC/MS selected-ion monitoring (SIM) chromatograms of the five ions of arsenolipids. ICP-MS as ^{75}As at *m/z* 75, LC-MS of *m/z* 122.9781 $[Me_2As(OH)_2]^+$, at *m/z* 104.9686 $[Me_2As]^+$, at *m/z* 363.1875 (As-FA) and at *m/z* 954.7102 (As-TAG), see also "Experimental". Abbreviations are explained in the text.

Although Fukuda et al. (2011), published the tR of AsPC and PC, this was not done for long chain lipids in which the methyl group was replaced by an arsenoyl group.

Analysis of long chain organic compounds containing a terminal dimethylarsinoyl group was performed by two analytical techniques (ICP-MS and ESI-MS). The effluent after NARP-HPLC was divided in the ratio of 20:80 for analysis by ICP-MS and positive and/or negative HR-ESI-MS. Arsenic was identified in ICP-MS as ^{75}A at *m/z* 75 and interference with $^{40}Ar^{35}Cl$ ion was not observed. The chromatograms are shown in Fig. 1. In the HR-MS, both positive (HR-ESI$^+$) and negative (HR-ESI$^-$) full scans were performed. Four ions at *m/z* 950.6, 363.2, 123.0, and 105.0 were used for the initial identification in selected ion monitoring (SIM) mode. All AsHC and AsFA in ESI$^+$ presented as highly abundant ions at *m/z* 122.9781 $[Me_2As(OH)_2]^+$ and at *m/z* 104.9686 $[Me_2As]^+$ (Amayo et al., 2014b; Arroyo-Abad et al., 2013; Lischka et al., 2013; Taleshi et al., 2014a, 2014b). The appropriate ions corresponding to As-FA (363.1875 Da) and to As-TAG (954.7102 Da) were selected because they represented abundant peaks (Fig. 1) that were identified previously and also their MS has been described (see Guttenberger et al., 2017a; Guttenberger et al., 2017b; Viczek et al., 2016).

In total, 98 peaks were identified containing long-chain compounds with terminal dimethylarsinoyl groups. The ion at *m/z* 363.2 ($[M+H]^+$) was scanned as an example of the presence of $Me_2As(O)$-15:0 (systematic name 15-(dimethylarsinoyl)pentadecanoic acid; $Me_2As(O)$ was used as an abbreviation for the dimethylarsinoyl group in the formulas). As shown in Table 1, $Me_2As(O)$-15:0 was present as follows: a free acid (tR = 19.70 min), AsLPC ($Me_2As(O)$-15:0-LPC, tR = 17.5 min), several molecular species of AsPC (e.g. $Me_2As(O)$-15:0/18:2-PC with tR = 73.65 min, $Me_2As(O)$-15:0/18:2-PC with tR = 60.56 min, etc.) and many AsTAG, for example $Me_2As(O)$-15:0/18:3/16:1 (tR = 180.52 min), $Me_2As(O)$-15:0/18:3/14:0 (tR = 181.69 min), $Me_2As(O)$-15:0/18:3/18:2 (tR = 183.16 min).

The last SIM in Fig. 1 demonstrates the presence of three molecular species at *m/z* 954.7 ($[M + NH_4]^+$), i.e. $Me_2As(O)$-15:1/18:2/16:1, $Me_2As(O)$-13:0/18:2/18:2, and $Me_2As(O)$-15:0/18:3/16:1.

For complete structural confirmation of the above arsenolipids, tandem MS was measured for all peaks. For example, the majority of phospholipids were $Me_2As(O)$-15:0/18:2-PC and $Me_2As(O)$-15:0/18:2-PE (Fig. 2).

