Title

Implementation in Lipid Extraction and Analysis from phytoplankton: *Skeletonema marinoi* as case study

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Abstract

Microalgae, dominating protists in aquatic systems, are rich in lipids, a complex molecular class regulating cell physiology and phytoplankton ecology. Investigation of microalgal lipid metabolism is increasing, but common strategies for comprehensive lipid analyses are still lacking. Major methodological gaps are represented by separate analytical procedures for extraction and characterization of lipid molecules, thus hindering accurate comparison of metabolic data. Herein we propose one unique methodology for sample collection, extraction and analysis of main lipid mediators (oxylipins, fatty acids, glycerolipids, sterols and sterol derivatives) from diatoms. Extractions relied on the MTBE/methanol method, which provides high lipid extraction yields, involving either MeOH or water preparation of samples. Experiments focused on *Skeletonema marinoi*, a diatom species often used as model organism in plankton chemical ecology. Extraction protocols were implemented to characterize oxylipins and glycerolipids from algal cells collected as pellets or accumulated on glass-fibre filters, thus providing a practical tool to study multiple lipid classes in natural phytoplankton.

Keywords: microalgae, diatoms, chemical extraction, mass spectrometry, glycerolipids, sterol sulphates, oxylipins, fatty acids.

55

56 **Abbreviations**

57 CHOS-d: cholesterol sulphate

58 DGDG: digalactosyldiacylglycerol

59 DMSO: dimethyl sulfoxide

60 EPA: eicosapentaenoic acid

61 ESI: electrospray ionization

62 FAME: fatty acid methyl ester

63 FFA: free fatty acid

64 GC-MS: gas chromatography-mass spectrometry

65 GF: glass-fibre

66 HPLC: high-pressure liquid chromatography

67 HTrA: hexadecatrienoic acid

68 HTtA: hexadecatetraenoic acid

69 LAH: lipolytic acyl hydrolase

70 LC-MS: liquid chromatography/mass spectrometry

71 LOFA: linear oxygenated fatty acid

72 LOX: lipoxygenase

73 MeOH: methanol

74 MEOH: MTBE/methanol extraction from cells re-suspended with methanol

75 MGDG: monogalactosyldiacylglycerol

76 MS: mass spectrometry

77 MTBE: methyl-*tert*-butyl ether

78 NMR: nuclear magnetic resonance

79 PC: phosphatidylcholine

PE: phosphatidylethanolamine

PG: phosphatidylglycerol

PI: phosphatidylinositol

PUA: poly-unsaturated aldehyde

PUFA: poly-unsaturated fatty acid

SQDG: sulfoquinovosyldiacylglycerol

StS: sterol sulphates

SULT: sulfotransferase

TFA: total fatty acid

TG: triglyceride

UHPLC: ultra-high-pressure liquid chromatography

WATER: MTBE/methanol extraction from cells re-suspended with water

Introduction

Diatoms are unicellular microalgae dominating plankton assemblages in terms of abundance and diversity (Armbrust, 2009; Malviya et al., 2016; Tréguer et al., 2018). Their biomass is largely constituted by lipids (Da Costa et al., 2016), an extremely complex class of molecules involved in regulation of cell physiology, energy homeostasis, formation and function of cell membranes and intra- and inter-cellular signalling (Gross and Han, 2011; Wenk, 2010).

Diatoms are major producers of oxylipins (Russo et al., 2020), low-molecular-weight lipids that can act as predation deterrents (Barreiro et al., 2011; Fontana et al., 2007a; Ianora et al., 2015; Lauritano et al., 2016; Miralto et al., 1999; Paffenhöfer et al., 2005; Ruocco et al., 2020, 2016; Russo et al., 2018) or signalling molecules (Cózar et al., 2018; Edwards et al., 2015; Russo et al., 2020) in plankton communities. Oxylipins embrace two major classes of molecules, namely poly-unsaturated aldehydes (PUAs) and linear oxygenated fatty acids (LOFAs), that are produced upon wound- or age-mediated lysis of diatom cells by a stepwise process including cleavage of fatty acids from glycolipids and phospholipids and subsequent oxygenation of free fatty acids by lipoxygenases (LOX) (Adelfi et al., 2019; Cutignano et al., 2006; d'Ippolito et al., 2018, 2009, 2006, 2005, 2004; Fontana et al., 2007b; Pohnert et al., 2002; Pohnert, 2010; Pohnert and Boland, 2002; Watson et al., 2009; Yi et al., 2017). While LOX-mediated oxygenation of eicosapentaenoic acid (EPA, C20:5) is the primary source of oxylipins in diatoms, hexadecatrienoic acid (16:3, ω-4) (HTrA) and hexadecatetraenoic acid (16:4, ω-1) (HTtA) can also serve as substrates for the synthesis of C16-derived oxylipins in few diatom species, such as *Skeletonema marinoi* and *Thalassiosira rotula* (d'Ippolito et al., 2006, 2005, 2004; Fontana et al., 2007a, 2007b). These fatty acids are major components of diatom glyceroglycolipids, in particular of monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs), that are integral parts of plastid membranes (Heydarizadeh et al., 2013).

130 More recently, the synthesis of another functionally relevant class of lipids, the sterol sulphates
131 (StS), has been described in several diatom species (Gallo et al., 2020, 2018, 2017; Nuzzo et al.,
132 2019). In diatoms, sterols are enzymatically synthesized through the mevalonate pathway, involving
133 cyclization of squalene to cycloartenol (Gallo et al., 2020). Sulfonation of sterols to their StS
134 counterparts is finally mediated by sulfotransferases (SULTs). StS have been demonstrated to
135 induce cellular apoptosis and culture demise in the plankton-dominating diatom *S. marinoi* (Gallo et
136 al., 2017), implying major involvement of these secondary metabolites in algal bloom termination
137 also in natural populations.
138 In recent years, an increasing number of field surveys has been investigating changes in lipid
139 profiles of natural phytoplankton (Bartual et al., 2018, 2014; Cózar et al., 2018; Grosse et al., 2019;
140 Ianora et al., 2015, 2008; Lauritano et al., 2016; Mayzaud et al., 2013; Morillo-García et al., 2014;
141 Ribalet et al., 2014; Russo et al., 2020; White et al., 2015; Wichard et al., 2008). However, the high
142 complexity of phytoplankton matrices often requires combination of different strategies for a)
143 sample collection, b) metabolite extraction, detection, identification and quantitation, c) final data
144 analysis.
145 Collection of natural phytoplankton is often a critical step in field surveys. In this perspective, water
146 filtration on glass-fibre (GF) filters is particularly advantageous, because it is quick and reduces
147 biases in phytoplankton composition introduced during sample collection. Moreover, GF filters
148 accommodate large water volumes and are compatible with organic solvents, facilitating subsequent
149 chemical extractions (Bidigare et al., 2003). Easy and unique procedures for lipid extraction and
150 analysis are still lacking. In fact, extraction of different lipid molecules is traditionally performed
151 through dedicated protocols, including Folch's (Folch et al., 1957) and methyl-*tert*-butyl ether
152 (MTBE)/methanol methods for glycerolipids and fatty acids (Adelfi et al., 2019; Cutignano et al.,
153 2016, 2006; d'Ippolito et al., 2004; Matyash et al., 2008), MeOH extraction for sterol sulphates
154 (Gallo et al., 2020, 2018, 2017; Nuzzo et al., 2019) and acetone-dichloromethane (Cutignano et al.,

155 2011; d'Ippolito et al., 2018; Gerecht et al., 2013; Miralto et al., 1999; Nanjappa et al., 2014) or
156 solid phase extraction (Rettner et al., 2018; Wichard et al., 2005a) for oxylipins.
157 Striving for a more comprehensive understanding of microalgal physiology, we have previously
158 reported a few methods to characterize different lipid classes from diatoms and natural
159 phytoplankton by combining traditional chromatographic approaches with mass spectrometry (MS)
160 (d'Ippolito et al., 2018; Gallo et al., 2018) and nuclear magnetic resonance (NMR) techniques
161 (Nuzzo et al., 2013). In the present study, we seek the implementation of a simple and general
162 methodology for extraction and analysis of phospholipids, glycolipids, StS, oxylipins and free fatty
163 acids (FFAs) from phytoplankton samples. To this aim, the analytical procedure was designed for
164 extraction of cell pellets or cells accumulated on GF filters by MTBE/methanol method which
165 offers high extraction efficiency, high volatility and low toxicity (Cajka and Fiehn, 2014; Calderón
166 et al., 2019; Matyash et al., 2008; Satomi et al., 2017). The methodology was tested on *Skeletonema*
167 *marinoi,* a model diatom in chemical ecology, whereas *Thalassiosira weissflogii* was used as
168 negative control to exclude non-enzymatic modifications of lipids during extraction procedures
169 (d'Ippolito et al., 2018, 2006, 2004; Gallo et al., 2020, 2018, 2017; Nuzzo et al., 2019; Wichard et
170 al., 2005b). For the extraction of glycerolipids, fatty acids and StS, cells were suspended in MeOH
171 to inhibit enzymatic reactions (treatment MEOH hereafter). Instead, according to the literature,
172 effective oxylipin extraction relied on suspension in water (treatment WATER hereafter) for
173 activation of the enzymes of the LOX pathways.

174 **Materials and methods**

175 *General*

176 All solvents were from Merk (Darmstadt, Germany). MeOH and MTBE were LC-MS grade, while
177 chloroform ($CHCl_3$) and dichloromethane ($CHCl_2$) were HPLC-grade. Milli-q water from a
178 Whatman apparatus (Merk, Darmstadt, Germany) was used throughout the experiments.
179 Diazomethane (diluted solution of diazomethane in diethyl ether) was prepared from Diazald as

described by (Black, 1983). Monogalactosyldiacylglycerol (MGDG 16:0/18:0), [D7]-cholesterol sulphate (CHOS-d), 16-hydroxyhexadecanoic acid and nonadecanoic acid, used as internal and external standards for glycerolipids, StS, LOFAs and FAs respectively, were purchased from Merk (Darmstadt, Germany).

LC-MS parameters

UHPLC–MS analysis for lipids and sterol sulphates was performed on Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific,Waltham, MA, USA) equipped with an Infinity 1290 UHPLC System (Agilent Technologies, Santa Clara, CA, USA), using a Kinetex Biphenyl 2.6 µm 150x2.1 mm column and a gradient of MeOH in water (Cutignano et al., 2016). Briefly, the elution program consisted of a gradient from 40 to 80% of MeOH in water in 2 min, then to 100% of MeOH in 13 min, holding at 100% for 7 min at the flow rate of 0.3 ml/min. Full MS scans were acquired over the range 200–1800 and the most intense peaks were selected for fragmentation with a stepped normalized energy of 25–28–35 and 20–40% in positive and negative ionization mode, respectively. The injection volume was 10 µl.

