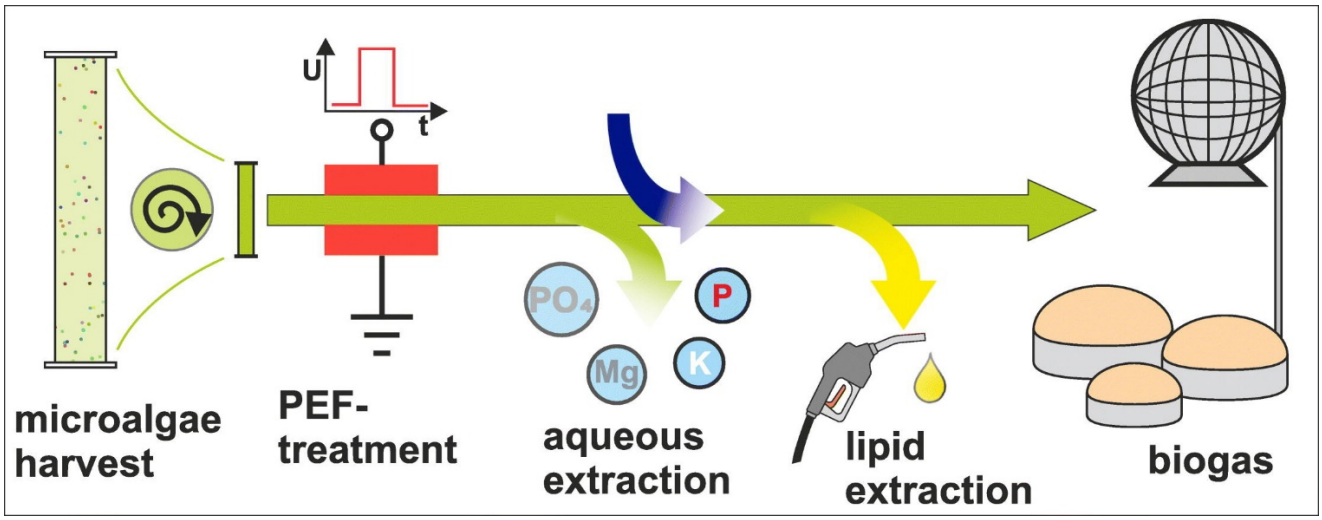
# Holistic exploitation of pulsed electric field (PEF)-treated and lipid extracted microalgae *Auxenochlorella protothecoides*, utilizing anaerobic digestion (AD)

**Graphical abstract**




**Abstract**

This study proposes a cascade processing of the lipid-rich microalgae *Auxenochlorella protothecoides*. Freshly harvested biomass is pre-concentrated to 100 g/kg$_{susp}$ and then pretreated using pulsed electric fields (PEF). PEF-treatment, which affects permeability of cells membrane, induces a spontaneous release of microalgal ingredients into the surrounding medium, mostly carbohydrates and micronutrients, which can be recovered. After separation of this aqueous fraction, lipid extraction is performed on the remaining wet pellet using an ethanol-hexane blend. After recovery of those different valuables, it was considered, to convert the rest biomass (cell envelope and most of the other remaining hard- or non-extractable components), to storable energy. Anaerobic digestion (AD) of the remains to biogas was considered to be a promising solution. AD-processes have the charm that unprocessed wet microalgal biomass, as well as wet organic biomass residues from previous extraction steps can be utilized directly without further purification or drying steps. The obtained biogas can then be upgraded to methane for an energetic use.

PEF-treated algal biomass showed a 10 % increased methane outcome (467 $mL_{norm}/g_{VS}$) compared to the untreated sample. PEF-treated microalgae, subjected to extraction of the aqueous fraction, demonstrated a 23 % increase in methane yield (558 $mL_{norm}/g_{VS}$), compared to control. Finally, the biomass, PEF-treated and subjected to extraction of water fraction and lipids, still achieved 41 % of methane potential (205 $mL_{norm}/g_{VS}$) during the AD process, compared to the untreated control.

Our study demonstrates the feasibility of cascade processing using PEF-treatment. Moreover, all the study was developed keeping in mind industrial requirements. In particular, our downstream approach did not require any drying, the pretreatment required low amount of energy, i.e. 1,5 $MJ/kg_{CDW}$ and the majority of the biomass was valorized. Since all techniques used, especially PEF-pretreatment, can be up-scaled, we believe this approach has great potential for implementation in industry.

**Keywords**

Microalgae exploitation, pulsed electric field (PEF)-treatment, lipid extraction, nutrient recycling, anaerobic digestion (AD), *Auxenochlorella protothecoides*.

## 1 Introduction

The usage of microalgae biomass for energetic utilization came into focus of industrial nations in late 20$^{th}$ century, as it became apparent that world crude oil resources will shorten in the medium-term. Situation might even get more critical since a permanent increase in oil demand is expected until 2040 and is presumed to peak at 106 mb/d (mega barrel per day) [1]. In this scenario the impact of the current COVID-19 pandemic and the Russian war of aggression against Ukraine on future energy requirements and energy supply, are not yet taken into consideration. In this context, calls for a more sustainable energy supply, are growing louder in the public and renewable energy resources are considered to have an important role to play. Considering the significant impact of fossil fuel combustion on climate, microalgae could contribute to the mitigation of greenhouse gas emissions as an alternative source for the production of biofuel. The main advantages are: the higher photosynthetic efficiency of 3-8 % compared to that of common crop plants (0.5-2 % [2]), the significant higher biomass yield and the

considerable higher $CO_2$-binding capacity which is 10-50 times larger than those of terrestrial plants [3]. An additional argument for the use of microalgae is the lack of competition with food- and feed crops, since no arable land is required for their growth. Since some microalgae species may store considerable quantities of lipids [4–9], which can be converted to biodiesel by transesterification, it was considered to exploit these organisms as a future biofuel resource. Research has shown that plain usage of lipids from microalgae for further processing into biofuels was not economically feasible at the current stage [10,11]. For that matter, biorefinery concepts, in which other microalgae ingredients such as proteins, carbohydrates, pigments, etc. are exploited, were investigated [12]. Promising applications, e.g. in the food-, feed- and pharmaceutical-industries were identified [13], which may significantly boost the valorization of whole microalgal biomass.

Numerous investigations regarding anaerobic digestion (AD) of algae in general and microalgae specifically, to produce methane, have been done since 1957 [14]. There was a significant revival of interest when it became evident that a biorefinery approach and therefore valorization of complete microalgae biomass was necessary to exploit microalgae in an economically viable way. After extracting all high value products, it was suggested to convert cell wall, cell membrane and most of the other remaining hard- or non-extractable components into storable energy. Therefore, anaerobic digestion of the remains from microalgae to biogas with help of appropriate microorganisms was considered to be a promising solution [15–28]. AD-processes entail the advantage that unprocessed wet microalgal biomass, as well as residual wet organic biomass from previous extraction steps, can be utilized directly without further purification or drying [29,30].

Microalgae are surrounded by a cell membrane, which is no restraint for decomposition by anaerobes used in AD, and by a cell wall, predominantly built up of cellulose, other polysaccharides and glycoproteins [28], which is much more recalcitrant to anaerobic degradation by microorganisms. For that reason, degradation or breaking down of long chain carbohydrates to carbon-based oligomers and finally monomers, is essential. Numerous pretreatment methods to support AD, mainly physical-, chemical- and biological methods and some of their combinations, were extensively documented [3,15,16,19,31–33]. Regarding **physical** methods, thermal pretreatment is the most popular one with either large temperature increases [34] or decreases [20] [35].Other physical methods that have been

tested, include **sonication** [21] [22], **microwave**-treatment [17] or **mechanical** means, such as French press [35], or bead milling [20]. The reported yields increased in a range between 20% and 200%, depending on the microalgae type and method, used. **Chemical** pretreatment of microalgae for AD by oxidizing-, alkaline-, acidic-agents, solvents and detergents is by far not as common as physical pretreatments, but a few studies nevertheless considered those type of preconditioning and demonstrated their beneficial impact with an increase of methane yield by up to 93% [23] [25]. Finally, another highly specific method to support AD, to cause damage to the cell wall and to hydrolyze microalgal biopolymers, is the **biological** pretreatment by individual enzymes or with enzyme blends [26] [27].

In general, it should be kept in mind that the impact, regarding biogas yield of a particular treatment method, even applied with identical parameters, might be strain-dependent. Indeed, cell structure, distribution and concentration of cellulose, hemicellulose, pectin, other polysaccharides and glycoproteins in the cell wall, are highly different from one species to another. The cell wall´s structure can be more complex and even the cellulose part is not always available for enzymes. In addition, lignin (or lignin-like algaenan) is not converted during AD [36–38]. All of these aspects cause significant differences in the response to a given treatment and therefore in the subsequent AD process [16,27,28,36].