The main problem in comparing our measured values of arsenolipids with previously published data is the lack of studies dealing with this problem. So far, it has been considered likely that, for example, marine unicellular algae cultured in an environment where the arsenic content reaches up to tens of mg/L have their detoxification mechanism superseded (Duncan et al., 2015). However, to our current knowledge, long chain arsenolipids (i.e., compounds containing a dimethylarsinoyl group at one end of the hydrocarbon chain) have been identified predominantly in marine fish while their analysis in macroalgae and unicellular algae has been extremely rare (Pétursdóttir et al., 2018). One possible reason the hypothesis of "overdosing" metabolic pathways with elements like arsenic need not apply is that no one has analyzed metabolites of the type described above. Methylated arsenical species (MA, DMA, TMA, etc.) or arsenoribosides, including arsenosugars and phospholipids, have been identified and quantified by many authors (Dembitsky and Levitsky, 2004; Duncan et al., 2013; Wang et al., 2015) whereas dimethylarsinoyl long chains have been nearly completely neglected because of analytical challenges connected with their analysis. However, Pétursdóttir et al. (2018) found that in marine macroalga *Ectocarpus*, the concentration of the AsHC360 hydrocarbon was in the tenths of mg/kg of lyophilized alga, whereas for AsPL958, the major AsPL, was ~10 times lower.

2.4. Identification of arsenic phospholipids by tandem MS

Tandem MS of AsPC provided a prominent ion at *m/z* 184.0733 (Fig. 2). This ion was detected as the base peak or one of the major ions in previous studies (Guttenberger et al., 2017a, 2017b; Viczek et al., 2016). Another ion found at *m/z* 805.4359 ($[M + H\text{-}59\ (Me_3N)]^+$) had a similar structure to the ion previously described by (Guttenberger et al., 2017a, 2017b), where it had a value of 781.4290 Da. Furthermore, neutral loss of 184.0733 Da resulted in an ion at *m/z* 681.4434. The series of four ions at *m/z* 602.2798, 584.2692, 520.3398, and 502.3292 can be described as $[M + H\text{-}Me_2As(O)\text{-}15:0]^+$ and $[M + H\text{-}18:2]^+$ and as dehydrated ions of the previous structures, i.e. ions having the structure $[M + H\text{-}C_{16}H_{29}CH{=}C{=}O]^+$ and/or [M + H-