HPLC-MS analysis for oxylipin detection and quantitation was performed on Micro-qToF mass spectrometer (Waters, Milford, MA, USA) with an electrospray ionization (ESI) source (positive mode) and coupled with a Waters Alliance HPLC system equipped with a C-18 Kromasil column (4.6x250 mm, 100 Å, Phenomenex Inc.). Fatty acid analysis was performed on an ion-trap GC-MS instrument (Thermo ITQ 700 Mass spectrometer interfaced with Thermo Focus GC Polaris Q; Thermo Fisher, Massachusetts, USA) mounting a 5% Diphenyl polysiloxane column (OV-5; Agilent Technologies, Santa Clara, CA, USA) in EI mode (70 eV) and using Helium as gas carrier.

Below the standard solutions prepared for the analysis: hydrogenated monogalactosyldiacylglycerol (MGDG 16:0/18:0) = solution 1 mg/ml in MeOH:$CHCl_3$ 4:1; [D7]-cholesterol sulphate (CHOS-d)

= solution 0.1 mg/ml in MeOH 100%, 16-hydroxyhexadecanoic acid = solution 0.1 mg/ml in MeOH 100%; nonadecanoic acid (19:0) = solution 1 mg/ml in DMSO 100%.

Algal culturing and harvesting

Four separate cultures of *Skeletonema marinoi* (CCMP2092) and one of *Thalassiosira weissflogii* (ICB-P09) were grown semi-continuously in sterile polycarbonate flasks containing f/2 medium. Cultures were subjected to a 12:12 h dark:light cycle and a light intensity of 100 µmol (m^{-2} s^{-1}). At the day of harvesting, concentrations of *S. marinoi* cultures ranged between 1.84-3.78 10^6 cells ml^{-1}, while *T. weissflogii* was harvested at a concentration of 1.87 10^5 cells ml^{-1}.

Each algal pellet replicate was obtained by transferring 15 ml of the diatom culture to a falcon tube. Algal cells were centrifuged with a swing-out centrifuge Allegra X-15R (Beckman Coulter, Pasadena, CA, USA) (4000 *g*, 4 °C, 10 min). The supernatant was discarded and wet pellets were immediately frozen in liquid nitrogen.

Alternatively, cells were accumulated on glass fibre GF/A filters (1.6 µm pore size) (Whatman-Merk, Darmstat, Germany). Each replicate was obtained by filtering 15 ml of the diatom culture. Filters were transferred to falcon tubes and immediately frozen in liquid nitrogen.

Samples were stored at -80 °C until analysis. A volume of 2 ml of each culture was used to determine cell density in a Bürker counting chamber (Merck, Darmstat, Germany; depth of 0.1 mm) under an inverted microscope.

Samples were prepared in triplicate for the different experimental procedures.

MTBE extraction - MEOH preparation

In this procedure, lipids were extracted through the application of the MTBE/methanol method (Matyash et al., 2008). Extraction consisted in the initial addition of MeOH, followed by addition of MTBE and water to a fixed volume ratio of 3:10:2.5 v/v/v.

In detail, cells on GF/A filters were re-suspended in 9 ml MeOH to cover the filter. Then, lipid standards (10 µg MGDG, 10 µg nonadecanoic acid, 1 µg CHOS-d, 1 µg hydroxyhexadecanoic acid)

were added. The sample was mixed by vortex and sonicated in ice bath for 30 seconds. After addition of 30 ml of MTBE, the sample was vortexed again and left at room temperature for 10 minutes. Water (7.5 ml) was added and the two-phase mixture was vigorously mixed before centrifugation at 4000 *g* at 4 °C for 10 minutes. The whole organic upper phase was collected with a glass Pasteur pipette and transferred into a glass round-bottomed flask. The aqueous residue was extracted again by repeating the procedure with other 10 ml MTBE. The organic phases were combined and dried by rotatory evaporator. The oily residue was recovered with MeOH/CH_2Cl_2 4:1 and transferred into a weighted vial. After removal of the solvent by nitrogen, the organic extract was weighted and kept at -80° until further analysis. On average, the dry organic extract from *S. marinoi* pellets was 1.06 mg, while the dry extract obtained from *S. marinoi* cells accumulated on filters was 1.5 mg. The dry extract obtained from *T. weissflogii* pellets was on average 0.81 mg. The extract was methylated by diazomethane (CH_2N_2) for 30 minutes for oxylipin and fatty acid analysis.

Extraction of pellets was performed through the application of the same protocol, but volumes of the solvents were different; in particular, 3 ml MeOH, 10 ml MTBE and 2.5 ml water were added. The second extraction step was performed by adding 10 ml of MTBE.

For each extraction, three replicates were considered.

MTBE extraction - WATER preparation

In this procedure, lipids were extracted in MTBE/methanol (Matyash et al., 2008), but with a specific sample preparation different from conventional method. Instead, samples were prepared through initial addition of water, followed by addition of MeOH and MTBE in a fixed ratio of 2.5:3:10 v/v/v.

Cells on GF/A filters were re-suspended with 7.5. ml of water to cover the filter and lipid standards (same amounts as reported above) were added. To allow cell lysis and oxylipin synthesis, the sample was vortexed, sonicated for 1 min in ice bath and left at room temperature for 30 min to let

253 enzymatic reactions to occur. Then, 9 ml of MeOH were added to stop enzymatic reactions and the
254 sample was vortexed again. After addition of 30 ml of MTBE, the sample was vigorously mixed
255 and then centrifuged at 4000 *g* for 6 min. at 4 °C. The upper organic phase was transferred into a
256 round-bottomed flask with a glass Pasteur pipette and the extraction was repeated by adding other
257 10 ml of MTBE to the water phase. The organic phases were combined and dried by rotatory
258 evaporator. The extract was re-suspended with MeOH/CH_2Cl_2 4:1, transferred into a weighted vial
259 and dried under nitrogen. As reported above, lipid extract was methylated by diazomethane
260 (CH_2N_2) for 30 minutes before oxylipin and fatty acid analysis.
261 Extraction of pellets was performed through the application of the same protocol, but with the use
262 of 3 ml MeOH, 10 ml MTBE and 2.5 ml water. The second extraction step was performed by
263 adding 10 ml of MTBE.
264 For each extraction, three replicates were considered.

265 *LC-MS/MS analysis of glycerolipids and sterol sulphates*

266 Extracts were carefully dissolved in 1 ml MeOH/CH_2Cl_2 4:1. An aliquot of 200 µl was transferred
267 into a glass vial and diluted to the final volume of 1 ml with MeOH (LC-MS grade). LC-MS
268 analysis was carried out injecting 5 µL of this solution. Chromatographic separation was performed
269 by Kinetex Biphenyl Column (130 Å, 2.6 µm, 2.1 x 150 mm; Phenomenex Inc) according to our
270 previous methods (Cutignano et al., 2016; Nuzzo et al., 2019). Peak identification and quantitation
271 was supported by LipidSearch® software (Thermo Scientific, Waltham, MA, USA).
272 Sterol sulphates were manually identified by using the Excalibur™ Software (Thermo
273 Scientific,Waltham, MA, USA) on the basis of their mass (*m/z*) and molecular fragmentation (Gallo
274 et al., 2018; Nuzzo et al., 2019). Absolute quantitation was determined on the basis of an external
275 calibration curve of the standard CHOS-d and hydrogenated monogalactosyldiacylglycerol (MGDG
276 16:0/18:0) for sterol sulphates and glycerolipids respectively. The calibration curves were estimated

277 in the concentration range between 10 ng/ml and 1000 ng/ml (CHOS-d: y=49812x, R^2=0.9981;
278 MGDG 16:0/18:0 ESI^+: y=6^7x, 0.9604; MGDG 16:0/18:0 ESI^-: y=3^7x, 0.9994, Figure S6).

279 *Analysis of linear oxygenated fatty acids (LOFAs)*

280 Dry extracts were carefully re-suspended with 100% MeOH to a final concentration of 1 mg/ml.
281 Analysis of LOFAs was performed by injecting 30 µl of this extract solution in a Micro-qTof mass
282 spectrometer (Waters, Milford, MA, USA) as described by d'Ippolito et al. (2018). Briefly,
283 oxylipins were eluted through a HPLC system in a gradient of MeOH and water (starting condition
284 75:25, v:v) and at a flow of 1 ml/min. Oxylipin derivatives of hexadecatrienoic (HTrA) and
285 eicosapentaenoic (EPA) acids were targeted for the analysis and were quantified as described by
286 Russo et al. (2020). In particular, oxylipin quantitation was calculated as follows: ng(x) = (a_x x
287 1000)/a_s, where "*x*" is the oxylipin species, "*S*" is the standard and "*a*" is the area of the peak in the
288 chromatogram.

289 *Analysis of free fatty acids (FFAs) and total fatty acids (TFAs)*

290 Analysis of fatty acids was performed on both MEOH and WATER extracts, before (FFAs) and
291 after saponification (total fatty acids, TFAs), in agreement with d'Ippolito et al. (2004). Fatty acids
292 were analysed as fatty acid methyl esters (FAMEs) with an ion-trap GC-MS instrument (Thermo
293 ITQ 700 Mass spectrometer interfaced with Thermo Focus GC Polaris Q; Thermo Scientific,
294 Waltham, MA, USA) injecting 2 µl of the extract solution 1 mg/ml in MeOH. The elution relied on
295 an increasing temperature gradient: 160 °C for 3 min, a subsequent increase of 3 °C/min to 260 °C,
296 followed by 7 min at 310 °C. Fatty acids were identified on the basis of retention time and MS/MS
297 fragmentation. Quantitation was achieved on the basis of the internal standard (nonadecanoic acid)
298 as described for oxylipins.

299 *Data analysis*

The amount of glycerolipids, StS, LOFAs and FFAs in the samples was normalized by the total number of cells harvested and the final value was expressed as fg-per-cell. The total amount of glycerolipids, StS, LOFAs and FFAs was calculated for each sample. Significant differences between the two preparations (MEOH and WATER) and the sample collection procedure (centrifugation and filtration) were evaluated through *t*-tests (N=6) performed on log-transformed values and considering Welch's correction for unequal variances. Statistical significance was set at α=0.01 to avoid type I error.

Statistical analyses and data representation were performed using R (version 3.6.1) implemented in RStudio.