**Pulsed electric field (PEF)**-treatment represents the application of electrical pulses on biological cells, which induces permeabilization of cell membranes [39–41]. It is already a well-established technology which is particularly efficient in extraction processes [42–45]. One of the main consequences of the PEF-treatment is a substantial release of intracellular ingredients [40,42,46,47] and an uptake of small molecules and ions into the cell [40,43,48]. In most studies focusing on microalgae, PEF was used as a pretreatment process for the exploitation of microalgal valuable constituents like lipids, proteins, polysaccharides, pigments, etc. [43,49,50]. Compared to other pretreatment methods, PEF technology was demonstrated to be particularly well suited for cascade processing since it preserves the overall structure of the microalgae which facilitates further downstream processing [51]. PEF-treatment is therefore well suited for integration in a biorefinery concept. Indeed, several demonstrations of cascade processing were already published, with extraction of water-soluble molecules followed by extraction of

lipids and finally hydrothermal liquefaction of the residual biomass [52]. The purpose of this work is to investigate the impact of PEF-treatment of *A. protothecoides* on subsequent AD. Biomethane potential (BMP) of fresh microalgae, of merely PEF-treated microalgae, of PEF-treated microalgae further processed with aqueous extraction and of microalgal residues obtained after PEF-assisted lipid extraction were compared, in order to evaluate the benefit of such a cascade processing.

Permeabilization of the cell´s membrane, induced by PEFs and subsequent transport of water soluble and digestible fractions outside the cell, are expected to improve the availability of fermentable material for the anaerobic microbial consortium and therefore, to enhance bio-methane production. It can be mentioned that in other investigations it was found that PEF-treatment of organic waste, e.g. landfill leachate enriched with bio-solids, vegetable scraps and fruit refuse, as well as waste sludges and effluents, could improve the AD process [53]. Similarly, it was shown that Focused Pulsed (FP)-treatment (an electric field-treatment method, derived from PEF-treatment) of waste activated sludge (WAS) and pig manure, could improve bio solid digestion and enhance methanogenesis [54] [55] [56].

## 2 Materials and Methods

### 2.1 Microalgae cultivation, harvesting and concentration

All experiments were performed with *A. protothecoides*, strain number 211-7a obtained from SAG, Culture Collection of Algae, Göttingen, Germany. To avoid long-term shifts of microalgal composition and cultivation behavior due to mutation, we ordered fresh *A. protothecoides* strain, twice a year, from SAG.

Autotrophic cultivation was performed, using *A. protothecoides* starter-cultures cultivated in Erlenmeyer flasks, mixotrophically in modified Wu medium for 5 days [43]. The starter culture was subsequently used as inoculant for our photobioreactor (PBR) of 25 L, filled with Tris–phosphate (TP) medium (recipe adapted from [57,58]; detailed composition given in [59]) supplemented with 40 µg/L Thiamine. The PBR is an annular bubble column reactor. It is made of a vertically installed cylindrical glass tube with a metal tube concentrically aligned inside, which serves for cooling. This arrangement is terminated at the top, by a metal disk, equipped with a number of sterile sealed throughputs for inoculation, sampling and measuring purposes. Another metal disk on the bottom of the glass tube, likewise sealed, allows

sterile harvesting. Preparation of the PBR consisted in autoclaving the reactor, filled with demineralized water and subsequently cooling down to the desired cultivation temperature and adding the sterile medium stock solution and inoculum. The quantity of inoculum was calculated in order to start the cultivation with an optical density (OD) of 0.1 (at $\lambda$ = 750 nm). The PBR was illuminated by LED (WU-M-500-840, 4000 K, Panasonic) with a Photosynthetic Photon Flux Density (PPFD) at the surface of the reactor of 200 µmol photons $m^{-2}$ $s^{-1}$ for the first 24 h and then 600 µmol photons $m^{-2}$ $s^{-1}$, until harvesting. The temperature was regulated at 25 °C and the $CO_2$ flow (3 % volume in a sterile airflow of 60 $Lh^{-1}$) was kept constant. The cultivation was daily monitored with OD measurement and microalgae were harvested after a period of approximately 21 days. Typical cell dry weight (CDW) one day before harvesting was between 4.5 and 5.5 $g/kg_{sus}$.

Prior to further biomass treatment, microalgae were concentrated by means of centrifugation [49], to obtain the desired CDW of 100 $g/kg_{sus}$. The exact final CDW was determined after the concentration-operation, immediately before further biomass-processing [43]. Average delay, from microalgae harvest to the start of following PEF-processing caused by concentration work of the microalgae, was roughly 2 h.

Detailed descriptions of OD measurement, microalgae harvest and successive pre-concentration procedure, are available in previous publications [43,49].

## 2.2 PEF processing

The samples were PEF-processed in a continuous flow treatment chamber comprising of plane-parallel arranged stainless steel-electrodes with 4 mm electrode gap, integrated in a polycarbonate housing. Treatment parameters were: magnitude of the electric field, E = 40 kV/cm, mass flux of concentrated microalgae suspension at CDW $\approx$ 100 $g/kg_{sus}$, $Q_{sus}$ = 0.1 $g_{sus}/s$, pulse repetition rate 3 Hz, pulse duration, $t_{Imp}$ = 1 µs and applied energy, $W_{spec}$ = 150 $kJ/kg_{sus}$. A more detailed description of the experimental setup and of the energy calculation can be found in our previous publications [42,43,49]. Control samples were just pumped through the treatment chamber, without application of PEFs.

## 2.3 Electric conductivity (K) measurements

Conductivity data of control- and PEF-treated samples were acquired without temperature compensation, using a WTW-conductivity meter, (Modell Cond-3310, WTW GmbH, Weilheim, Germany). Temperatures of investigated microalgae suspensions were systematically recorded for later calculation of temperature compensated conductivities. The equivalent conductivity at T = 25 °C, $K_{25}$ [µS/cm], was calculated using equation (1) where $\alpha_{25}$ is the temperature coefficient of variation at T = 25 °C [60]. The compensation coefficient $\alpha_{25}$ was experimentally determined by measuring conductivities of microalgae suspensions at various temperatures (data not shown) and had a value of 2.8% $°K^{-1}$.

$$K_{25} = \frac{K_T}{1 + \alpha_{25}(T - 25)} \qquad equation\ (1)$$

## 2.4 Total lipid determination

Total lipid extraction was performed on bead-milled (Mixer-Mill, MM400, Retsch, Haan, Germany) and freeze-dried (freeze dryer - Christ Alpha 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) microalgae, with a commercial Soxhlet apparatus (behrotest® Kompakt-Apparatur KEX 30 from Behr Labortechnik) using n-hexane, for at least 3 h, corresponding to at least 20 extraction cycles. For detailed description of crushing, freeze-drying and total lipid extraction of microalgae, the reader can refer to our previous publication [43].

## 2.5 Determination of carbohydrates

Determination of microalgal carbohydrate content was performed, using the Anthrone Sulfuric Acid assay. Fresh starch aqueous solutions with concentrations ranging from 0.02 g/L to 0.4 g/L were prepared from starch powder (Merck No. 1.01257). They were used as standards and processed like the samples. The freeze-dried biomass was resuspended in distilled water and diluted to a concentration ranging between 0.1 and 0.4 g/L. All samples were processed in triplicates. The anthrone reagent was prepared on the day of experiment, by dissolving anthrone (Merck No. 1.01468) in 95 % sulfuric acid (AnalaR NORMAPUR: VWR Chemicals No. 20700) at a final concentration of 0.1 % w/v.

The preparation was eagerly shaken by hand and kept on ice for at least 5 min. Then, 400 µL of diluted sample or standard were transferred into a 1.5 mL Eppendorf Safe Lock tube. Hereafter, 800 µL of anthrone reagent was added and homogenized by carefully inverting the sample solution a couple of times. After 5 min of incubation on ice, the mixed solution was transferred into a thermo-incubator (Thermoshaker with cooling for microtubes and microplates, Grant Instruments$^{TM}$ PCMT, Cambridge, United Kingdom), pre-heated to 95 °C, subsequently shaken at 300 rpm for 16 min and then cooled down on ice. Optical density of the cooled samples was measured at λ = 625 nm and carbohydrate concentration was calculated using the standard curve, considering the dilution factors.

### 2.6 Determination of total protein content

Total protein content of microalgal suspensions was determined in two ways, depending on availability of respective measuring capacity. For the first method, measurement was preceded by a chemical extraction process at high temperatures. For this, a volume of fresh, concentrated microalgae suspension, containing 5 mg of dry microalgal biomass, was resuspended in 2 mL sodium hydroxide 1 M and incubated at 95 °C for 1 h [61]. Afterwards, extraction suspension was centrifuged at 10000 g for 10 min and then, supernatant was processed for protein determination, following a modified Lowry assay [62] (DC™ Protein Assay, Bio-Rad Laboratories GmbH, Munich, Germany), using bovine serum albumin (BSA) as standard. For the second method, protein content was determined using the Merck Direct Detect infrared spectrometer (Direct Detect® Infrared Spectrometer, Merck KGaA, Darmstadt, Germany). For this, cell suspension was diluted to a microalgae-concentration in the range between 2.5 – 5.0 mg/mL and then, 2 µL of this suspension was measured directly with the Merck-device.

### 2.7 PEF assisted lipid extraction.

PEF assisted lipid extraction was either performed with small samples of 0,5 g of biomass, as in our previous publications, in order to check the general characteristics of biomass, or in larger scale, in order to provide enough biological substance for further AD. For small sample extraction, please refer to [43,49] for detailed method.