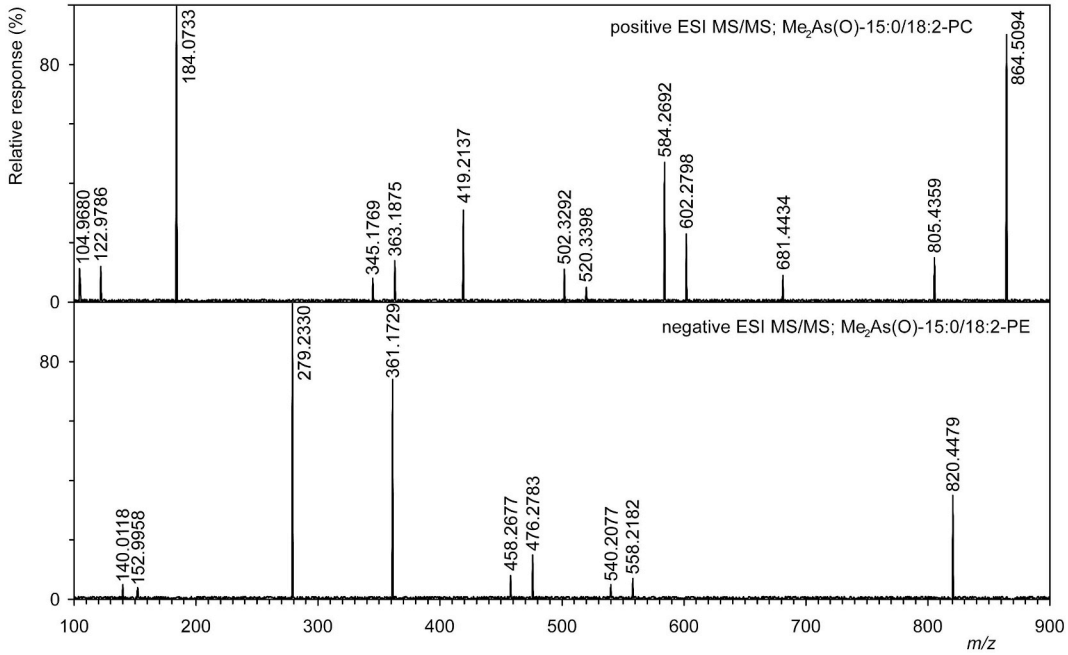

Fig. 2. Positive- (upper) and negative-ion (bottom) full-scan ESI tandem mass spectra of As-phosphatidylcholine ($Me_2As(O)$-15:0/18:2-PC) and As-phosphatidylethanolamine ($Me_2As(O)$-15:0/18:2-PE), see also "Experimental". Abbreviations are explained in the text.

$Me_2As(O)$-$C_{13}H_{26}$-CH=C=O]$^+$. Also ions of free AsFA (Table 1S), have been identified; in particular the ion at *m/z* 363.1875 and its dehydration product at *m/z* 345.1769 were among the most abundant pairs in the mass spectrum. Further, ions at *m/z* 122.9786 and *m/z* 104.9680 (Table 1S) were present in the MS spectra of all long-chain compounds containing the $Me_2As(O)$ group (Amayo et al., 2011, 2014a; Arroyo-Abad et al., 2013; Lischka et al., 2013; Taleshi et al., 2014a, 2014b).

An ion at *m/z* 820.4479, i.e. [M-H]$^-$ and a series of four ions of the common types [M-H-R_xCOOH]$^-$ and [M-H-R′$_x$CH = C=O]$^-$, where x is 1 or 2 (substitution on the primary or secondary hydroxyl of the glycerol backbone) were identified in the mass spectrum of AsPE, e.g. $Me_2As(O)$-15:0/18:2-PE (Fig. 2). The structure of these four ions at *m/z* 558.2182, 540.2077, 476.2783, and 458.2677 was as follows: [M-H-$C_{17}H_{31}$COOH]$^-$, [M-H-$C_{16}H_{29}$CH=C=O]$^-$, [M-H-$Me_2As(O)$-$(CH_2)_{14}$COOH]$^-$ and [M-H-$Me_2As(O)$-$(CH_2)_{13}$CH=C=O]$^-$. The ions at 361.1729 and 279.2330 had the structures of free acids, i.e. $Me_2As(O)$-$(CH_2)_{14}COO^-$ and linoleic acyl, respectively.

Other molecular species of additional phospholipids were identified similarly. In total, 15 PC, 8 PE, 6 PI, 2 PG, and 5 lipids of unknown structures were obtained. Based on HR-ESI-MS we assume that the latter were derivatives of ceramides. Because they were represented in small amounts, even tandem MS could not confirm their structure.

2.5. Identification of arsenic triacylglycerols by tandem MS

Nearly 40 molecular species of TAGs were separated and identified, see Table 1. Fig. 1 shows SIM of an ion at *m/z* 363.2 ([$Me_2As(O)$-15:0]$^+$). This ion is characteristic of all TAGs containing AsFA. In this way, all TAGs containing at least one AsFA were identified.