Results

Experimental setup

The experimental setup was designed to optimize and validate methods for phytoplankton sampling and lipid extraction through MTBE/methanol. In particular, we tested variations in lipid (oxylipins, fatty acids, glycerolipids and StS) extraction yields after MEOH and WATER treatments of diatom cells. Also, we analysed differences in lipid extraction depending on cell collection methods, i.e. centrifugation (algal pellets) and filtration on glass-fibre filters (Table 1).

First of all, we tested if MEOH and WATER preparations affected extraction yields of glycerolipids, sterol sulphates (StS), oxylipins and free fatty acid (FFAs) in two identical diatom biomasses. Two ecologically relevant strains of marine diatoms, namely *Skeletonema marinoi* and *Thalassiosira weissflogii*, were accumulated as pellets (i.e. through centrifugation). These two species were selected because they are characterized by distinct lipid metabolism (Cutignano et al., 2006; d'Ippolito et al., 2018, 2006, 2004; Fontana et al., 2007a, 2007b). As *S. marinoi* is known to possess strong hydrolytic and lipoxygenase activity, we expected MEOH and WATER preparations for MTBE/methanol extraction to give significant differences in the extraction yields of lipids (glycerolipids, StS, oxylipins and FAs). On the contrary, we hypothesized that MEOH and WATER

preparations from *Thalassiosira weissflogii* did not lead to significant differences in the amount of glycerolipids, StS, oxylipins and FFAs, because of the weak hydrolytic and lipoxygenase activity of this diatom (Wichard et al., 2005a).

In agreement with our hypothesis, analysis did not reveal occurrence of LOFAs or differences in lipids of *T. weissflogii* whereas there was a clear change between MEOH and WATER preparations of *S. marinoi*. This experiment proved that cell preparation is crucial for the enzymatic activities responsible for the synthesis of LOX products, whereas extraction by MTBE/methanol did not affect the composition of the samples. This latter result was also confirmed for MEOH and WATER preparations of *S. marinoi* cells collected on GF/A filters. Finally, MTBE/methanol extraction from cell pellets and cells collected on GF/A filters did not lead to different yields of glycerolipids, StS, and FFAs, while significant differences in LOFA concentrations were observed between extracts of pellets and GF/A filters from WATER preparations.

Oxylipins (LOFAs)

In accordance to the literature (d'Ippolito et al., 2018, 2006, 2004; Wichard et al., 2005b), lipoxygenase products were detected only in *S. marinoi* and not in *T. weissflogii* (Table 2, Table S1). Consistently with previous reports, these results also highlighted a significantly higher concentration of total LOFAs in *S. marinoi* pellets extracted after WATER preparation in comparison to those extracted after MEOH preparation ($t=-9.35$, $d.f.=2.78$, $p<0.01$, $N=6$) (Figure 1A, Figure S1, Table 2, Table S1). Quantitative analysis of extracts from *S. marinoi* cells collected on GF/A filters gave the same results with major differences in LOFAs between WATER and MEOH preparations ($t=-5.88$, $d.f.=3.99$, $p<0.01$, $N=6$) (Figure 1B, Table S1).
On the other hand, LOFA concentrations were significantly higher in extracts obtained from GF/A filters than pellets ($t=-17.67$, $d.f.=3.6$, $p<0.001$, $N=6$) (Figure 1C, Table 2, Table S1).
In good agreement with the literature (d'Ippolito et al., 2018, 2004, 2003, 2002), LOFAs detected after MTBE/methanol extraction were mostly composed of hydroxy-eicospentaenoic acid (HEPE)

and epoxy-hydroxy-eicosatetraenoic acid (EHETE), while hydroxy-hexadecatrienoic acid (HHTrE) and epoxy-hydroxy-hexadecadienoic acid (EHHDE) were present as minor components. Overall, these data supported the reliability of LOFA extraction by MTBE/methanol of samples activated in WATER.

Glycerolipids

A higher amount of glycerolipids was detected in MEOH than in WATER preparations from pellets of *S. marinoi* and *T. weissflogii* (Table 2, Figure S2, Table S1) even if significant differences in the total amount of these lipids (fg-per-cell) were found only in *S. marinoi* pellets ($t=12.91$, $d.f.=2.22$, $p<0.01$, $N=6$). We identified eight different glycerolipid classes: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and triglyceride (TG). Major differences (90% reduction in WATER) between MEOH and WATER preparations were observed for MGDGs, SQDGs, PGs and PCs (Table S1). TGs and PEs showed minor changes while DGDGs and PIs were below the detection limit in WATER preparations.

Significant differences were also observed in total glycerolipid concentration between MEOH and WATER preparations of *S. marinoi* collected on GF/A filters ($t=17.96$, $d.f.=2.22$, $p<0.01$, $N=6$) (Figure 2C, Table 2). As reported for pellets, WATER preparations of filters showed major depletions in MGDG, SQDG and PG concentrations in comparison to MEOH samples. Moreover, also for GF/A filters, TGs were less affected by the preparation method and DGDG levels were below the detection limit in samples from WATER preparation (Figure 2C, Table S1).

Finally, no variation in composition and no significant difference in the levels of glycerolipids were found in samples from *S. marinoi* cells of pellets and GF/A filters ($t=3$, $d.f.=3.44$, $p>0.01$, $N=6$). However, a slightly higher glycerolipid concentration was generally observed from pellets than filters (Figure 2D, Table S1).

375 *Sterol sulphates (StS)*

376 In general, preparation and collection methods did not affect composition and extraction yields of
377 StS (Figure S3, Table 2, Table S1). Therefore, we did not detect significant changes in StS
378 concentration between MEOH and WATER preparation in both *S. marinoi* pellet (t=-0.51,
379 d.f.=2.04, p>0.01, N=6) (Figure 3A) and *S. marinoi* cells on GF/A filters (t=-2.03, d.f.=2.03,
380 p>0.05, N=6) (Figure 3C), as well as in *T. weissflogii* pellet (t=-0.71, d.f.=2.04, p>0.05, N=6)
381 (Figure 3B; Table 2). Furthermore, no significant difference was observed in extracts of cells
382 collected by centrifugation or filtration (t=3.72, d.f.=3.88, p>0.01, N=6) (Figure 3D, Table 2).
383 Overall, characterization of StS identified two animal sterols (the cholesterol sulphate and
384 desmosterol sulphate) and three phytosterols (the 24-methylene-cholesterol sulphate,
385 dihydrobrassicasterol sulphate and 24-ethyl-cholesterol sulphate), in agreement with literature
386 information (Gallo et al., 2020).

387 *Fatty acid methyl esters (FAMEs)*

388 Free fatty acids (FFAs) did not significantly vary after MEOH and WATER preparation in both *S.*
389 *marinoi* (t=1.02, d.f.=4, p>0.05, N=6) and *T. weissflogii* (t=0.43, d.f.=2.65, p>0.05, N=6) pellets
390 (Figure 4A, B, Table 2, Table S1). Analogously, no significant variation was observed in *S. marinoi*
391 cells collected on GF/A filters (t=4.22, d.f.=3.63, p>0.01, N=6) (Figure 4C, Table 2, Table S1).
392 Moreover, cell harvesting procedures (i.e. centrifugation and filtration) did not cause significant
393 differences in FFAs concentrations in *S. marinoi* (t=-1.19, d.f.=2.11, p>0.05, N=6) (Figure 4D,
394 Table 2, Table S1).
395 In general, ten FA species were identified in *S. marinoi* and *T. weissflogii* including C14:0, C16:0,
396 C16:1, C16:3, C16:4, C18:0, C18:2, C18:3, C20:5 and C22:6. As expected, levels of these chemical
397 species were rather constant in *T. weissflogii* (Figure S4) whereas reduction in C16:4, C16:3 and
398 C20:5 was found in extracts from *S. marinoi* cells from WATER preparations (Figure 4A and 4C,

399 Table S1). These results were confirmed by analysis of total fatty acids (TFAs) after saponification
400 of *S. marinoi* pellets analysed (Figure S4, S5).

401 **Discussion**

402 Results highlighted that MTBE/methanol extraction is reliable for the identification and quantitation
403 of different lipid classes from diatom samples obtained after centrifugation (i.e. cell pellets) or
404 filtration on GF/A filters. The choice of *Skeletonema marinoi* as a model diatom for our
405 experiments was motivated by the extensive evidence reporting that the genus *Skeletonema* is
406 cosmopolitan and it forms large blooms in coastal regions across the globe (Canesi and Rynearson,
407 2016). *S. marinoi* (formerly reported as *Skeletonema costatum*) is currently considered as a model
408 diatom species in chemical ecology, in respect of its high oxylipin and StS synthesis potentials
409 (Barreiro et al., 2011; d'Ippolito et al., 2018, 2004; Fontana et al., 2007a, 2007b; Gallo et al., 2020,
410 2018, 2017; Ianora et al., 2004; Nuzzo et al., 2019).
411 In analogy with other extraction procedures, *S. marinoi* samples analysed after MTBE/methanol
412 extraction from MEOH and WATER preparations significantly differed in terms of total LOFA and
413 glycerolipid concentrations. These results were consistent between experiments performed on cells
414 from pellets or GF/A filters, thus suggesting that harvesting and extraction did not affect lipid
415 composition. In particular, higher glycerolipid concentrations were observed after MEOH
416 preparation of *S. marinoi* samples (both pellets and GF/A filters), while higher LOFA
417 concentrations were measured after WATER preparations in these same cultures. FFA
418 concentration was not affected by the sample preparation procedure (i.e. MEOH or WATER), but
419 this result was mostly dependent on the abundance of C16:0 and C16:1 FAs, which were not further
420 metabolised to form oxylipins. Instead, polyunsaturated fatty acids acting as oxylipin precursors
421 (mainly C16:3 and C20:5) were strongly depleted in *S. marinoi* samples extracted after WATER
422 preparation (Figure 4A, C). This hypothesis was further supported by GC-MS/MS analysis of FFAs
423 and TFAs, that showed selective reduction of those fatty acids that are known precursors of

oxylipins in *S. marinoi*, such as C16:3 and C20:5 poly-unsaturated fatty acids (PUFAs) (Cutignano et al., 2006; d'Ippolito et al., 2018, 2004; Fontana et al., 2007b, 2007a; Orefice et al., 2015). It is interesting to note that other polyunsaturated fatty acids such as C18:2 and C18:3 occurred at similar concentrations after WATER and MEOH preparation, in agreement with previous reports that these fatty acids are not recognized as substrates by diatom lipoxygenases.