For large scale extraction, 280 mL (4 x 70 mL) of PEF-processed microalgae were centrifuged in a Sigma 8 k centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), with a swinging-

bucket rotor, at 3200 g, for 12 min. Supernatant was discarded and pellets transferred to four glass bottles with a nominal volume of 1 L each, mixed up with 380 mL ethanol and 154 mL n-hexane each and vigorously stirred for 21 h, for lipid extraction. Extracted microalgae/solvent-mixtures were transferred to four centrifuge bottles, with a nominal volume of 1 L each and centrifuged at 4400 g, for 12 min. Subsequently, 10 mL supernatant from each centrifuge bottle were pipetted to 50 mL-falcons, already filled with 5 mL demineralized water. Falcons were shaken vigorously by hand. Thereafter, 20 mL n-hexane was added into each falcon and violently shaken for 30 s in the Mixer-Mill (Retsch Mixer Mill MM400), mentioned above, with ensuing 10 min retention time, for phase separation. 15mL of the upper phase of the mixture were transferred to small glass bottles and hexane was evaporated by nitrogen flushing under the laboratory fume hood (Gefahrstoffarbeitsplatz GAP-Line mit Frischluftschleier, asecos GmbH, Gruendau, Germany). The weight of the remaining lipid was determined by analysis on scale Mettler AE 163 (Mettler-Toledo GmbH, Greifensee, Switzerland).

In order to eliminate any residues of organic solvent from the extracted biomass, the extracted microalgae pellets were then placed in a laboratory fume hood (Gefahrstoffarbeitsplatz GAP-Line mit Frischluftschleier, asecos GmbH, Gruendau, Germany) and subjected to a cautious, fan powered drying procedure, until constant weight was achieved, i.e. for approximately 2-3 hours. The obtained, dried microalgae remnants were collected from the centrifuge bottles, mixed together, pulverized manually by mortar, pestle and finally homogenized.

### 2.8 Determination of ion concentrations in the aqueous phase

Concentrations of cations and anions, present in extracellular media of concentrated microalgae samples were examined by ion chromatography with help of high-performance liquid chromatography (HPLC) and by optical emission spectrometry (OES), supported by an inductively coupled plasma (ICP).

Sample preparation: All samples were centrifuged at 10000 g for 10 min and then purified with syringe filters (LLG-Spritzenvorsatzfilter CA, cellulose acetate, haeberle No. 9.055 511 3203, haeberle Labortechnik GmbH & Co. KG, Lonsee-Ettlenschiess, Germany) with 0.20 µm pore size. Dilution was done with either highly purified water, $\kappa$ = 0.055 µS/cm (Millipore Gradient, Merck Millipore, Darmstadt,

Germany) or with according eluent, before introduction to the analyzer. Calibration of ion chromatographs was performed by means of external standards.

Cation determination (ammonium, sodium, potassium, magnesium und calcium) was implemented by ion chromatography (Ionenchromatograph 690, Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany), measuring range $meas_{range}$ = 1 - 50 mg/L. Eluent was 30 mL 0.1 mol/L $HNO_3$ + 100 mL acetone, in 1 L highly purified water ($\kappa$ = 0.055 µS/cm), operating at a flow of $Q_{eluate}$ = 1.0 mL/min (HPLC Compact Pumpe, BISCHOFF Analysentechnik u. -geraete GmbH, Leonberg, Germany). Prepared samples were injected into the autosampler (autosampler 838, Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany). Column was Metrosep C3 (Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany) working at T = 30 °C. Sensor was conductivity detector Metrohm 690 (Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany).

Anion determination (fluorine, chlorine, nitrogen dioxide, bromine, nitrogen trioxide, phosphate, sulfate) was done by Metrohm Suppressor 753, Interface 830 and Separationcenter 820 (Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany), measuring range $meas_{range}$ = 1 - 50 mg/L. Eluent was 381 mg $Na_2CO_3$, 337 mg $NaHCO_3$, 300 mL Acetonitril in 2 L highly purified water ($\kappa$ = 0.055 µS/cm), operating at a flow of $Q_{eluate}$ = 0.8 mL/min (HPLC Compact Pumpe, BISCHOFF Analysentechnik u. -geraete GmbH, Leonberg, Germany). Prepared samples were injected into the autosampler 838. Column was Metrosep Dual 2 (Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany), working at ambient temperature ($T_{ambient}$ = 22 - 25 °C). Sensor was conductivity detector Metrohm 690.

For a more precise detection of cations at lower concentrations in aqueous solutions, at $ion_{conc}$ < 1 mg/mL, ICP, together with OES was applied (Agilent 725 ICP-OES spectrometer, Agilent, Santa Clara, California, USA), with simultaneous Echelle spectrometer and radial plasma observation, measuring range 0.1 – 50 mg/L. Sample feeding: 1.3 mL/min. Preparation was the same as for cation/anion determination, described above. Plasma gas was argon, excitation frequency was 40 MHz at 2 kW power-input.

## 2.9 Total carbon (TC), total inorganic carbon (TIC), total organic carbon (TOC)-concentration-determination in the aqueous phase

Evaluation of carbon fractions in cultivation media of samples was performed by difference method, measuring TC and TIC (Dimatoc 2100, Dimatec, Essen, Germany) and subsequent calculation of the amount of TOC (see equation 2), according to TOC-analytics, described in DIN EN 1484 [63] and DIN EN 15936 [64]. Measuring ranges were $meas_{range\ 1}$ = 10 - 100 mg/L and $meas_{range\ 2}$ = 100 - 1.000 mg/L, carrier gas was oxygen, working at a flow of $Q_{oxygen}$ = 8.000 mL/h. Samples need to be filtered before measuring, to avoid particles in the system with diameters of 80 µm and bigger. To ensure this, all samples were purified in using syringe filters (LLG-Spritzenvorsatzfilter CA, see exact description above) with d = 0.20 µm pore size. Working principle was measurement of $CO_2$ in the infrared range, after thermo-catalytic oxidation of carbon in aqueous solutions. For quantification, Dimatec standards 1.000 ppm TOC and 1.000 ppm TIC has been applied, with potassium hydrogen phthalate as reference.

$$TOC = TC - TIC \qquad equation\ (2)$$

## 2.10 Evaluation of total nitrogen bound (TNb) in the aqueous phase

Determination of TNb (Dimatoc 2100, with connected TNb-module, Dimatec, Essen, Germany) in cultivation media of samples was done, according to TNb-analytics, described in DIN EN 12260 [65]. Measuring range of used instrument was $meas_{range}$ = 10 - 100 mg/L, working with oxygen as carrier gas at a flow of 8.000 mL/h. Samples need to be filtered before measuring, to avoid particles with diameters of 80 µm and larger, all examined samples were purified, using syringe filters with 0.20 µm pore size (LLG-Spritzenvorsatzfilter CA, see exact description above). Working principle was detection of $NO_2$ in the VIS spectrum (chemiluminescence), after thermo-catalytic oxidation of nitrogen in aqueous solutions. For quantification, Dimatec standard 1.000 ppm was applied, with potassium nitrate/ammonium sulfate (stabilized) as reference.

## 2.11 Determination of carbon (C)-, hydrogen (H)-, nitrogen (N)- and sulfur (S)- fractions in the solid phase

For determination of C-, H-, N- and S- fractions, concentrated microalgal biomass was pre-frozen in the freezer at -22 °C (Liebherr, model GN 5215, Liebherr-International Deutschland GmbH, Biberach an der Riss, Germany) and then lyophilized for 24 h (freeze dryer - Christ Alpha 1-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at reduced pressure of $p_{absolute}$ = 63 mbar (vacuum pump - Leybold Trivac D4B, Leybold GmbH, Köln, Germany) and thereafter evacuated and shrink-wrapped (la.va V.300, Landig + Lava GmbH & Co. KG, Bad Saulgau, Germany) and stored in freezer at T = -22 °C, until later evaluation. For C-, H-, N- and S-value determination, three identical samples, with $m_{sample}$ = 4 - 6 mg each, for every evaluated parameter-set, were weighed and subsequently tightly wrapped in tin-foil, before transfer to the elemental analyzer (vario EL cube, Elementar Analysensysteme GmbH, Langenselbold, Germany). Samples were burned in the elemental analyzer at T = 1150 °C. Determination of present elements C, H, N and S, in m-% of investigated sample-mass, was performed by inspection of the infrared spectrum of exhaust gas phase from the incinerator, with sulphanilic acid as reference.

## 2.12 Determination of calcium (Ca)-, magnesium (Mg)-, phosphorus (P)-, sodium (Na)- and potassium (K)- fractions in the solid phase

For investigation of Ca-, Mg-, Na-, K- and P-fractions in the solid microalgal phase, samples need to experience a preceding acid digestion with $HNO_3$(65 %), HCl(30 %), HF(40 %) and $H_2O_2$(35 %), with purity grade Merck Suprapur (Merck Nos. 100441-$HNO_3$, 101514-HCL, 100335-HF, 107298-$H_2O_2$). Treatment was done in Teflon pressure-vessels, in the lab-microwave (Multiwave 3000, both: Anton Paar GmbH, A-8054 Graz, Austria). The acidic solution was analyzed in an ICP-OES spectrometer (Agilent 725 ICP-OES, as described in detail, above in section ´Determination of ion concentrations in the aqueous phase´).