In the positive LC-ESI/MS, NH_4^+ ions were present in the mobile phase as ammonium acetate and the ions [M + NH_4]$^+$ were produced as major ions in the mass spectra. They determine the molecular weight. This fact is important, especially when it comes to undescribed or minor TAGs, which undoubtedly include triacylglycerols containing AsFA. In addition, due to NH_4^+ ions in the mobile phase, the formation of ions of the type [M+H]$^+$, [M+Na]$^+$, [M+K]$^+$, etc. was suppressed. This simplifies the interpretation of the mass spectra. In addition to [M + NH_4]$^+$ ion at *m/z* 978.7102, a triacylglycerol having the structure $Me_2As(O)$-15:0/18:2/16:0 (Fig. 3) produced a similar structure of ions as non-AsTAG. In particular, these include an ion at *m/z* 961.6836 ([M + NH_4-H_2O-NH_3]$^+$) and an ion at *m/z* 943.6730 ([M + NH_4-$2H_2O$-NH_3]$^+$). The central area of the mass spectrum features three ions with the structure [M + NH_4-18:2-NH_3]$^+$ at *m/z* 699.4539, the dehydration product [M + NH_4-18:2-NH_3-H_2O]$^+$ at *m/z* 681.4434 and another ion at *m/z* 599.5034 resulting from loss of $Me_2As(O)$-15:0 from [M+H]$^+$, more specifically the ion [M + NH_4-$Me_2As(O)$-15:0-NH_3]$^+$.

The ions at *m/z* 699.4539, 681.4434 and 599.5034 are structural analogues (differing only in side chain lengths, the polar head (s) remains the same) of ions at *m/z* 657.44379, 675.45410, and 551.50299 described by (Guttenberger et al., 2017b).

Two ions at *m/z* 437.2243 and 363.1875 and their dehydration products, i.e. ions at *m/z* 419.2137 and 345.1769 were also identified. These four ions are identical to the fragment ions previously identified by Guttenberg and colleagues (2017b). The ion at *m/z* 263.2369 (acyl chain, i.e. linoleic acid ([RC = O]$^+$ with loss of H_2O) was of course also present.

Similarly, further major AsTAGs, i.e. $Me_2As(O)$-15:0/18:2/16:0 have been identified, see Fig. 3. Based on these findings, it is possible to show that the mass spectra of AsTAG are similar to the spectra of non-arsenic TAGs, only the dehydration ions are more abundant due to the polar end of the hydrocarbon chain ($Me_2As(O)$ replaces the CH_3 group).

Identifying the positions of the double bonds or their geometric configurations in the FA did not cause any difficulties. Unfortunately, in the case of AsFA, neither the position nor the geometric configuration have been determined and are assumed to be the same as in the case of arsenic-free FA. The same applies to the regioisomers of both phospholipids and triacylglycerols, i.e. compounds having the same general formula and differing only in the position of the acyl group on the glycerol backbone. For instance, the ion type [M + H-R′$_2$CH=CO]$^+$, resulting from loss of substituent at the *sn*-2 position (secondary OH group), was more abundant than the ion [M + H-R′$_1$CH=CO]$^+$ (Hsu and Turk, 2009). A similar rule applies to TAG (Byrdwell, 2005; Mottram et al., 1997; Rezanka and Sigler, 2014). Unfortunately not even Guttenberger et al. (2017b) who synthesized two different regioisomers ($Me_2As(O)$-15:0/16:0/16:0 versus 16:0/$Me_2As(O)$-15:0/16:0), found different abundances of key ions, e.g. 657.44379 Da and/or 675.45410 Da for compound 3-((15-(dimethylarsinoyl)pentadecanoyl)oxy)propane-1,2-diyl dipalmitate and 657.44354 Da and/or