S. marinoi extracts analysed after MEOH preparation were consistently enriched in glycolipids (DGDGs, MGDGs and SQDGs) and phospholipids (PGs, PIs, PEs), but as expected these glycerolipid classes showed evident reduction after WATER preparation. Cell lysis induced by sonication exposed glycolipids and phospholipids to the activity of lipolytic acyl hydrolases (LAHs) prior to LOX-dependent synthesis of LOFAs (Adelfi et al., 2019; Cutignano et al., 2006; d'Ippolito et al., 2004). This process was strongly favoured when cells were prepared through the addition of water (i.e. WATER preparation), while it was strongly inhibited when samples were prepared through the initial addition of MeOH (i.e. MEOH preparation), thus explaining the observed differences in the two treatments. Interestingly, our results also confirmed that lipolytic activity mainly focused on glycolipids and phospholipids, while triglycerides (TGs) were less affected (Adelfi et al., 2019; Cutignano et al., 2006; d'Ippolito et al., 2004).

No significant variations in the lipid profiles of *T. weissflogii* between MEOH and WATER preparations were observed. This result was due to the very low hydrolytic and lipoxygenase potential of this diatom, also supported by the absence of oxylipins (Wichard et al., 2005b). These results were useful to offer a first validation of MTBE/methanol extraction procedures, highlighting that differences in glycerolipid, oxylipin and FFA concentrations observed in *S. marinoi* were mostly due to enzymatic reactions modulated by extraction procedures.

StS species quantified in *S. marinoi* and *T. weissflogii* were in good agreement with sterol composition reported for these diatoms in previous studies (Gallo et al., 2020, 2018, 2017; Nuzzo et al., 2019). Although no significant differences were observed between the preparation methods, MTBE/methanol extraction involving MEOH preparation seems particularly suitable for sterol

sulphate analysis, because concentrations of some species (e.g. desmosterol sulphate) were slightly reduced after WATER preparations.

Concentration of glycerolipids, StS and FFAs showed no significant differences between cell pellets and GF/A filters of *S. marinoi*. However, MTBE/methanol extraction supported higher recovery of glycerolipids and StS from pellets, whereas LOFAs were found at higher concentrations when extracted from filters. This result was probably due to cell lysis occurred during cell filtration with consequent triggering of enzymatic reactions leading to lipid degradation, free fatty acid production and final oxylipin synthesis. Thus, filters might be particularly advantageous to estimate LOX products in diluted natural phytoplankton communities.

Conclusions

Comprehensive lipid analysis is arising as a promising approach to investigate plankton physiology and ecological dynamics at the community-level (Da Costa et al., 2016; Gross and Han, 2011; Heydarizadeh et al., 2013; Pohnert, 2010; Pohnert et al., 2007; Schwartz et al., 2016; Wenk, 2010). Our results support substantial methodological improvement in phytoplankton sampling and lipid analysis. Our methodology reduces the number of protocols to be applied for the extraction of multiple lipid classes and allows more consistent analysis on the basis of coherent extractions based on MTBE/methanol. In particular, effective extraction of glycerolipids and StS was achieved when cells were suspended in MeOH, while effective extraction of oxylipins and FFAs relied on the initial addition of water to activate enzymatic reactions.

These protocols can represent a reference methodology for characterization of lipids in natural phytoplankton communities. In this perspective, cell collection on GF/A filters is particularly advantageous when studying phytoplankton occurring at low cell densities in the field. In comparison to centrifugation, cell collection on GF/A filters allows harvesting larger water volumes, is less time consuming and reduces biases in sample composition due to supernatant discard (Bidigare et al., 2003; Rodríguez-Ramos et al., 2014). Moreover, the biphasic

MTBE/methanol extraction protocol allows effective removal of GF/A filter residues produced after sonication and vortex agitation (Mayzaud et al., 2013), thus reducing loss of sample while enabling direct chromatographic analysis of the extracts and reliable quantitation of lipids.

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718 **Table 1**. Summary of main differences in the lipid composition of cells of *Skeletonema marinoi* and
719 *Thalassiosira weissflogii* by MTBE/methanol extraction from different preparations (see main text).
720 Condition 1 = extraction from MEOH and WATER preparation of pellets of *S. marinoi* or *T.*
721 *weissflogii*; Condition 2 = extraction from MEOH and WATER preparation of *S. marinoi* cells on
722 glass-fibre filters; Condition 3 = extraction from MEOH preparation of pellets and filters of *S.*
723 *marinoi*; Condition 4 = extraction from WATER preparation of pellets and filters of SKE.
724 GLY=glycerolipids, StS=sterol sulphates, LOFAs=linear oxygenated fatty acids, FAME=fatty acid
725 methyl esters. Black circles indicate the analyses that were performed in each condition. Asterisks
726 indicate significant differences as indicated by t-tests considering Welch's correction for unequal
727 variances (α=0.01). n.d = not detected. n.a. = not analyzed.

Condition	*Diatom*	*Preparation*	*Harvesting*	*Variables*			
				GLY	StS	LOFAs	FAME
1	*S. marinoi*	MEOH vs WATER	Pellet	●*	●	●*	●
1	*T. weissflogii*	MEOH vs WATER	Pellet	●	●	n.d.	●
2	*S. marinoi*	MEOH vs WATER	GF/A Filter	●*	●	●*	●
3	*S. marinoi*	MEOH	Pellet vs GF/A filter	●	●	n.a.	n.a
4	*S. marinoi*	WATER	Pellet vs GF/A filter	n.a	n.a	●*	●

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731 **Table 2**. Mean concentration (±s.d., *n=3*) of lipids (fg-per-cell) measured in *S. marinoi* and *T. weissflogii*. Experimental conditions and

732 abbreviations are according to Table 1. Abbreviations: n.d = not detected. n.a. = not analyzed

	Condition 1				**Condition 2**		**Condition 3**		**Condition 4**	
	S. marinoi		***T. weissflogii***		***S. marinoi***		***S. marinoi***		***S. marinoi***	
Cells/ml	1.84 10^6		1.87 10^5		2.5 10^6		3.78 10^6		2.25 10^6	
Preparation	MEOH	WATER	MEOH	WATER	MEOH	WATER	MEOH	MEOH	WATER	WATER
Cell Collection	Pellet	Pellet	Pellet	Pellet	Filter	Filter	Pellet	Filter	Pellet	Filter
GLY	10973±669	1494±395	33288±1170	28336±2709	8248±1480	1356±5	8572±2131	5009±838	n.a.	n.a
StS	1535±47	1335±76	1569±105	1742±555	1929±144	2166±172	954±28	879±22	n.a.	n.a.
LOFAs	1.98±1.8	302±97	n.d.	n.d.	14.58±4	53.81±13	n.a.	n.a.	6.37±1	39±4
FFAs	2613±456	2249±430	11805±1941	11203±748	245±33	164±16	n.a.	n.a.	4940±237	6204±1842

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739 **Figure 1**. Concentration (fg-per-cell) of linear oxygenated fatty acids (LOFAs) in *S. marinoi*. **A**)
740 LOFA abundance and composition in *S. marinoi* pellets extracted with MTBE/methanol after
741 MEOH and WATER preparation. **B**) LOFA abundance and composition in *S. marinoi* cells
742 collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER preparation. **C**)
743 LOFA abundance and composition in *S. marinoi* pellets and GF/A filters extracted with
744 MTBE/methanol after WATER preparation. Bar plots summarise average concentration (±s.d.) of
745 the detected oxylipins in the three cultures. Box plots are log-transformed values of total oxylipin
746 concentrations (mean±s.d.); asterisks indicate t-test significance between extracts (*=p<0.01;
747 **=p<0.001). Abbreviations: HEPE=hydroxyeicosapentaenoic acid, EHETE=epoxy-hydroxy-
748 eicosatetraenoic acid, EHHDE=epoxy-hydroxy-hexadecadienoic acid,
749 HHTrE=hydroxyhexadecatrienoic acid.
750
751 **Figure 2**. Glycerolipid concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**)
752 glycerolipid abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with
753 MTBE/methanol after MEOH and WATER preparation. **C**) glycerolipid abundance and
754 composition in *S. marinoi* cells collected on GF/A filters extracted with MTBE/methanol after
755 MEOH and WATER preparation. **D**) glycerolipid abundance and composition in *S. marinoi* pellets
756 and GF/A filters extracted with MTBE/methanol after MEOH preparation. Bar plots summarise
757 average concentration (±s.d.) of the detected glycerolipid classes in the diatom cultures. Box plots
758 are log-transformed values of total glycerolipid concentrations (mean±s.d.); t-test significance
759 between the two extracts is indicated by the asterisks (ns=not significant; *=p<0.01). Abbreviations:
760 MGDG=monogalactosyldiacylglycerol, DGDG=digalactosyldiacylglycerol,
761 PC=phosphatidylcholine, PE=phosphatidylethanolamine, PG=phosphatidylglycerol,
762 PI=phosphatidylinositol, SQDG=sulfoquinovosyldiacylglycerol, TG=triacylglycerols.
763

764 **Figure 3**. Sterol sulphate (StS) concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**)
765 StS abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with
766 MTBE/methanol after MEOH and WATER preparation. **C**) StS abundance and composition in *S.*
767 *marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER
768 preparation. **D**) StS abundance and composition in *S. marinoi* pellets and GF/A filters extracted
769 with MTBE/methanol after MEOH preparation. Bar plots summarise average concentration (±s.d.)
770 of the detected StS classes in the diatom cultures. Box plots are log-transformed values of total StS
771 concentrations (mean±s.d.); t-test significance between the two extracts is indicated by the asterisks
772 (ns=not significant). Abbreviations: 24-M-CHO=24-methylene cholesterol sulphate,
773 CHO=cholesterol sulphate, D_BRASS=dihydrobrassicasterol sulphate, DES=desmosterol sulphate,
774 24_E_CHO=24-ethylcholesterol sulphate.
775
776 **Figure 4**. Free fatty acids (FFAs) concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**)
777 FFA abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with
778 MTBE/methanol after MEOH and WATER preparation. **C**) FFA abundance and composition in *S.*
779 *marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER
780 preparation. **D**) FFA abundance and composition in *S. marinoi* pellets and GF/A filters extracted
781 with MTBE/methanol after MEOH preparation. Bar plots summarise average concentration (±s.d.)
782 of the detected FFA species in the three cultures. Box plots are log-transformed values of total FFA
783 concentrations (mean±s.d.); t-test significance is between the two extracts is indicated by the
784 asterisks (ns = not significant).