## 2.13 Sample shipping

Control and PEF treated microalgae suspensions as well as the dried fraction from lipid extracted microalgae, were sent to the partner laboratory, The Helmholtz-Centre for Environmental Research

GmbH – UFZ in Leipzig-Halle, Germany, for anaerobic digestion (AD), on the day following PEF-treatment i.e. once the lipid-extracted sample was ready. Before shipment, other samples than lipid extracted ones, were stored in the fridge at T= 7 °C. For express-shipment, samples were first wrapped up in bubble wrap and then tightly packed into a styrofoam box with 50 mm wall thickness, for thermal isolation, together with a pre-cooled collection of ice-packs. The styrofoam-box with the microalgae inside was additionally packed inside a card board and shipped by DHL express delivery service, with a 19 h delivery period from door to door. The temperature inside the styrofoam box was $T_{box}$ = 11 °C on time of arrival. Samples were stored in the fridge, at 7 °C, in our partner laboratory, the UFZ in Leipzig upon arrival, during the incubation period of three weeks before final AD.

### 2.14 Preparation and AD of microalgal biomass

The total solid (TS) and volatile solid (VS) content of the microalgae preparations were determined gravimetrically by heating and drying the samples at 105 °C (Binder oven, Germany) for 24 h followed by burning the organic content at 550 °C (P300 Nabertherm furnace, Germany) for 6 h. Degassed digestate from a large-scale biogas plant operating with maize silage and cattle manure as a main substrate was used as inoculum for the batch experiments. The biochemical methane potential (BMP) of various microalgae preparations was determined according to VDI 4630 (2016) [66] using an Automatic Methane Potential Test System II (Bioprocess Control, Sweden) under mesophilic temperature (38 ± 1 °C) during 38 days. The experiments were conducted under anaerobic conditions by initially flushing the batch reactors with nitrogen. To minimize potential inhibitory effect of the substrate, the ratio of substrate:inoculum (based on VS) was set between 0.36 and 0.38 to include much higher amount of inoculum compared to substrate. The microalgae preparations were added as well vortexed suspensions, while lipid-extracted biomass was added as dried powder.

### 2.15 Experimental planning for different microalgae sample preparations used in anaerobic digestion and details of the process

The objective of the study was to evaluate biomethane production either from complete biomass or, as in a typical bio refinery process, from residual biomass remaining after extraction of valuable

components. In particular, it was tested how cascade processing, using PEF-pretreatment method prior to extraction, could influence the biomethane production. Four different biomass samples were therefore examined, with respect to their potential impact on biomethane production (see Figure 1):

(1) Untreated microalgae, just pumped through the treatment chamber, without application of PEFs, hereafter denoted as control.
(2) PEF-processed microalgae.
(3) Rest biomass obtained after applying PEF-treatment on the microalgae and proceeding with an aqueous extraction. For the extraction, the extracellular medium (containing aqueous extract) was simply removed and replaced by deionized water, in the course of 2.5 hours after PEF-treatment.
(4) Rest biomass obtained after applying PEF-treatment on the microalgae, removing the aqueous fraction and proceeding with a solvent extraction of the lipids.

Microalgae samples (1) and (2) were stored in the fridge, at T = 9 °C, immediately after concentration step and pumping for control or PEF-treatment for sample (2), respectively, without any additional modification, until AD.

In sample (3), in the course of an aqueous extraction, extracellular medium was removed by centrifugation and replaced by deionized water in two different ways, before storage in the fridge, at T = 9 °C:

(3) $_{T0}$): extracellular medium replaced directly after PEF-treatment, without delay and

(3) $_{T150}$): extracellular medium replaced after a 150 min incubation period after the PEF-treatment, meanwhile kept at room temperature (T = 22-24 °C)

Sample (4) was submitted to lipid extraction, 2 h after PEF-treatment. After the extraction, the residual microalgal biomass was dried fan-forced, at room temperature, to remove any traces of volatile organic components from the biomass. This is essential, since remaining organic solvent compounds might inhibit anaerobic microorganisms in the subsequent AD-process and reduce biomethane yield, as known from literature [67–70]. The resulting dry microalgal biomass was homogenized by hand, with help of a ceramic lab-mortar and conclusively sent to our partner laboratory, the Helmholtz-Centre for

Environmental Research GmbH – UFZ in Leipzig-Halle, Germany, for anaerobic digestion (AD), in the next treatment step.

Since in a cascade process, biomass cannot always be processed directly in one go, we decided to evaluate the impact of a time lag on microalgal biomass evolution and subsequent AD, and we therefore inserted an arbitrary three-week storage period after the PEF-treatment of microalgae and before the subsequent anaerobic digestion.

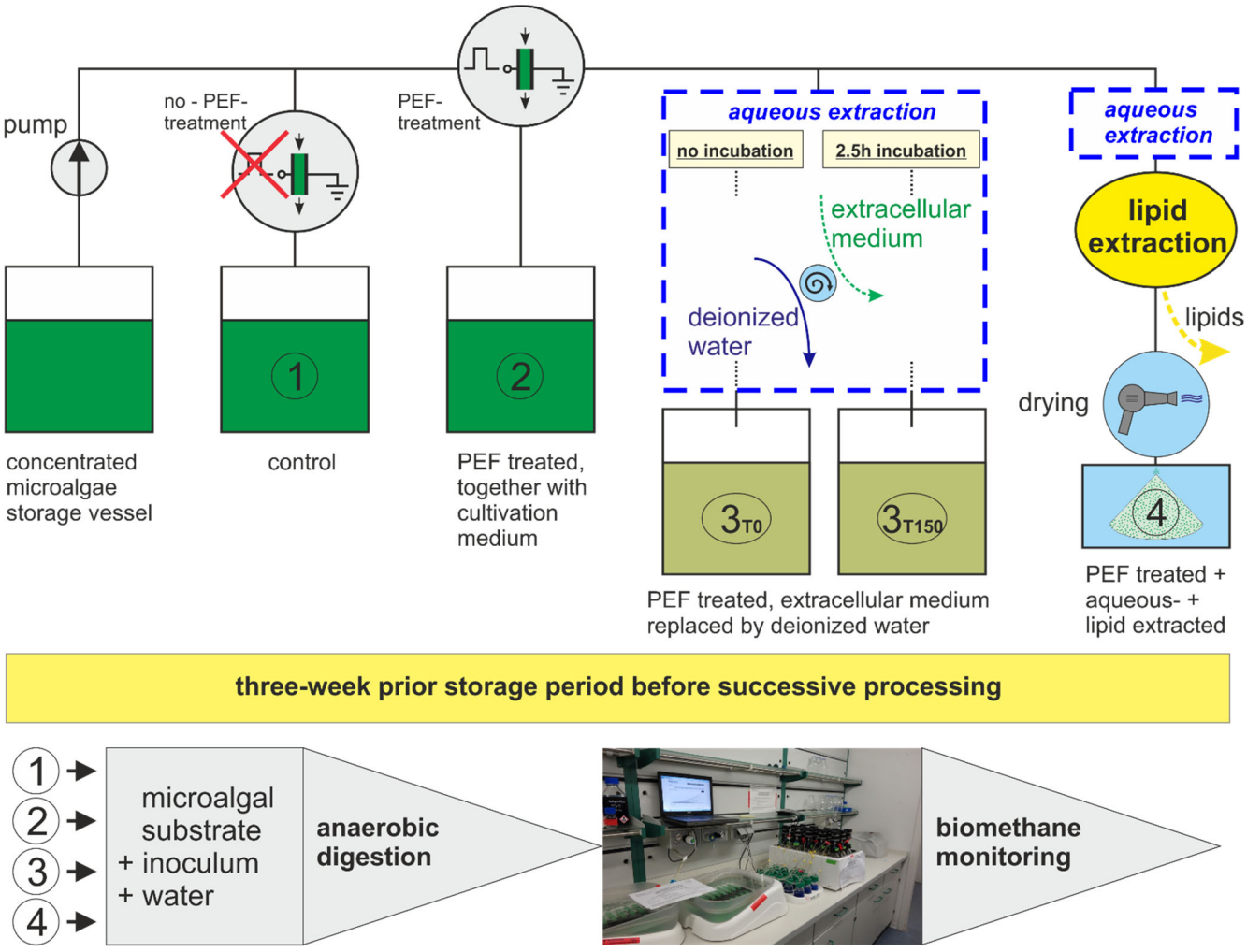



*Figure 1: Description of different microalgae sample preparations and detailed process flow.*

## 2.16 Statistical Analysis

Results reported in the manuscript were obtained from n independent experiments (n = 2 to 6) with internal duplicates, triplicates or quadruplicates, depending on the respective experiment. Multiple values of replicates were averaged for one output value per experiment. These output values were used to calculate averages and standard deviations (SD) on n samples displayed in the graphs.