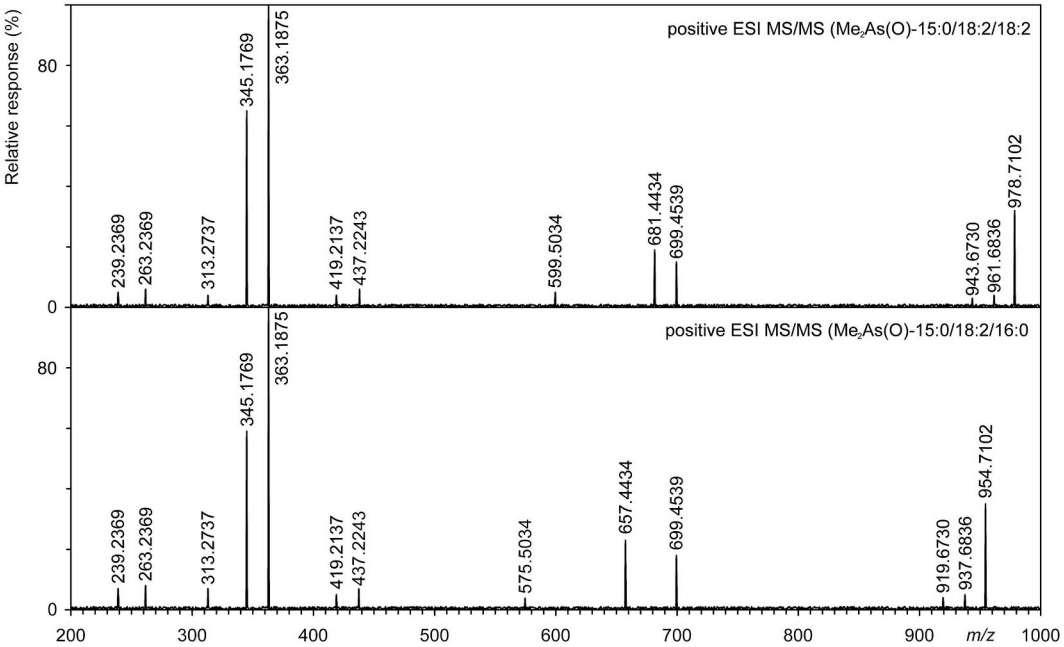

Fig. 3. Positive-ion full-scan ESI tandem mass spectrum of arsenic triacylglycerols, i.e. $Me_2As(O)$-15:0/18:2/18:2 and $Me_2As(O)$-15:0/18:2/16:0, respectively, see also "Experimental". Abbreviations are explained in the text.