Figure S1

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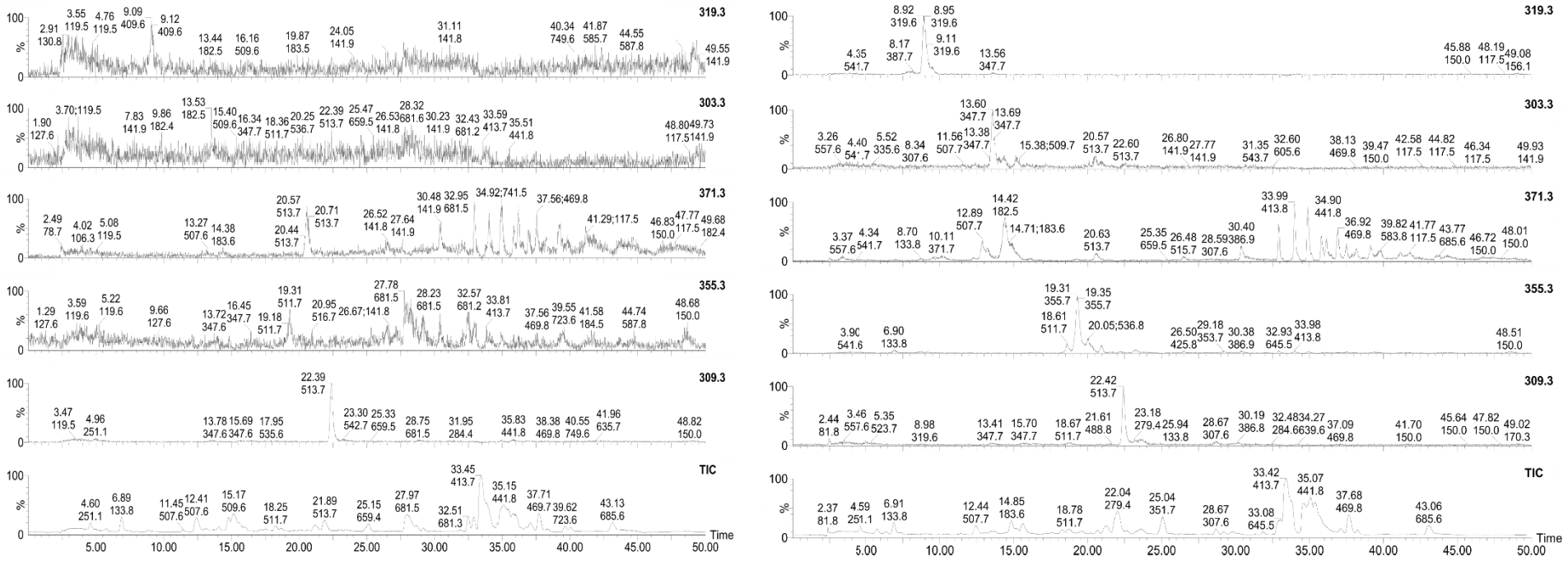


Figure S1. LC-MS chromatograms of oxylipins obtained for SKE pellets after MEOH preparation (left) and WATER preparation (right). One single replicate is displayed for each extraction procedure. Total ion current (TIC) and targeted oxylipin peaks are visualized. 309.3=Standard (16-hydroxyhexadecanoic acid, Retention time, Rt: 22.4), 355.3=HEPE (Rt: 19.31), 371.3=EHETE (Rt: 14.4), 303.3=HHTrE (Rt:13.6), 319.3=EHHDE (Rt:8.9).

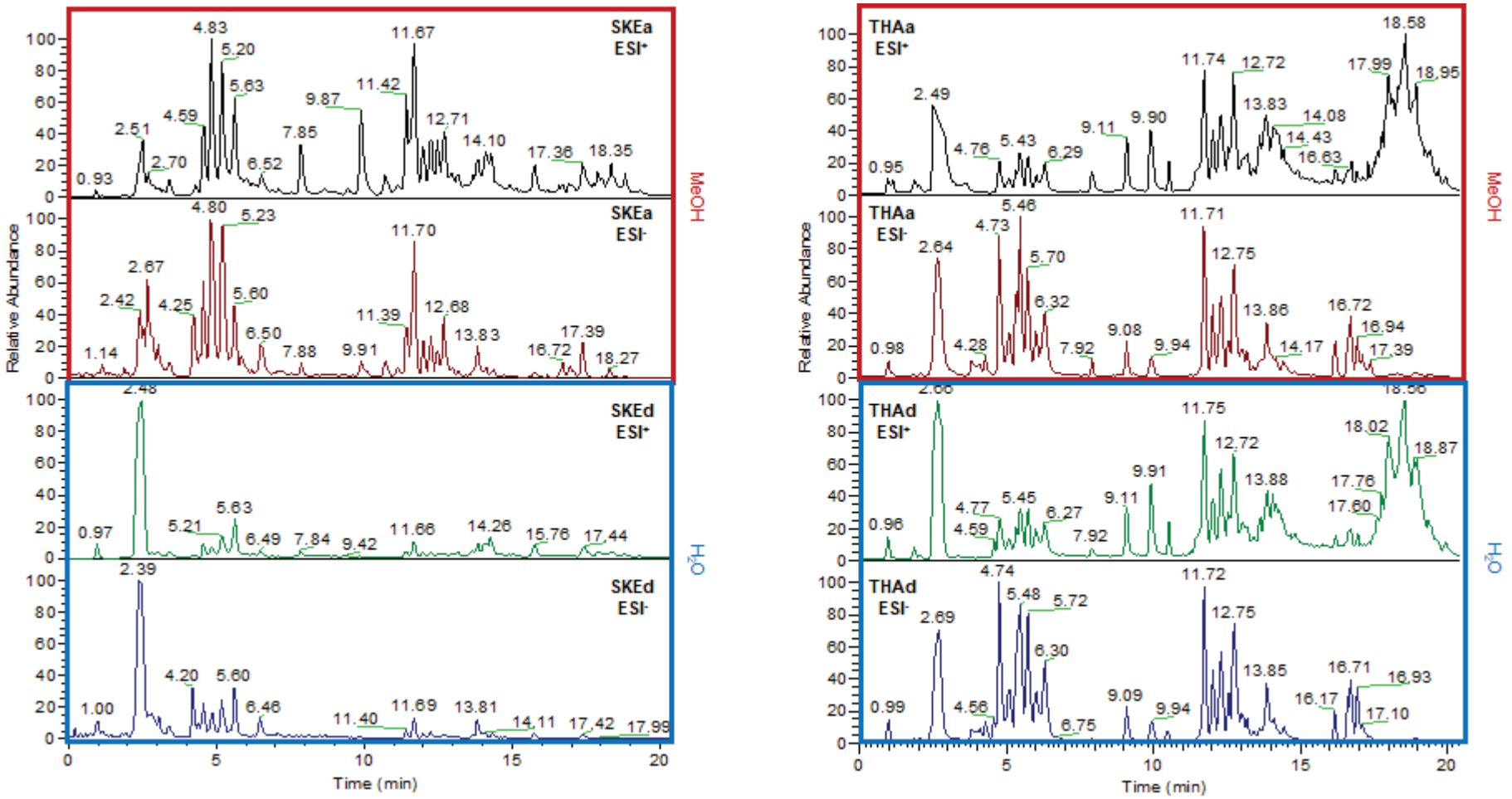


Figure S2. LC-MS (ESI^+ and ESI^-) chromatograms of glycerolipids obtained from SKE (left) and THA (right) after MEOH (SKEa and THAa) and WATER (SKEd and THAd) preparations. For a better visualization, molecules with a m/z between 450 and 1000 were selected. Retention time is reported in abscissa, while ordinates report the relative abundance of the peaks.

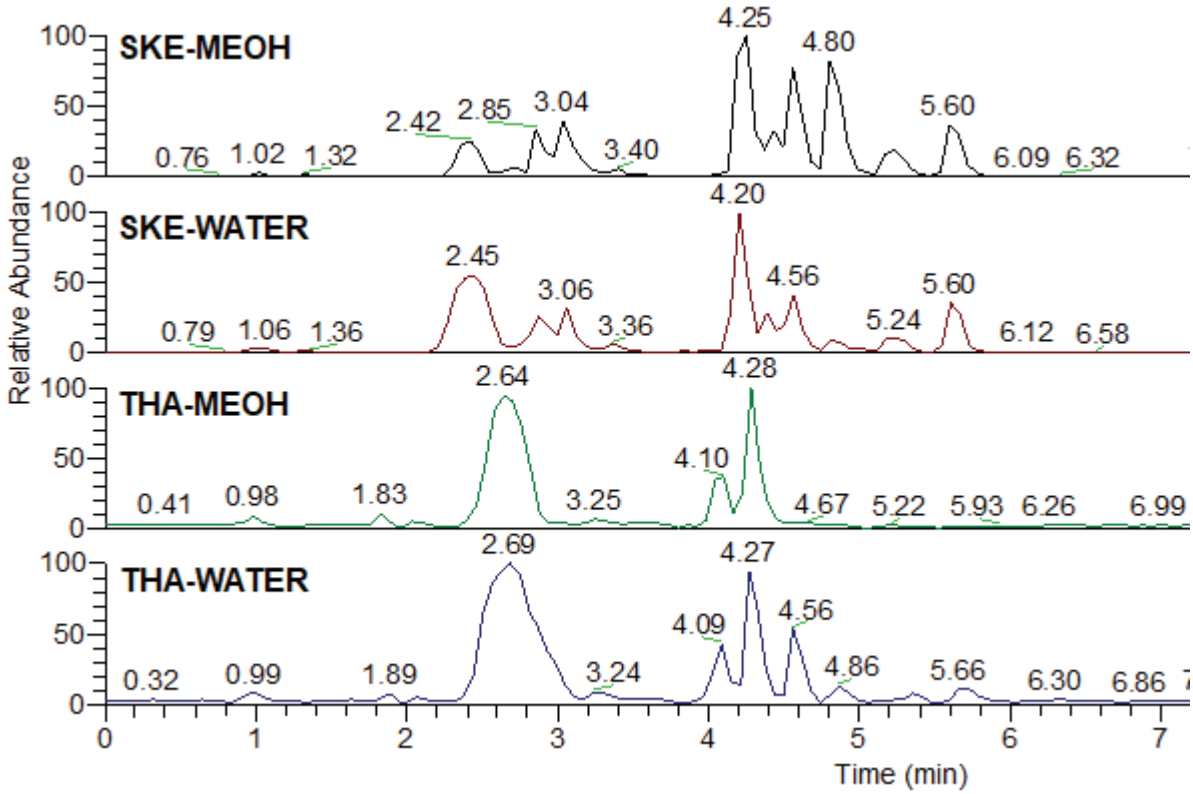


Figure S3. LC-MS chromatograms (ESI^-) of sterol sulphates obtained for SKE and THA after MEOH and WATER preparation. For a better visualization, molecules with a m/z between 450 and 500 and with retention time between 0 and 7 min were selected.