Statistical significance was evaluated using the unpaired t-test. P-values greater than 0.05 are rated as not significant. P-values less than 0.05 are indicated with (✶), less than 0.01 with (✶✶) and less than 0.001, with (✶✶✶). Relationships between groups which are evaluated as not significant, are not shown. This improved readability and fosters the evaluation of the statistical analysis.

## 3 Results and discussion

### 3.1 Harvesting of microalgae and determination of basic composition

Microalgae *A. protothecoides* were harvested from the 25 L photobioreactor (PBR). The CDW was 5.82 g/$kg_{sus}$ ±0.39. Following the harvest, the microalgae were systematically concentrated. The average CDW obtained was 103.5 ±8.1 g/$kg_{sus}$. This concentration step makes subsequent extraction processes more economic [42]. The average composition of the biomass on harvest day is given in Table 1. Due to a very good reproducible cultivation procedure, concerning the technical aspects of cultivation (cultivation media, lighting, pH- and temperature-control), the composition of microalgal biomass varies within narrow bounds. The biomass contains a high amount of lipids (39.8 ±0.9%), and of carbohydrates (23.9 ±6.2%), while the amount of proteins is much lower (9.1 ±1.6%). The percentage of ashes is as expected very low(1.4 ±0.5%).

Note that the addition of the various estimated components (i.e. lipids, proteins, carbohydrates and ashes) do not sum up to 100 % but only to 74,2 % of the total biomass. The missing fraction is suspected to belong largely to the cell wall, since some molecules withstands all type of hydrolysis [26,36–38,71]. This was however not investigated in this study.

| component | lipids [m-% CDW] | proteins [m-% CDW] | carbohydrates [m-% CDW] | minerals/ashes [m-% CDW] |
|---|---|---|---|---|
| mean | 39.8 | 9.1 | 23.9 | 1.4 |
| SD | 0.9 | 1.6 | 6.2 | 0.5 |

*Table 1: composition of microalgae A. protothecoides harvested from 25L-PBR. Numbers are as mean and standard deviation, obtained from three independent cultivations.*

### 3.2 Effect of PEF-treatment

The concentrated microalgae suspensions were treated with rectangular shaped PEFs, at a field intensity of E = 40 kV/cm, a pulse duration of $t_{pulse}$ = 1 µs, a repetition rate $f_{rep}$ = 3 Hz, with an energy of $W_{PEF}$ = 150 kJ/$kg_{sus}$ i.e. 1.5 MJ per kg dry cell mass (DCM). An additional control suspension was pumped through the treatment cell, without being exposed to PEF-treatment.

#### 3.2.1 PEF-impact on electric conductivity

Evaluation of efficiency of the PEF-treatment was assessed in first approximation by measuring the electric conductivity-changes induced by the treatment. Conductivity (K) of the control sample i.e. sample simply pumped through the device, was stable over time (K~1070 µS/cm, data not shown). This behavior is confirmed by earlier publications from our group, throughout an observation period of 150 min and beyond [42,72]. Therefore, no liberation of microalgal ingredients is expected to be induced simply by the pumping process.

The evolution of the conductivity of the PEF treated sample normalized to $T_{sus}$ = 25 °C as well as the temperature of the suspension, are displayed on Figure 2. With the beginning of PEF-treatment, an instant rise of conductivity was measured and the conductivity almost doubled, reaching K = 2122 µS/cm. This is a well-known phenomenon, which is due to the fact that PEF-treatment permeabilizes the external membrane of cells and therefore enables a spontaneous release of ions and small charged molecules. During the following hours, further microalgal intracellular ingredients are liberated, but to a much smaller extent than in the initial phase. For that reason, the conductivity still rises very slowly and converges to approximately K = 2290 µS/cm. Those data therefore confirm the efficiency of the PEF-treatment i.e. the permeabilization of the cells membrane and therefore release of intracellular ions and charged molecules. In the same time, the temperature rises immediatly after PEF-treatment from 23 °C to 33 °C due to ohmic heating. Temperature then decreases steadily, attaining a final level of T = 24 °C after 150 min i.e. going back to the initial temperature.

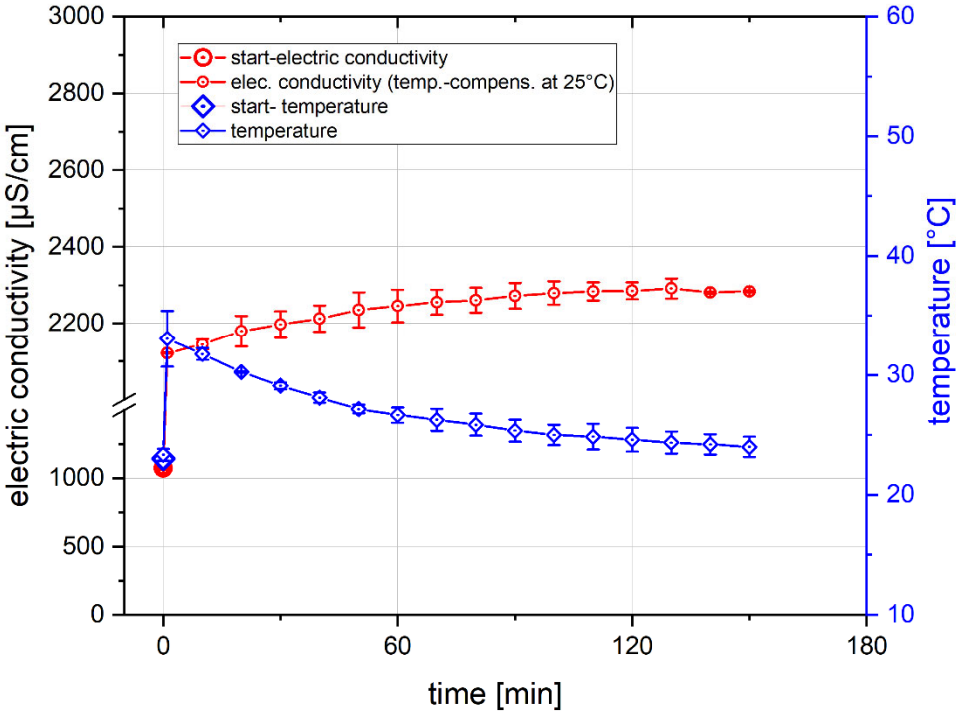



*Figure 2: Evolution of temperature (blue diamonds) and conductivity (red circles) after PEF-treatment, obtained from two independent experiments. PEF-treatment was applied at time t = 0. The graph displays the conductivity compensated to the reference temperature of T = 25 °C.*

### 3.2.2 PEF-impact on microalgal lipid extraction

The total lipid content of the concentrated microalgae, used in the two experiments with larger extraction volumes ($V_{conc.\ microalgae}$ ~ 300 mL), was 43,3 ±2.0% of CDW. In order to perform lipid extraction, the concentrated microalgae suspension was centrifuged directly after PEF-treatment in order to separate the microalgae pellet from the supernatant, which was discarded. The microalgae pellet was resuspended in an organic solvent mixture, comprising of ethanol and n-hexane as detailed in the material and methods-section. Results of the PEF-supported lipid extraction are presented in Figure 3. As can be seen, extraction of lipids from untreated samples (control) is unsuccessful and only 6.2 ±0.9 % of the available lipids (~ 2.4 ±0.9 % of CDW) were extracted. When extraction is performed on PEF- treated samples, extraction rate is much higher although some variations are observed depending whether the experiment is performed on small or large volumes despite, the fact that the

biomass : solvent- ratio was always kept constant (see material and methods). Indeed, when the standard protocol of our laboratory is used, i.e. extraction is performed in small Teflon tubes containing not more than 5 mL of concentrated biomass, approximately 98 % of the total lipids (~ 38.5 ±0.2 % of CDW) were extracted [43,72]. However, in the two lipid extraction experiments that were performed at larger scale ($V_{conc.\ microalgae}$ ~ 300 mL) for the need of anaerobic digestion, the lipid yields were slightly lower with extraction of only 59.5 % (~ 27.0 ±6.1 % of CDW) and 78.7 % (~ 32.3 ±2.3 % of CDW) of the total available lipids. It is supposed that the reduced lipid extraction efficiency with larger microalgae volumes is owed to the upscaling step. The volume of concentrated microalgae in the conducted experiments was approx. 60 times larger than the volumes, normally used for lipid extraction on small laboratory-scale. There has been no attempt to optimize the lipid extraction procedure with regard to larger extraction volumes, as it was not an objective of this investigation. Improvement of several technical issues such as mixing procedure during the extraction therefore might have the potential for a noticeable increase of extraction efficiency. Nevertheless, the samples were further processed by anaerobic digestion since a total lipid extraction was not required for such an experiment. A fatty acid methyl ester (FAME)-profile of the used microalgae is available in earlier publications [43,72].

