Supporting Information

Effective lipid extraction from algae cultures using switchable solvents

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Chemicals. All solvents and chemicals used in this study were obtained from Sigma-Aldrich (purities ≥ 98%) and were used without purification.

Microorganism and culture conditions.

Desmodesmus communis was isolated from a water sample collected in Forlì-Cesena (Italy) in 2009. The algae was grown in a modified Chu13 medium[1] corresponding to the following composition (g L^{-1}): KNO_3 (0.2), KH_2PO_4 (0.04), $MgSO_4$ $7H_2O$ (0.1), $CaCl_2$ $6H_2O$ (0.08), Fe citrate (0.01), citric acid (0.1); micro elements: B, Mn (both at 0.5 ppm), Zn (0.05 ppm), Cu, Co, Mo (0.02 ppm).

Tetraselmis suecica (CCAP 66/4) was and *Nannochloropsis gaditana* (Lubián CCMP 527) were obtained from the University of Almeria; both algae were supplied with $NaNO_3$ and KH_2PO_4, plus micronutrients and vitamins of the F/2 medium.[2] To create the marine environment, artificial sea salt was used (Instant Ocean, Spectrum Brands, Atlanta, US) at a concentration of 25 g L^{-1}.

D. communis was cultured in a 70 L column photobioreactor (M2M Engineering, Italy) with internal illumination. The microalgae were cultivated using variable CO_2 concentrations (1.25-7%) in air to maintain the pH value to 7, at room temperature with light intensity at 350 µmol m^{-2} s^{-1} and a 16h light: 8h dark cycle. *N. gaditana* and *T. suecica* were cultured in a 25 L column photobioreactor made of transparent acrylic tube and operated under non-axenic conditions in a greenhouse at room temperature with a light intensity of 250 µmol m^{-2} s^{-1} at the surface of the PBR. The source of light was continuously provided (24 h per day, 7 days per week) by metal

halide lamps (Philips HPI-T). The pH was not controlled, and bubbling air was continuously supplied through diffusers at an aeration rate of about 0.5 vvm (volume of gas per volume of mixed culture per minute).

The absence of biological contamination in the cultures was periodically checked by visual inspection with light microscopy.

Fresh culture aliquots (50 mL) for the culture extraction experiments were collected routinely, maintaining the culture in a semi-continuous system with maximum cell density around 2 g L^{-1}, by removal of part of the culture and replacement with fresh medium. Dry weight of the algal biomass was determined filtering aliquots of 30-40 mL of algal suspension through a pre-weighted, pre-combusted (105°C, 24 h) glass fibre filter (Whatman GF/F, 47 mm, nominal pore size 0.7 mm). The filters were then dried at 105°C for 1-2 h to obtain a constant weigh.

Concentrated algae pastes (about 80% water content in the final samples) aliquots (50 mL) for the wet biomass extraction experiments were harvested every 2-3 days using a Westfalia centrifuge (Model: OTC 3-03-107) operated at 10000 rpm and recovered manually from the inside bowl.

GC-MS analysis. GC-MS analyses were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 mm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12-600 m/z range. The temperature of the column was increased from 50°C up to 180°C at 50°C min^{-1}, then from 180°C up to 300°C at 5°C min^{-1}. Tridecanoic acid and methyl nonadecanoate were utilized as internal standards for quantification of free and bounded fatty acids converted into fatty acid methyl esters (FAMEs). The relative response factors used for the quantitation were obtained by injecting solutions of known amounts of methyl tridecanoate, methyl nonadecanoate and commercial FAMEs mixture.

Extraction and characterization of lipids. The determination and the characterization of lipid content was done on algal dried samples obtained by centrifugation of cultures at 4000 rpm for 20 min; the lipid oils were obtained by extracting 500 mg of dried algal samples with 45 mL of $CHCl_3$/MeOH mixture (2/1 v/v) under reflux for 2 h for three times; after filtration over celite, the

combined $CHCl_3$/MeOH layers were evaporated under vacuum. Total lipids (TLs) were measured gravimetrically and reported as percentage on algae dry weight basis.

Each algal lipid extract was also analyzed by ^1H NMR and by GC-MS to determine the amount of FAMEs.