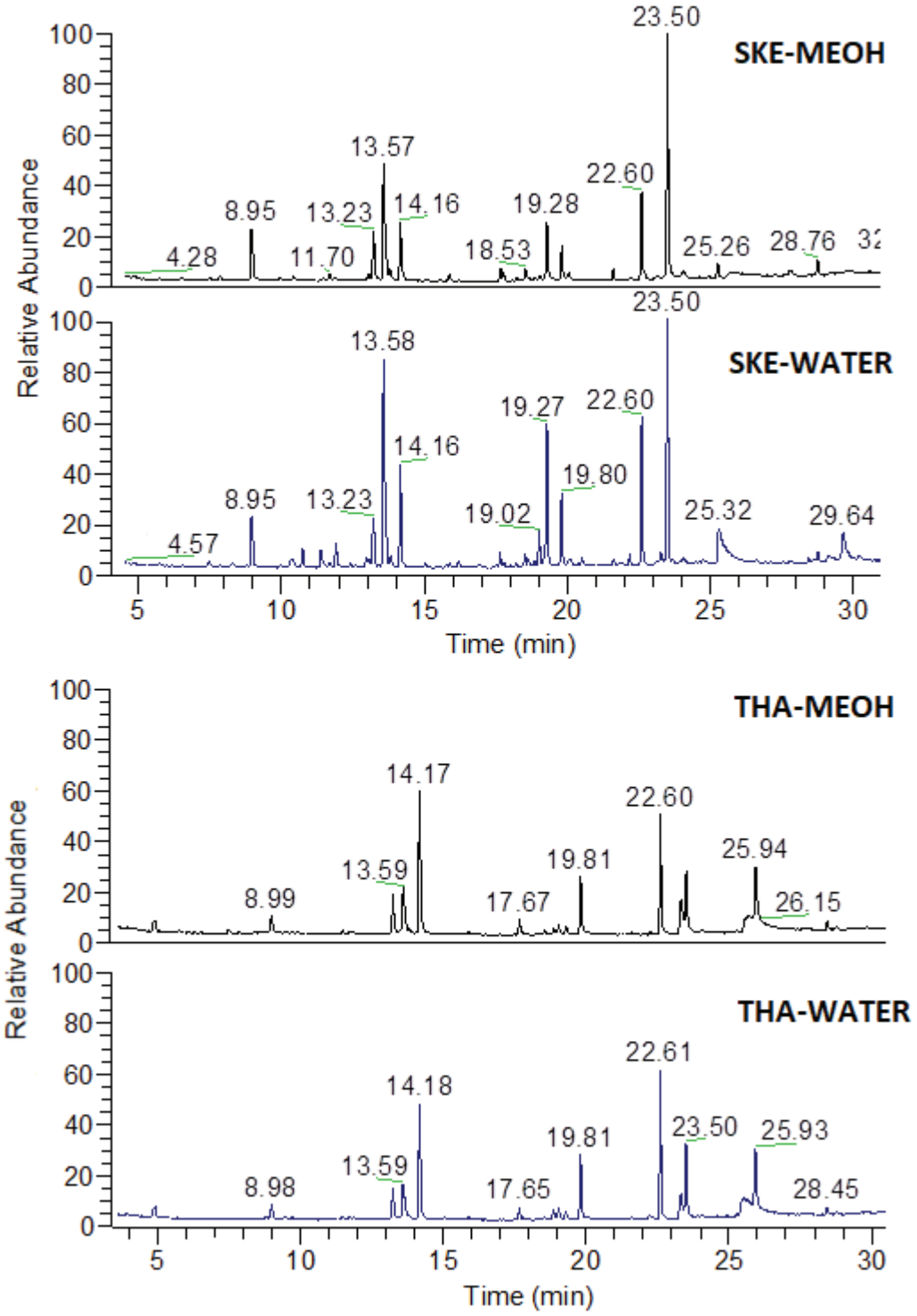


Figure S4. GC-MS chromatograms of fatty acid methyl esters (FAME) obtained for SKE and THA after MEOH and WATER preparation of samples. Total ion current (TIC) chromatograms ranging between 5 and 30 minutes are displayed. Retention time is reported in abscissa, while ordinates report the relative abundance of the peaks.

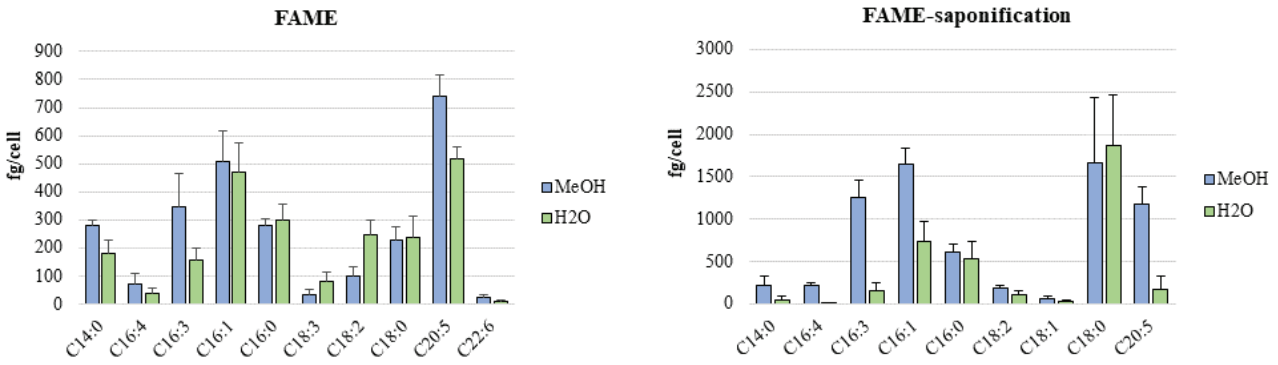
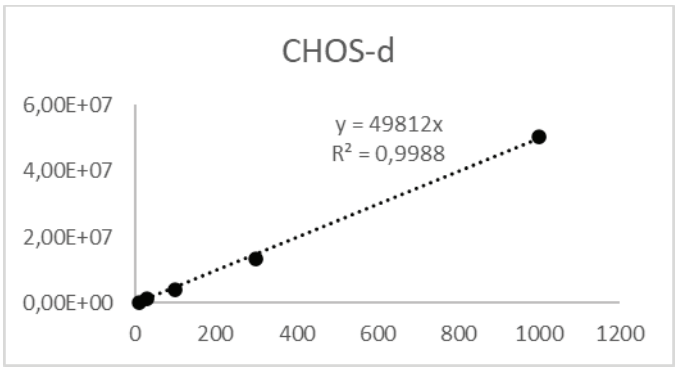


Figure S5. GC-MS/MS quantitation (fg-per-cell) of free fatty acids (FFAs, left) and total fatty acids (TFAs, right) analyzed as fatty acid methyl esters (FAMEs) in SKE extracts obtained after MEOH or WATER (H_2O) preparation.

A)



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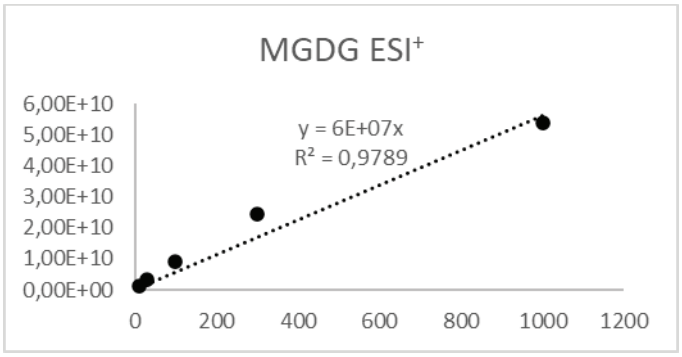

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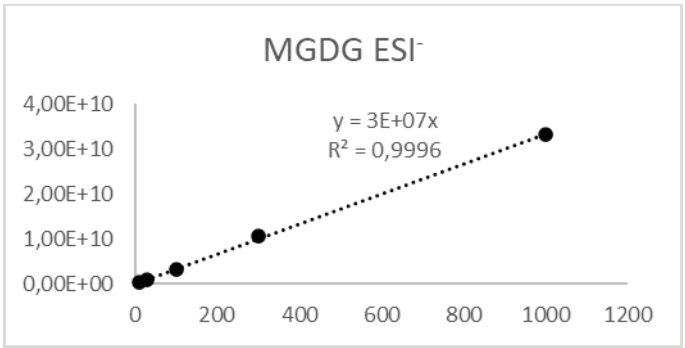

Figure S6. Calibration curve to sterol sulfates (A) and hydrogenated MGDG in ESI^+ (B) and ESI^- (C) in the range between 10 ng/mL to 1000 ng/mL.

Figure 1. Concentration (fg-per-cell) of linear oxygenated fatty acids (LOFAs) in *S. marinoi*. **A**) LOFA abundance and composition in *S. marinoi* pellets extracted with MTBE/methanol after MEOH and WATER preparation. **B**) LOFA abundance and composition in *S. marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER preparation. **C**) LOFA abundance and composition in *S. marinoi* pellets and GF/A filters extracted with MTBE/methanol after WATER preparation. Bar plots summarise average concentration (±s.d.) of the detected oxylipins in the three cultures. Box plots are log-transformed values of total oxylipin concentrations (mean±s.d.); asterisks indicate t-test significance between extracts (*=$p<0.01$; **=$p<0.001$). Abbreviations: HEPE=hydroxyeicosapentaenoic acid, EHETE=epoxy-hydroxy-eicosatetraenoic acid, EHHDE=epoxy-hydroxy-hexadecadienoic acid, HHTrE=hydroxyhexadecatrienoic acid.

Figure 2. Glycerolipid concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**) glycerolipid abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with MTBE/methanol after MEOH and WATER preparation. **C**) glycerolipid abundance and composition in *S. marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER preparation. **D**) glycerolipid abundance and composition in *S. marinoi* pellets and GF/A filters extracted with MTBE/methanol after MEOH preparation. Bar plots summarise average concentration (±s.d.) of the detected glycerolipid classes in the diatom cultures. Box plots are log-transformed values of total glycerolipid concentrations (mean±s.d.); t-test significance between the two extracts is indicated by the asterisks (ns=not significant; *=$p<0.01$). Abbreviations: MGDG=monogalactosyldiacylglycerol, DGDG=digalactosyldiacylglycerol, PC=phosphatidylcholine, PE=phosphatidylethanolamine, PG=phosphatidylglycerol, PI=phosphatidylinositol, SQDG=sulfoquinovosyldiacylglycerol, TG=triacylglycerols.