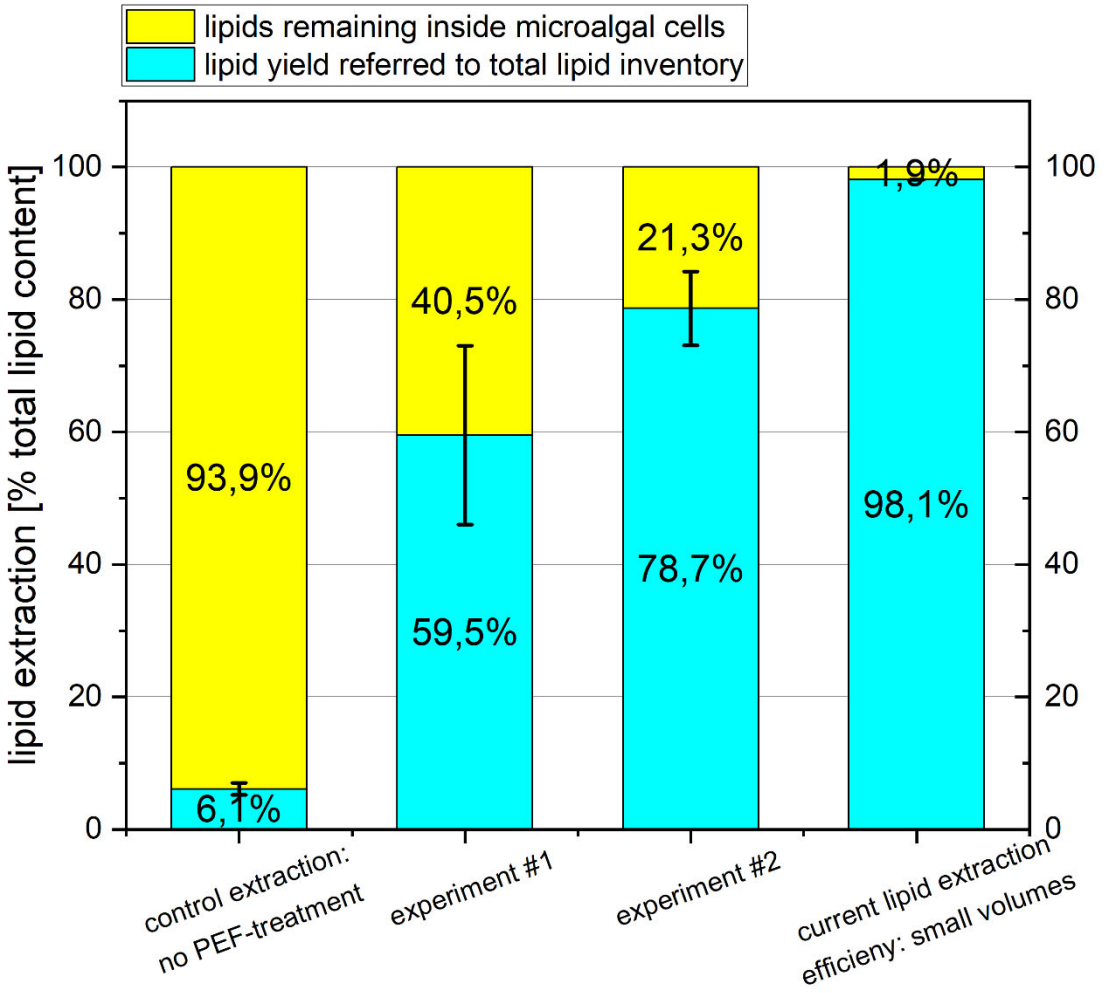



*Figure 3: Extraction of lipids (cyan) related to total lipid content of microalgae and lipids, remaining inside the microalgal cells after lipid extraction (yellow). Control extractions of lipids (w/o PEF-treatment, left column), were performed with small volumes (5 mL) and the results are the average of 3 independent experiments with internal duplicates. PEF-assisted lipid extractions conducted with large volumes (approx. 300 mL) as precursor for successive AD was performed twice in 2 independent experiments with internal quadruplicates and are shown separately (two columns located in the center). Right column represents the standard lipid extraction protocol of our laboratory for small volumes (5 mL), obtained from 3 independent experiments, with internal duplicates.*

## 3.3 Characterization of different microalgae sample preparations as precursors for successive AD

### 3.3.1 Characterization of carbon content and distribution

Carbon is one of the main building blocks for the synthesis of methane ($CH_4$). Using microalgae suspension as substrate for anaerobic digestion, carbon is present inside microalgal cells (solid phase) and in the extracellular medium (liquid phase). These two fractions are different regarding carbon

content and accessibility. The easy access to the organic carbon dissolved in the extracellular medium is granted, due to the lack of shielding by cell membrane and cell wall. Carbon inside microalgal cells is not instantly available for anaerobic microorganisms, because two barriers need to be overcome, cell wall and cell membrane. Additionally, despite the fact that it contains big amounts of carbon, the cell wall of many green microalgae is hard to penetrate and to hydrolyze for anaerobic microorganisms. This is due to the composition of the cell wall, comprising mainly of cellulose, hemicellulose, pectin, other polysaccharides and glycoproteins; therefore it is rather recalcitrant against hydrolysis in the first step of AD according to previous studies with *Chlorella sp.* and other green microalgae [19,25,36,71,73]. The cell membrane, as second barrier, is comparatively easy to penetrate and to hydrolyze.

Figure 4 show the carbon content of microalgae (on the left) and of extracellular medium (on the right) for the different samples. It can be seen that PEF-treatment induces a release of almost 10 % of organic carbon from microalgal cells compared to control with a carbon content in the microalgae of 59.1 g/L in the control, going down to 54.9 g/L in the PEF-treated sample (Figure 4, left). In the course of the incubation period of three weeks (3 wks) after the day of experiment (DOE), all microalgae samples began to hydrolyze. This induced a further reduction of the carbon content inside the microalgal cells (Figure 4, left) and therefore an increase of the carbon content in the extracellular medium (Figure 4 on the right). This effect is more visible on PEF treated samples (carbon content decreasing from 54.9 g/L down to 51.6 g/L) than on the control samples (carbon content decreasing from 59.1 g/L down to 58.4 g/L). The last sample on the graph, identified with number 3, corresponds to a sample submitted to PEF-treatment and to a subsequent aqueous extraction. The aqueous extraction consisted in leaving the microalgae suspension for 2.5h after the PEF-treatment and then subsequently replacing the extracellular medium containing aqueous extracts by demineralized water (see materials and methods). In these samples, the amount of available organic carbon and other microalgal ingredients, is lower than with the other sample preparations, as part of the released components are stripped off by the aqueous extraction. For this reason, organic carbon content of microalgae was reduced to 51.9 g/L on DOE and furthermore to 51.2 g/L after the 3 wks incubation period (Figure 4, left).

In the extracellular medium, presented in Figure 4, right, the control sample supplies easily accessible TOC of 1.5 g/L on DOE and 2.2 g/L after the 3 wks incubation period. The PEF-treated sample (2) reveals larger amount of available organic carbon on the day of the experiment (5.7 g/L) and after the incubation period of three weeks (9.0 g/L). Sample (3), with no surprise, has less carbon available in the extracellular medium, since it underwent an aqueous extraction. This means that the medium, containing most of the released organic carbon, was removed after the aqueous extraction period and replaced by demineralized water (1.6 and 3.6 g/L organic carbon before and after 3 wks incubation period).

Overall, PEF-treatment contributes to an effective liberation of available microalgal carbon, i.e. 7.1 % (4.2 g/L) of organic carbon could be released by PEF-treatment, with regard to the control on the DOE. This value was even boosted by the three-week incubation period, which induces a further release of 6.0 % (3.3 g/L) of organic carbon in relation to the carbon content of the PEF-treated sample on DOE. Located inside microalgal cells and in the cell wall, is a much bigger carbon reservoir, amounting between 50 and 60 g/L (Figure 4, left), with delayed access and even partially inaccessible for microorganisms during a subsequent AD.

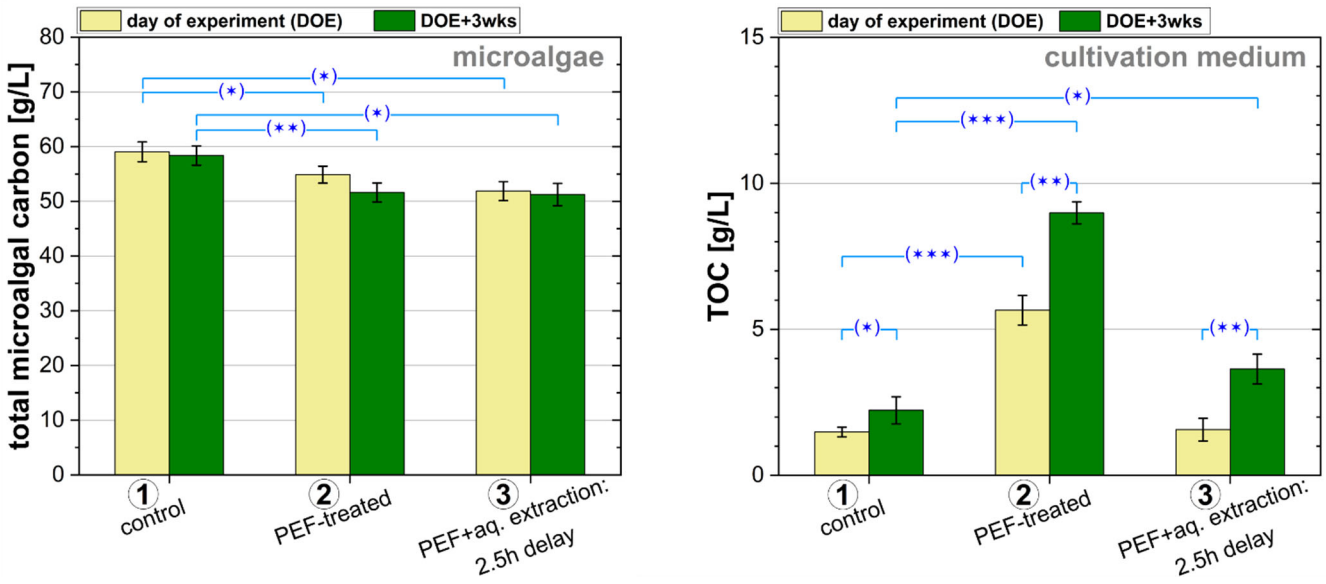



*Figure 4: Total microalgal carbon content of pure microalgae fraction (solid phase), on the left and TOC, present in cultivation medium (liquid phase), on the right, both on day of experiment (DOE) and DOE + 3 wks of incubation time. Values determined in at least 3 independent experiments, performed in triplicate, each. Statistical significances are indicated, compared to control and among each other, for samples with same treatment parameters but 3 wks incubation time in between.*