NMR Spectra were recorded in CD_3OD using a 5 mm probe on a VARIAN Inova 600 spectrometer.

The determination of FAMEs was performed according to the literature.[3] Lipid samples (about 2 mg) were dissolved in dimethylcarbonate (0.4 mL) containing tridecanoic acid (0.02 mg). 2,2-dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL) were then added; the samples were placed in an incubator at 90°C for 30 min. After cooling for 5 min to room temperature, 1.3 M BF_3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (0.02 mg) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-dimethylcarbonate layer, containing FAMEs, was transferred to vials for GC-MS analysis. Each analysis was repeated in duplicate.

Fractionation of lipids. TLs (about l00 mg) were also separated into the different classes, namely neutral lipids (NL), glycolipids (GL) and phospholipids (PL).[4] The separation was achieved on a chromatographic column (1 x l5 cm) packed with silica gel (200~300 mesh); the individual classes were eluted with $CHCl_3$ (200 mL), acetone (400 mL) and MeOH (200 mL) according to the procedure reported in the literature.[5] The isolated lipids in each eluate were firstly monitored on TLC silica gel plates according to the procedure reported in the literature,[4] then measured gravimetrically after evaporation of the solvent and reported on algal dry weight basis.

The mobile phases used for the detection of the lipid classes were hexane:diethyl ether:acetic acid (70:30:0.5 v/v) for NL; $CHCl_3$: MeOH:H_2O (75:25:4) for GL; $CHCl_3$: MeOH:H_2O (65:25:4) for PL.

The spray reagents used for the detection were: 5% (w/v) molybdophosphoric acid solution in ethanol, followed by heating for 10 min at 180°C for NL and PL; 0.5 % (w/v) naphthol solution in methanol/water (1:1 v/v), followed by drying the plate, spraying with 95% v/v H_2SO_4 and then heating at 120°C for 5 min for GL.

The presence of NL and PL in GL fraction was checked by using the spray reagent for NL and PL for the GL fraction. For all the tested algae, NL in GL fraction were revealed just in traces, whereas no PL were found in this fraction.

The presence of GL in NL and PL fractions was checked by using the spray reagent for GL for both the NL and PL fractions. For all the tested algae, both PL and NL fractions did not shown any GL traces.

Wet biomass extraction with DMCHA. Wet samples of each alga were added to DMCHA (about 250 mg with a water content of 80% to 1 mL for the biomass/DMCHA ratio 50 mg mL^{-1}; 50 mg with a water content of 80% to 5 mL for the biomass/DMCHA ratio 2 mg mL^{-1}) and magnetically stirred at 250 rpm at room temperature for 24 h (aliquots for analysis were withdrawn after 3 and 6 h). After centrifuging the solutions to remove algal cells, DMCHA layer, containing algal lipids, was split in two parts: the first was used to determine the *lipid extraction efficiency* of DMCHA by evaporating the amine. TLs obtained after evaporation of DMCHA were measured gravimetrically, reported as percentage on algae dry weight basis, fractionated as described above and compared with the results obtained through the extraction with $CHCl_3$/MeOH. Each DMCHA lipid extract was also analyzed by GC-MS to determine the amount of FAMEs (Table 1). Each extraction condition was replicated for three times.

The second part of DMCHA layer was used to determine the *lipid recovery efficiency* from the hydrogen carbonate ammonium salt ($DMCHAH^+$ HCO_3^-): a doubled volume of H_2O was added to DMCHA and CO_2 was bubbled into the mixture for 45 min to obtain a solution containing $DMCHAH^+$ HCO_3^-. Green lipids drops floated on the top of the colorless $DMCHAH^+$ HCO_3^- solution in water. Hexane (1 mL) was added to recover the lipid phase; after separation hexane was removed at room temperature by blowing N_2 and residual FAMEs analyzed as described above. $DMCHAH^+$ HCO_3^- was finally reconverted to DMCHA by stirring and heating at 70-80°C for 1 h in order to remove CO_2.