Figure 3. Sterol sulphate (StS) concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**) StS abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with MTBE/methanol

after MEOH and WATER preparation. **C**) StS abundance and composition in *S. marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER preparation. **D**) StS abundance and composition in *S. marinoi* pellets and GF/A filters extracted with MTBE/methanol after MEOH preparation. Bar plots summarise average concentration (±s.d.) of the detected StS classes in the diatom cultures. Box plots are log-transformed values of total StS concentrations (mean±s.d.); t-test significance between the two extracts is indicated by the asterisks (ns=not significant). Abbreviations: 24-M-CHO=24-methylene cholesterol sulphate, CHO=cholesterol sulphate, D_BRASS=dihydrobrassicasterol sulphate, DES=desmosterol sulphate, 24_E_CHO=24-ethylcholesterol sulphate.

Figure 4. Free fatty acids (FFAs) concentration (fg-per-cell) in *S. marinoi* and *T. weissflogii*. **A-B**) FFA abundance and composition in *S. marinoi* and *T. weissflogii* pellets extracted with MTBE/methanol after MEOH and WATER preparation. **C**) FFA abundance and composition in *S. marinoi* cells collected on GF/A filters extracted with MTBE/methanol after MEOH and WATER preparation. **D**) FFA abundance and composition in *S. marinoi* pellets and GF/A filters extracted with MTBE/methanol after MEOH preparation. Bar plots summarise average concentration (±s.d.) of the detected FFA species in the three cultures. Box plots are log-transformed values of total FFA concentrations (mean±s.d.); t-test significance is between the two extracts is indicated by the asterisks (ns = not significant).

Figure 1

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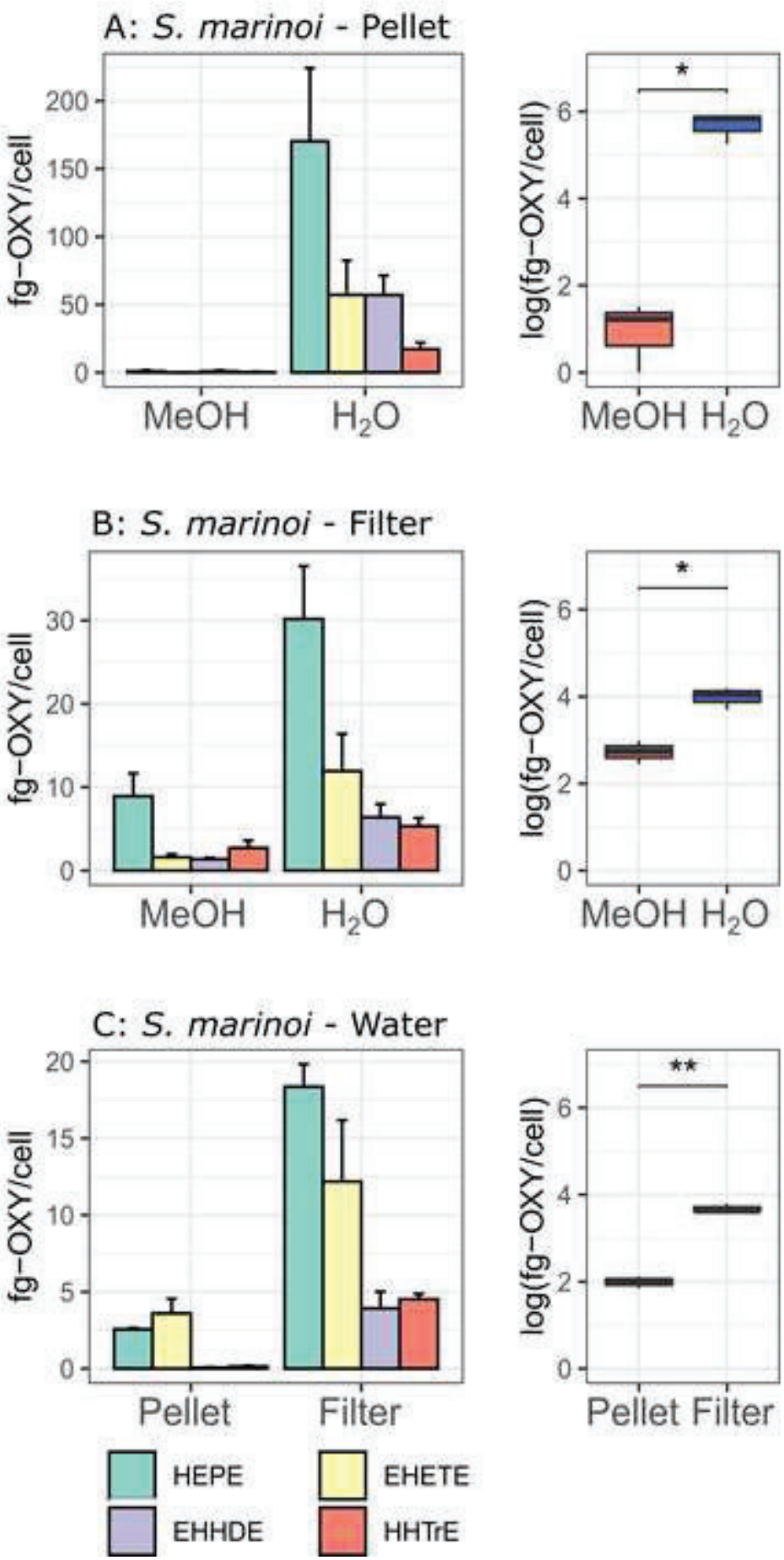

Figure 2

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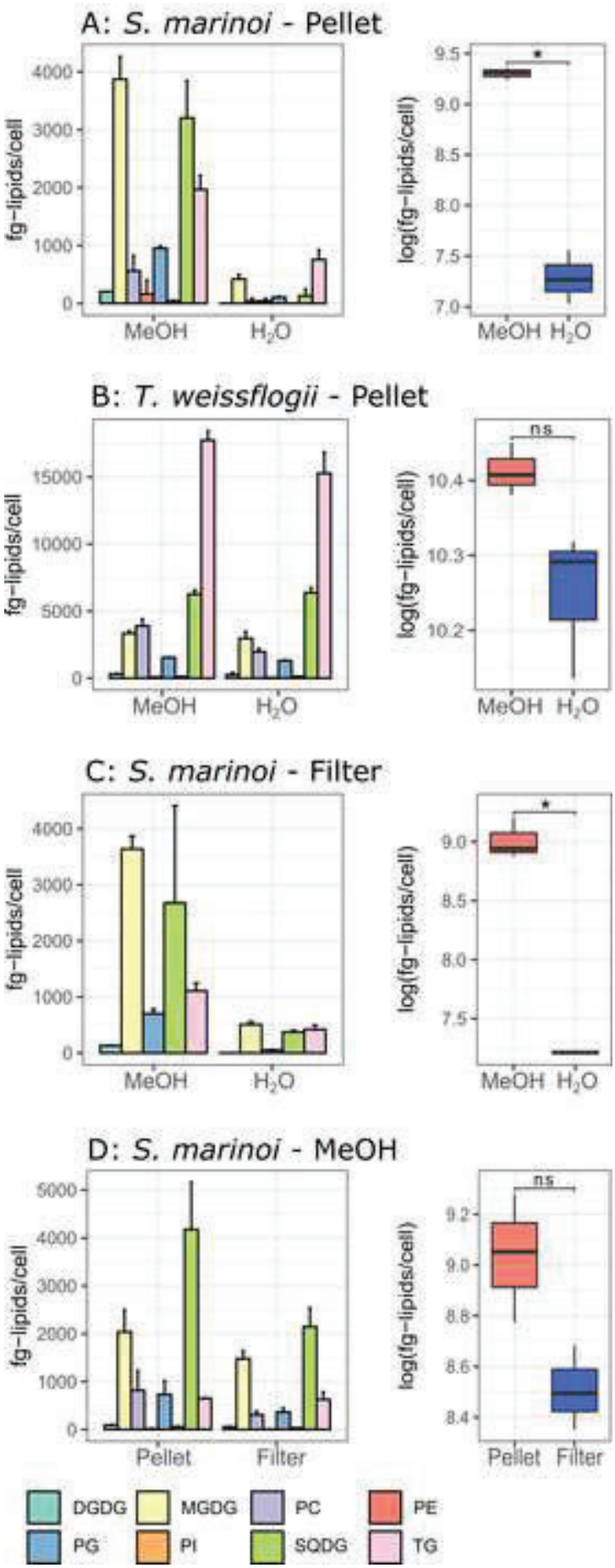