### 3.3.2 Characterization of carbon to nitrogen ratio

Carbon and nitrogen found in cultivation media represent approx. 10 % of the total carbon and nitrogen of the concentrated microalgal biomass (concentrated microalgal cells + extracellular medium). Most of the C- and N-fraction is indeed located in microalgal cells (structural components and membranes). An appropriate C:N-ratio is essential to provide a sufficient amount of building blocks for biomethane-generation (carbon) and for reproduction of anaerobic consortium (e.g. nitrogen) [74–77]. A too high nitrogen-fraction needs to be prevented, as it could lead to the formation of ammonia, which may results in inhibition or even breakdown of the AD-process[19,78,79]. No precise singular value can be given as an absolute optimal C:N-ratio, and the most suitable conditions will depend on the used substrate, the anaerobic consortium, the digester type, pH and temperature, etc. Favorable C:N-ratios mentioned in the literature are between 16 and 35. However, preferred ranges are C:N = 20-35 (shaded areas in Figure 5).

Figure 5, left represents the C:N-ratio in the solid fraction of the samples. The untreated *A. protothecoides (1)*, used in this investigation, exhibits a C:N-ratio, of about 27, i.e. perfectly located in the desired area between 20 and 35 (Figure 5, left). This is also the case for PEF treated microalgae (2) (C:N = 28.4) and samples submitted to aqueous extraction after 2.5 h (3) (C:N = 32.1), respectively. Note that this ratios are much more favorable than the ones typically found in the literature for other microalgae strains, with values typically around C:N = 10 [11,19,80]. The reason for this beneficial C:N-ratios might be the high lipid content and therefore low protein content of the investigated microalgae. This assumption is supported by the fact that lipid extracted samples (4) show considerably reduced C:N-ratios and fall below the mentioned optimal range, with a C:N-value of 15.9 (Figure 5, left).

C:N-ratios in the cultivation medium (liquid phase) are lower than observed in the solid phase, typically in the region C:N = 5-14 (Figure 5, right) and might affect the AD-process in the initial phase. Indeed, the substances in the cultivation medium are instantly available without prior hydrolyzation of cell wall and cell membrane. The found C:N-ratios are in principle too low for an efficient biomethane generation. The control sample shows with C:N = 5.0 the lowest value. Note that PEF-treatment of microalgae result in doubling of the C:N-ratio in the liquid phase, compared to control (Figure 5, right). Subsequent

aqueous extraction creates a minor improvement and C:N ratio reaches 10.6. Mind that 3 weeks incubation time, additionally increases C:N-ratios by 22-33 %.

The data on the distinct C:N ratios in the biomass and in the medium are of interest to better understand the composition of samples. Nevertheless, microalgal cells and cultivation medium, both containing organic components, are subjected to the anaerobic microorganisms simultaneously and contribute both to methane yield. Different components, located in different compartments reveal their influence only within a limited period of time; e.g. in the initial phase of AD in case of components in the cultivation medium. For that reason, it is reasonable to assume, the less favorable C:N-ratios of the cultivation medium have no adverse effect on the final AD-results.

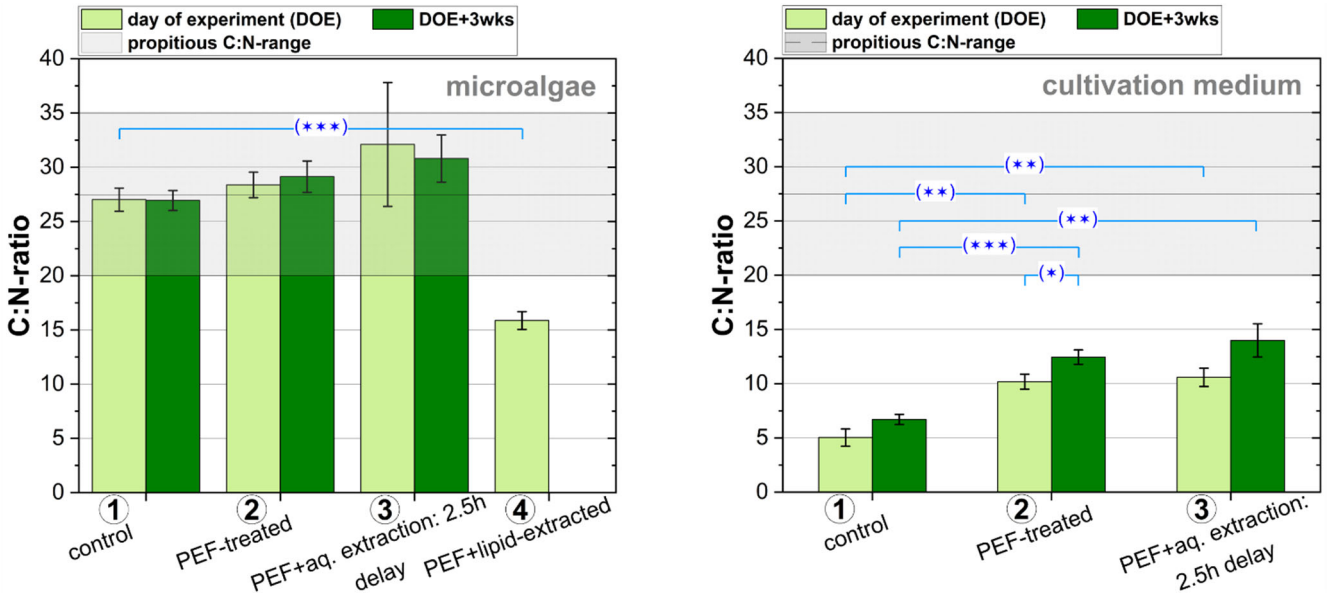



*Figure 5: C:N-ratios of pure microalgae fractions (solid phase), on the left, and present in cultivation media (liquid phase), on the right, both on day of experiment (DOE) and DOE + 3 wks of incubation time. Values obtained from 3 independent experiments, with internal duplicates. Statistical significances are indicated, compared to control and among each other, for samples with same treatment parameters but 3 wks incubation time in between.*

### 3.3.3 Release of micronutrients ($PO_4$, $SO_4$, $NH_4$, P, S, K, Mg, Ca)

Beside basic components for synthesis of biomethane (carbon, nitrogen, hydrogen, etc.), microalgae and their cultivation medium contain a wide range of molecules and ions. Most of these micronutrients are essential for the growth of microalgal biomass. Furthermore, some of those ingredients may influence the growth of anaerobic microorganisms involved in the AD-process and thus the generation

of biomethane itself. The available concentrations of the individual micronutrients need to meet the requirements of the anaerobic microorganisms as well, to avoid inhibition of the AD-process. Since PEF-treatment used in this investigation induces a release of microalgal ingredients, this issue needs a closer inspection, regarding its possible impact on the anaerobic consortium. An excerpt of the released intracellular components is presented in Figure 6, left (on day of experiment (DOE) and Figure 6, right (DOE +3 weeks incubation time). As expected, PEF-treatment induced a considerable increase of the concentration of all micronutrients in the extracellular medium. For example, the concentration of phosphates in the control is only 40 mg/L and grows to 529 mg/L immediately after PEF-treatment. Similarly, the concentration of phosphorus starts in the control sample at 1 mg/L, reaching 501 mg/L after PEF-treatment. Finally, the potassium concentration in the control was 39 mg/L, which increased to 531 mg/L after PEF-treatment. Similar behavior can be found for all the micronutrients that were measured as can be seen in more detail in both diagrams of Figure 6.

Regarding the samples that underwent aqueous extraction, it can be seen that significant amounts of micronutrients, extracted during the 2.5 h aqueous extraction time, were removed together with the cultivation medium (see Figure 6, both graphs), as they were replaced by deionized water. For example, phosphates are reduced by 330 mg/L (62 %), phosphorus by 390 mg/L (78 %) and potassium by 224 mg/L (61 %), due to the aqueous extraction. Values in brackets represent the quantity of removed substance in relation to total available substance after PEF-treatment and before aqueous extraction.