Table 1. FAMEs yields on dry weight basis obtained through DMCHA extraction of wet biomass (mean ± standard deviation, n = 3).

	biomass/DMCHA ratio 2 mg mL^{-1}			biomass/DMCHA ratio 50 mg mL^{-1}		
Time (h)	*N. gaditana*	*T. suecica*	*D. communis*	*N. gaditana*	*T. suecica*	*D. communis*
3	10.4±0.6	5.7±0.6	3.1±0.4	9.4±1.8	4±0.8	4.3±0.5
6	10.6±0.4	5.8±0.3	4.4±0.1	10.8±1.1	4.3±0.6	5.3±0.3
24	11.1±0.6	7.2±0.5	5.9±0.2	11±0.9	5.4±0.6	6.1±0.7

Culture extraction with DMCHA. An aliquot (5 mL) of cultures (biomass concentration about 2 g L^{-1}) of each alga was added to DMCHA (2.5 mL for the 1/1 culture/DMCHA volume ratio; 5 mL for the 2/1 culture/DMCHA volume ratio). The biphasic mixture was stirred at 500 rpm at room temperature for 24 h (aliquots for analysis were withdrawn after 3 and 6 h) and then centrifuged to recover the upper organic phase.

Upper DMCHA layer, containing algal lipids, was treated as described for wet biomass extraction experiments, but avoiding the addition of water in the *lipid recovery efficiency* phase. Results of lipid extraction efficiency are reported in Table 2. Each extraction condition was replicated for three times.

Table 2. FAMEs yields on dry weight basis obtained through DMCHA extraction of cultures (mean ± standard deviation, n = 3).

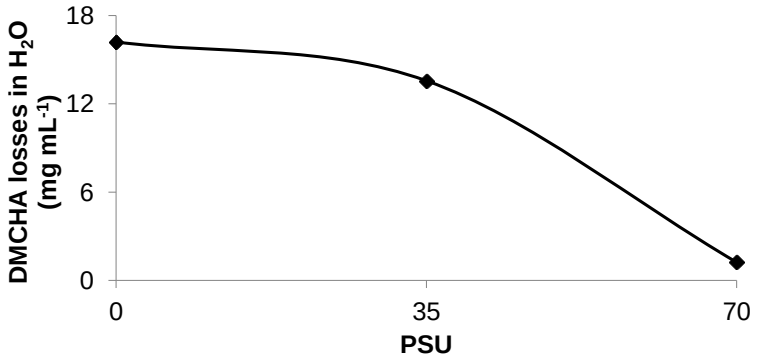
	1/1 culture/DMCHA volume ratio			2/1 culture/DMCHA volume ratio		
Time (h)	*N. gaditana*	*T. suecica*	*D. communis*	*N. gaditana*	*T. suecica*	*D. communis*
3	9.9±1.0	4.6±0.4	0.3±0.1	10.4±0.1	4.2±0.6	0.5±0.2
6	10.4±0.3	5.5±0.3	0.5±0.1	10.8±0.5	5.0±0.7	0.7±0.3
24	10.8±0.1	6.1±0.6	3.1±0.4	11.9±1.5	5.8±1.3	1.7±0.9

Determination of DMCHA losses. To check the amount of DMCHA lost in aqueous phase, 1 mL of DMCHA was added to 1 mL of D_2O; after 24 h of contact ^1H NMR quantitative analysis was performed by using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt as internal standard. The same procedure was repeated by adding NaCl to D_2O to get 35 and 70 psu; DMCHA solubility by varying salinity of the water phase was shown in Fig. 1 in †ESI.

To check the amount of DMCHA lost in the lipid phase, after switching DMCHA (5 mL) into $DMCHAH^+$ HCO_3^- with CO_2 and H_2O (5 mL), lipid layer was extracted with *D*-14 hexane (1 mL); then ^1H NMR quantitative analysis was performed by using dimethylterephthalate as internal standard. DMCHA loss was 2.9±0.07 mg per mL of *D*-14 hexane, corresponding to 0.9% of DMCHA used for the extraction.

Spectra were recorded in D_2O or hexane-d_{14} using a 5 mm probe on a VARIAN Mercury 400 spectrometer.

Fig 1. DMCHA losses in H_2O by varying the salinity of the medium.



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