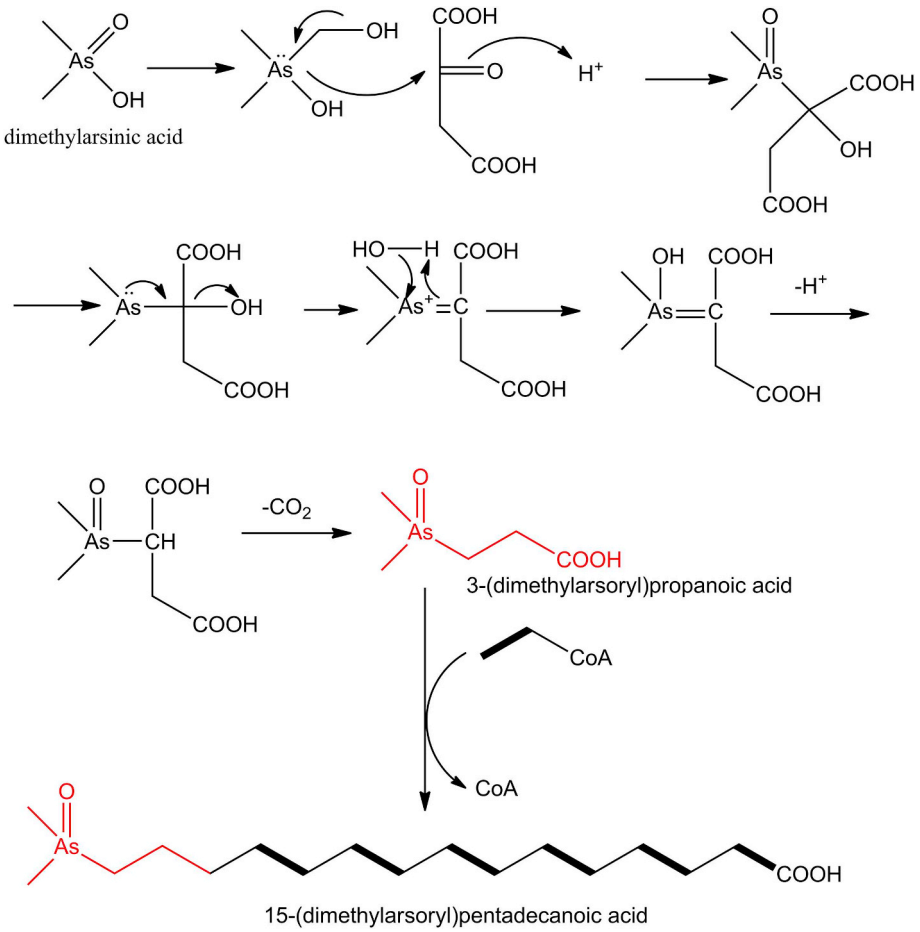

Fig. 4. Probable biosynthesis of dimethylarsinoylpropionic acid (3-dimethylarsinoyl) propanoic acid; $(CH_3)_2As(O)\text{-}CH_2CH_2COOH$).

675.45398 Da for the compound 2-((15-(dimethylarsinoyl)pentadecanoyl)oxy)propane-1,3-diyl dipalmitate. In addition, similar abundances of structurally similar ions were found, see Figs. 2 and 3, and this did not allow us to determine the position of AsFA on the glycerol backbone of both phospholipids and TAG.

Further (see Fig. 4), a biosynthetic scheme compiled based on previous studies is suggested (Edmonds et al., 1997; Rumpler et al., 2008; Sele et al., 2012; Sloth et al., 2005). This biosynthetic scheme explains why naturally occurring AsFA have an odd number of carbon atoms, although it is worth mentioning that minor AsFA with even numbers of carbon atoms were identified (Arroyo-Abad et al., 2013; Lischka et al., 2013; Viczek et al., 2016). Deciphering metabolic pathways involved in the incorporation of inorganic arsenic into the biomass of primary producers is crucial for an understanding of arsenic cycling in aquatic food chains.

3. Conclusion

The use of techniques already known in the area of analysis of arsenolipids, along with novel approaches such as MTBE extraction from algal biomass grown at extremely high arsenic concentrations, direct analysis of total lipids without any prior concentration and fractionation of arsenolipids, the use of non-aqueous reversed phase chromatography in two columns connected in series, and the use of a mobile phase similar to that used for arsenic-free lipids, enabled us to separate and identify nearly 100 molecular species of arsenolipids in algal biomass. In total, 39 AsTAG, 15 AsPC, 8 AsPE, 7 AsDAG, 6 AsPI, 2 AsPG, and 5 lipids of unknown structures were revealed. Based on HR-ESI-MS, we assume that the latter are derivatives of ceramides. Because they were present in small amounts, even tandem MS could not confirm their structures.

4. Experimental

4.1. Cultivation

The experiment was performed with three different *Coccomyxa* strains: *Coccomyxa elongata* Chodat & Jaag (strain CCALA 427, GenBank: KX809908.1) isolated from a pool in the Czech Republic and acquired from the Culture Collection of Autotrophic Organisms at the Institute of Botany CAS, Třeboň, *Coccomyxa elongata* (strain S3) found growing in a laboratory as contaminant in Na_3AsO_4 salt solution (concentration 207 mg/L) and *C. onubensis* Garbayo et al. ex Fuentes et al. isolated from a burning coal spoil heap near Ostrava (Czech Republic), see Barcyte et al. (2018) for details. The two latter strains were kept in a working collection at the Department of Ecology, Charles University, Prague. The strains were cultivated in liquid circumneutral Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) at an arsenic concentration of 10 g/L (133.5 mmol). Arsenic was added as sodium arsenate dibasic heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$) at a concentration of 41.64 g/L. The volume of algal suspension in Erlenmeyer flasks was 1000 ml. The cultures were kept in a Q-Cell 200 incubator (PolLab, Poland) with continuous light of 20 μmol photons m^{-2} s^{-1} provided by a cool white 8 W fluorescent tube and the cultivation temperature was 20 °C. The strains were cultivated for 40 days and finally harvested by centrifugation at 4400 rpm for 10 min. The samples were stored frozen until analysis (Dembitsky et al., 1992).