Figure 3

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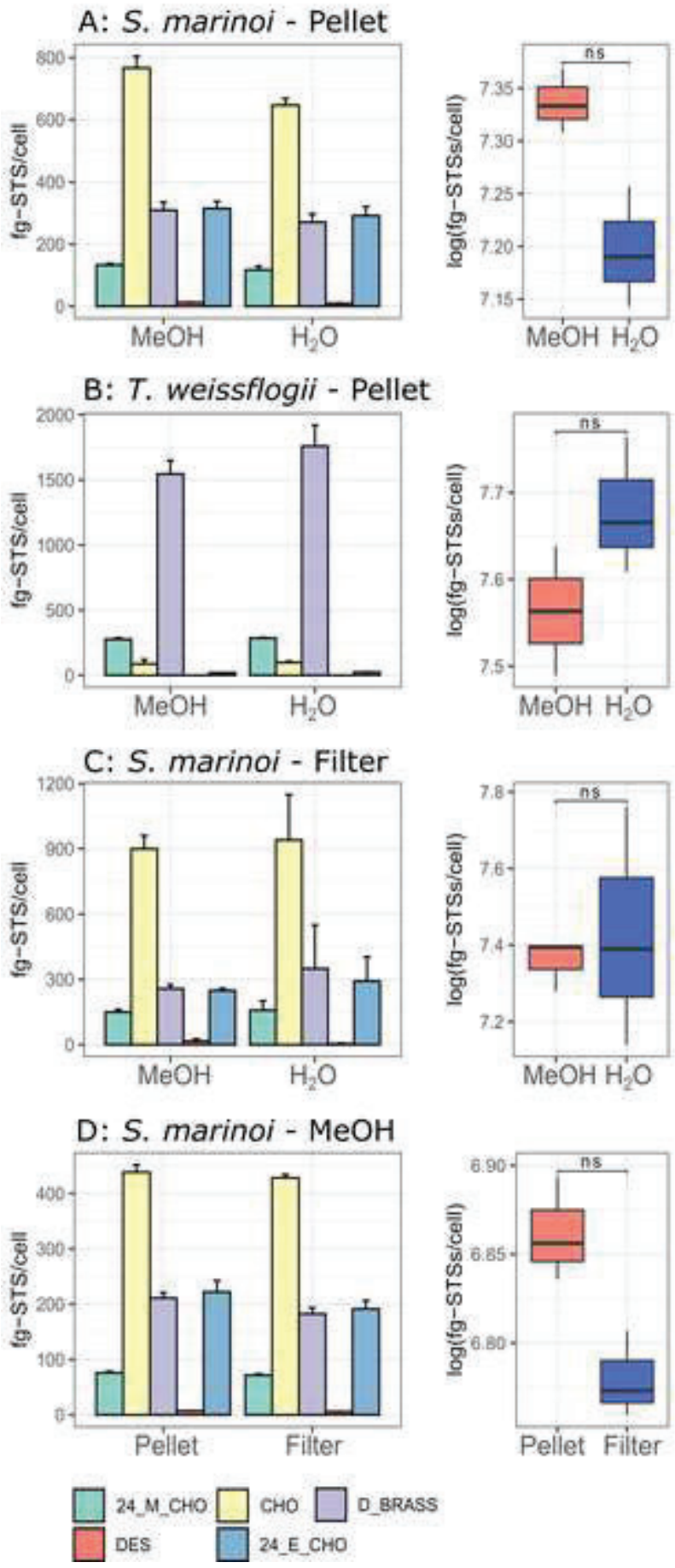

Figure 4

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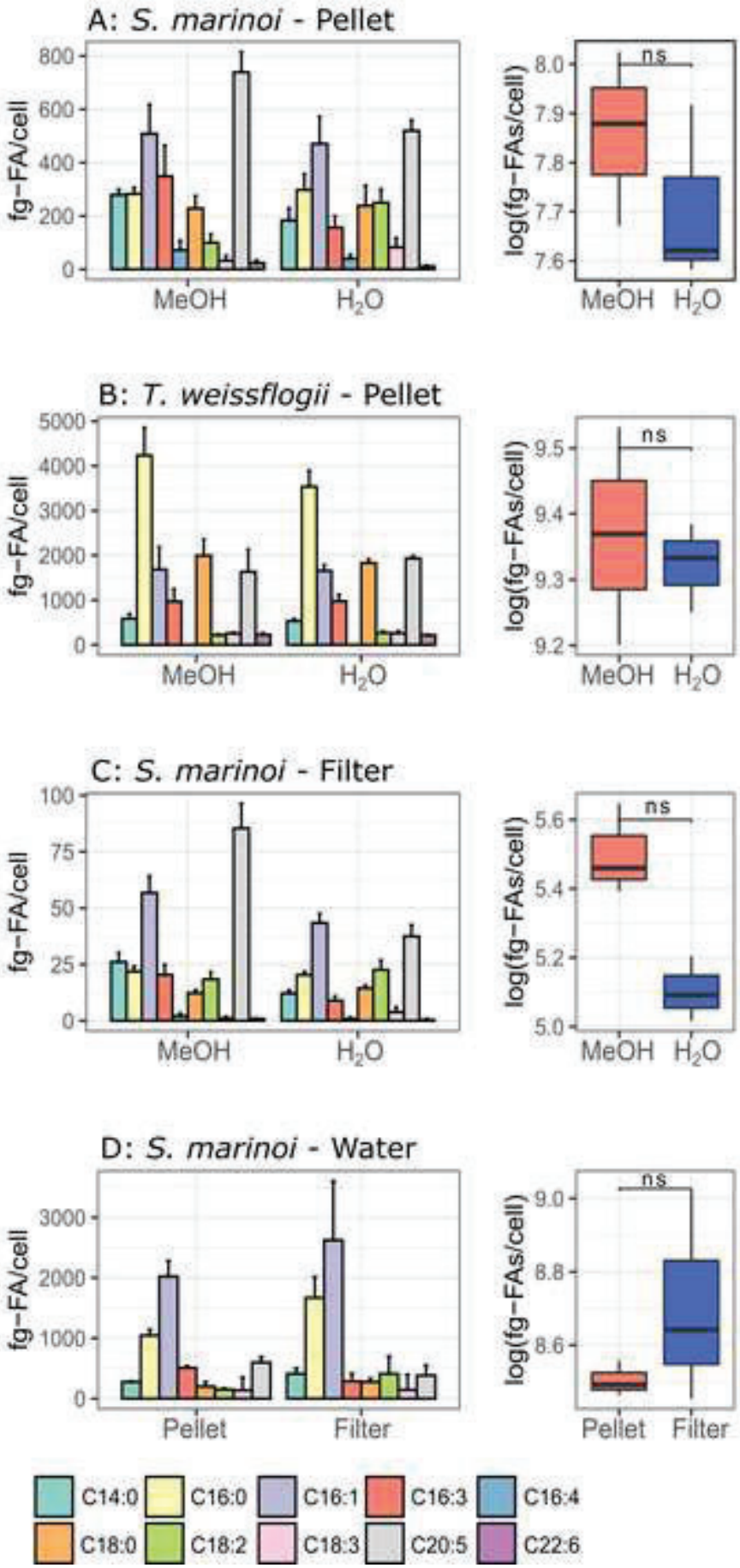

Table S1. Mean concentration (fg per cell±sd) of the single chemical species quantified for each lipid class in the different experiments. In the first experimental condition, lipid variations between MEOH vs WATER preparation of *Skeletonema marinoi* (SKE) and *Thalassiosira weissflogii* (THA) pellets were inspected. In experimental condition 2, lipid variations between MEOH vs WATER preparation of SKE cells accumulated on GF/A vs filters were inspected. In third experimental condition, differences in lipid quantitation between SKE cells accumulated as pellets or on GF/A filters were inspected. Abbreviations of glycerolipids: DGDG= digalactosyldiacylglycerol, MGDG= monogalactosyldiacylglycerol, SQDG= sulfoquinovosyldiacylglycerol, PG= phosphatidylglycerol, PC= phosphatidylcholine, PI= phosphatidylinositol, PE= phosphatidylethanolamine, TG= triacylglycerol. Abbreviations of sterol sulphates: Cholesterol-s= cholesterol sulphate, Desmosterol-s= desmosterol sulphate, 24-methylch.-s= 24-methylene cholesterol sulphate, Dihydrobrass.-s= Dihydrobrassicasterol sulphate, 24-ethylch.-s= 24-ethylcholesterol sulphate. Oxylipin (linear oxygenated fatty acids, LOFAs) abbreviations: HEPE= hydroxy eicosapentaenoic acid, EHETE= epoxy hydroxy eicosatetraenoic acid, HHTrE= hydroxy hexadecatrienoic acid, EHHDE= hydroxy epoxy hexadecadienoic acid. FAMEs=fatty acid methyl esters.

	Mean concentration (*fg per cell mean ± sd*)							
	Condition 1				**Condition 2**		**Condition 3**	
	SKE pellet		**THA pellet**		**SKE filter**		**SKE pellet and filter**	
	MEOH	WATER	MEOH	WATER	MEOH	WATER	Pellet	Filter
Glycerolipids								
DGDG	204±4	0	322±38	258±176	130±4	0	92.9±10	57.6±7
MGDG	3872±397	419±75	3336±190	2975±491	3637±230	506±49	2038±455	1477±166
SQDG	3204±640	130±117	6266±313	6375±379	2673±1734	373±27	4176±984	627±394
PG	952±39	106±14	1535±26	1299±70	701±78	54.9±7	730±284	363±85
PC	560±265	38.7±53	3897±530	1975±236	n.d.	n.d.	815±411	304±80

PI	46.6±7	0	117±8	127±6	n.d.	n.d.	56.8±12	30.8±2
PE	164±237	44.1±43	74±19	48.9±3	n.d.	n.d.	12.3±13	4.3±7
TG	1967±245	754±172	17738±688	15276±1576	1105±140	420±78	649±12	627±161
Sterol sulphates								
Cholesterol-s	766±38	646±21	87.7±34	98.2±10	899±61	834±142	438±13	428±6
Desmosterol-s	121	9.3±1	n.d.	n.d.	16±9	5.1±0.4	7.2±0.3	5.8±0.1
24-methylch.-s	132±5	117±12	276±11	285±6	149±13	135±31	75.9±3	71.4±3
Dihydrobrass.-s	309±26	270±26	1545±102	1758±160	256±19	234±42	210±10	182±11
24-ethylch.-s	314±23	291±29	19.4±1	24.2±2	248±11	229±38	221±20	191±15
LOFAs								
HEPE	0.9±0.8	170±54	n.d.	n.d.	8.9±3	30.1±6	2.5±0.1	18.3±1
EHETE	n.d.	57±2	n.d.	n.d.	1.5±0.4	11.9±4	3.6±1	12.1±4
HHTrE	0.19±0.3	17.1±5	n.d.	n.d.	2.7±1	5.3±1	0.14±0.1	4.5±0.4
EHHDE	0.85±0.8	57±1	n.d.	n.d.	1.3±0.2	6.3±2	0.06±0.03	3.9±1
FAMEs								
C14:0	278±21	183±46	585±108	531±52	26±4	12±1	276±14	411±89
C16:0	282±24	297±59	4234±627	3537±359	21±2	20±1	1045±96	1669±346

C16:1	508±110	470±102	1686±502	1655±134	56±7	43±4	2021±258	2624±976
C16:3	348±115	156±43	970±278	975±149	20±4	9±2	508±30	286±127
C16:4	71±36	40±15	n.d.	n.d.	2±1	0.8±0.7	n.d.	n.d.
C18:0	228±47	238±75	1995±365	1830±87	12±1	14±1	200±74	275±60
C18:2	99±33	249±50	218±24	266±41	18±3	22±4	n.d.	410±288
C18:3	32±19	82±34	253±27	257±54	1±1	4±2	138±212	144±250
C20:5	739±76	519±40	1633±498	1932±62	85±11	37±5	598±383	383±168
C22:6	23±10	11±3	226±37	216±14	1±0.2	0.4±0.6	n.d.	n.d.