4.2. Isolation of arsenolipids - extraction with methyl tert-butyl ether (MTBE)

Extraction with MTBE was performed according to Matyash et al (2008). Briefly, 1 mL of methanol and 100 mg of lyophilized cells were vortexed, 4 mL of MTBE was added, and the mixture was shaken for 1 h at room temperature. Then, 1 mL of water was added and the mixture was centrifuged at 1000 g. The top layer was separated and the lower layer was again extracted. The combined organic extracts were evaporated to dryness under a stream of nitrogen and redissolved in 1:1 acetonitrile/methanol for further analysis.

4.3. Non-aqueous reversed phase separation and identification of arsenolipids by mass spectrometry

Separation and identification of molecular species of arsenolipids was performed using non-aqueous reversed-phase (NARP) coupled simultaneously to ICP-MS and HR-ESI-MS. The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA), and two columns HIRPB-250AM (Hichrom Limited, UK) - 250 × 2.1 mm ID, 5 µm particle size, in series were used and chromatography was performed with the following parameters: flow rate of 1 mL/min, injection volume of 10 µL, column temperature of 25 °C, a solvent gradient program with acetonitrile (ACN) containing 5 mM aqueous ammonium acetate 2-propanol (iPrOH) also containing 5 mM aqueous ammonium acetate as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 10 min to 230 min of ACN/iPrOH 30:70 (vol/vol); held for 10 min. In negative ESI mode, the same mobile phase was used; formic acid (2 mM) was used instead of ammonium acetate.

The eluent from the HPLC column was split so that 20% of the flow was introduced to ICP-MS and 80% of the flow was introduced to ESI-MS. ICP-MS at *m/z* 75 confirmed the presence of arsenical organic compounds. At the same time, detection of HR-ESI$^+$-MS and HR-ESI$^-$-MS using single ion monitoring (SIM) of selected ions allowed the identification of arsenic-containing organic molecules.

An Agilent 7500ce ICP-MS system (Agilent Technologies Österreich GmbH, Wien, Austria) was fitted with an Agilent MicroMist glass concentric nebulizer. Typical ICP-MS conditions were used for the analysis of arsenic: RF power 1550, plasma gas flow 15 L/min (Ar), carrier gas flow 0.9 L/min, makeup gas flow 0.16 L/min, optional gas 12% (20% O_2 in Ar, in order to avoid the formation of carbon on the interface cones).

Separation using NARP-HPLC on two columns coupled in series was used to allow complete separation of Cl^- anions, and non-organic polar compounds containing arsenic were eluted from the column with a retention time higher than 10 min. This eliminated the interference of $^{40}Ar^{37}Cl$ and ^{75}As as confirmed by the absence of the signal at *m/z* 77, i.e., $^{40}Ar^{37}Cl$ (chlorine is in nature represented by two isotopes, i.e. ^{35}Cl and ^{37}Cl in a ~3:1 ratio).

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. ESI–MS analysis was performed in the Fourier transform positive ion mode. MS spectra were acquired with target mass resolution of R = 105,000 at *m/z* 800. The ion spray voltage was set at 3.5 kV and the scan range of the instruments was set to *m/z* 200–2000. Nitrogen was used as a nebulizer gas - set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for collision-induced dissociation (CID) experiments. A CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected in high resolution FT mode. Further source conditions in the positive mode were: vaporizer temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; tube lens voltage, 170 V.

The same apparatus was used for negative ion mode analyses. MS spectra were acquired with a target mass resolution of R = 110,000 at *m/z* 400. The ion spray voltage was set at −2500 V and the scan range of the instruments was set to *m/z* 200–2000. Nitrogen was used as a nebulizer gas – set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxilliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the parent ion fragmentation. The MS/MS product ions were detected in high resolution FT mode. The HESI temperature was set to 250 °C.

Notes The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2019.05.002.

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