Figure 6, on the right, shows that 3 weeks incubation time results in an additional, in part considerable increase of micronutrients in most cultivation media of the tested sample preparations. Phosphates e.g. peaks at 2058 mg/L, phosphorus finishes at 768 mg/L and potassium was 606 mg/L after the 3 weeks incubation period. A reason for the latter could be decomposition of the microalgal structures (cell wall, membranes and inner microalgal organelles) by endogenous enzymes. This process is referred to as autolysis and is described for different yeasts [81–83] and *Saccharomyces cerevisiae*, specifically [84]. In this context, membranes, permeabilized by PEF-treatment, could foster the release of still active enzymes, initiate and even accelerate autolysis of microalgae.

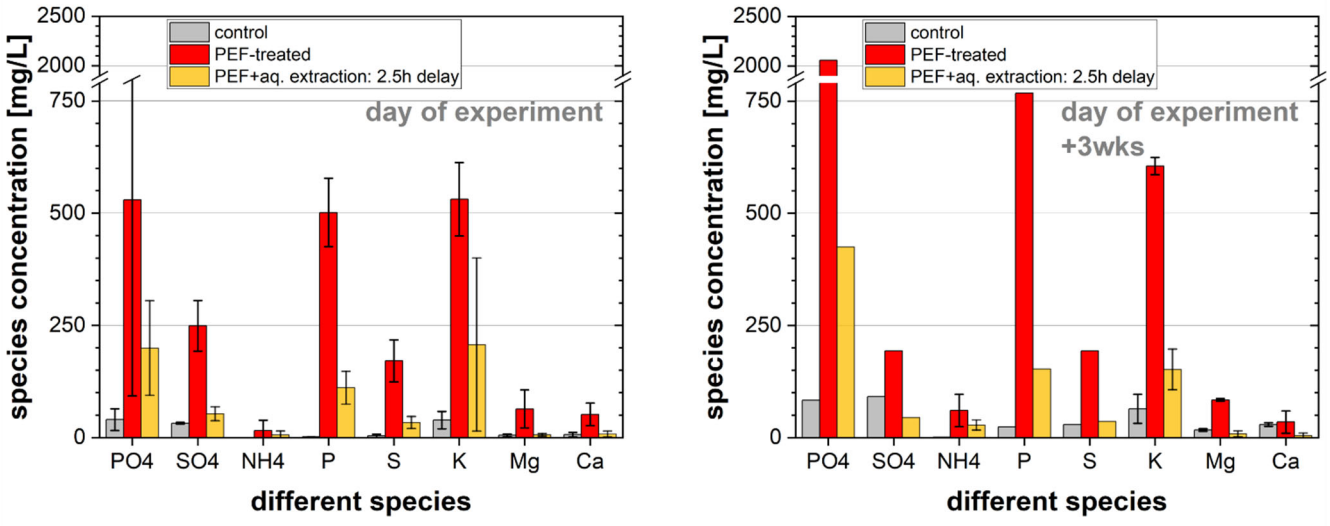



*Figure 6: Micronutrient concentrations in extracellular medium (liquid phase), on day of experiment (DOE), on the left and on DOE + 3 wks of incubation, on the right. Values obtained from 6 experiments, performed in triplicate, with 1 to 5 samples considered for statistic purposes, in respective categories.*

### 3.4 Biomethane yields by anaerobic digestion (AD) of different microalgae preparations

Biomethane yields from two different experiments, performed in duplicate are plotted in Figure 7 and Figure 8. Contributions caused by the inoculum were subtracted from the measured gross biomethane volumes, so that only net biomethane values are presented in the respective diagrams. We can first notice that in the **second experiment** (Figure 8), methane production of all investigated sample preparations started significantly earlier than in first experiment. Control sample in first experiment met the specific methane yield level of 350 $mL_{norm}/g_{VS}$, after 6 days, while it took only 4 days in the second experiment to achieve that. A specific methane yield of 400 $mL_{norm}/g_{VS}$ was achieved after 6 days, with PEF treated samples in experiment 2, while the same level was attained on day 9 in experiment 1. Apart from this overall shift, both experiments behave in a very similar manner.

In the **first experiment** (Figure 7) control and PEF-treated samples converged in their methane yield after 28 d of AD to a level of roughly 470 $mL_{norm}/g_{VS}$, with different dynamics at the beginning. Within the first week of AD the control sample produced methane faster, at a higher level (398 $mL_{norm}/g_{VS}$),

compared to the PEF-treated samples (154 $mL_{norm}/g_{VS}$). Control- and PEF-treated samples of second experiment showed a similar behavior in the initial phase of AD. For example, on the fourth day, specific methane yield of control was on a level of 362 $mL_{norm}/g_{VS}$, while the PEF-treated sample remained at 183 $mL_{norm}/g_{VS}$. PEF-treated samples also had a lag period, compared to the control, but it was substantially shorter than those in the first experiment. Our hypothesis is that the observed delay is due to the liberation of some inhibitors induced by PEF-treatment. The high phosphate- and phosphorus-concentrations in the microalgae medium after PEF-treatment (Figure 6, right), which are immediately available for the anaerobic consortium, could be a reason, for an initially delayed methane production. Indeed, there are hints that phosphate concentrations from 20 mM, which resembles the phosphate concentration of the PEF-treated sample preparations in our experiments, could inhibit methanogenesis [85]. However, considering the dilution by the inoculum, the concentration of phosphate in the digester is considerably lower than the inhibitory level cited in the literature. LCFAs are plausible candidates as inhibitors liberated by PEF-treatment since it is known that they inhibit AD, but can also contribute to the methane production once they are degraded by β-oxidation, a slow degradation process [86], which works much slower than hydrolyzation [86]. This would therefore explain the delay between the control and the PEF-treated sample and at the same time it would explain why both samples have similar final values of the methane yields.

Directly after PEF-treatment, a selection of samples was subjected to an aqueous extraction, in two different ways. The concept was to examine the impact of different aqueous extraction periods ($t_{extract}$ = 0 min and $t_{extract}$ = 150 min) and the responding release of different amounts of algal ingredients, on AD. These aqueous extracted samples, demonstrated highest methane yields in all conducted experiments, reaching roughly 550 $mL_{norm}/g_{VS}$ after 38 d of AD, in both experiments. In first experiment, both samples, aqueous extracted at different extraction periods, showed similar, in part even identical methane yield progression. That suggests, there are no significant differences in the composition of these two samples. This assumption was proved by another experiment with aqueous extractions performed from $t_{extract}$ = 0 min to 150 min, in 30 min-steps (data not shown). For that reason, it was decided to realize only one aqueous extraction period ($t_{extract}$ = 150 min) in the second

experiment. In this sample, significant amounts of micronutrients have been removed in the course of the aqueous extraction (see Figure 6, right). For that reason, it is assumed that components, currently unspecified, which are seriously inhibitive for the anaerobic consortium in the AD-process, have been extracted before anaerobic digestion. These findings and the fact that the high lipid inventory in the microalgal cells is still available for AD, result in high methane yields.

Instant availability of C, N and other components in the cultivation medium, resulting in an early metabolization, should reveal their influence in the initial phase of AD. In the performed experiments, the low C:N-ratios in the cultivation medium, rising constantly from sample type (1) C:N = 6.7, via sample type (2) C:N = 12.5, to sample type (3) C:N = 13.99 (see Figure 5, right for more details, regarding investigated species), are associated with increased delay of methane yields in the initial phase of AD. Indeed, the control samples in both experiments had the best start values in methane yield, even though they had the lowest C:N-ratios in the cultivation medium. The cultivation media of samples (2) and (3) (see Figure 5, right), with higher C:N-ratios, which are closer to the optimal C:N-range, had lower performances in the initial phase of AD (see Figure 7). These observations suggest that C:N-ratios in the cultivation medium are not playing a major role in the start of the AD process and support the interpretation, mentioned above, regarding the other inhibitors such as LCFAs, which are dominating in the initial phase of AD.

PEF-treated and subsequent lipid extracted samples and the control samples showed comparable, intensive performances in both experiments, in the first couple of days. The absence of a lag-phase in this samples is coherent with the fact that after lipid extraction the presence of the suggested inhibitive LCFAs is unlikely. Additionally, it was observed that in the lipid extracted samples, the methane yield slowed down drastically after 4 days (first experiment) and after 2 days (second experiment), with only marginal increase of methane yield throughout the remaining 5 weeks of AD. This can be explained by the fact that the lipids which were extracted, are large contributors to methane formation. Final methane yields in both experiments were comparable, with 205 $mL_{norm}/g_{VS}$ in first experiment and 181 $mL_{norm}/g_{VS}$ in second experiment (compare respective graphs in Figure 7 and Figure 8). The methane yield in the second experiment was marginal lower, which is attributed to two factors. First of all, microalgae used in first experiment had a lipid inventory which was 4 % of CDW higher when available in the second

experiment. Secondly, the lipid extraction efficiency with the second experiment was 19 % higher, compared to first experiment, with respect to the total existing lipid inventory of the respective microalgae (see Figure 3).

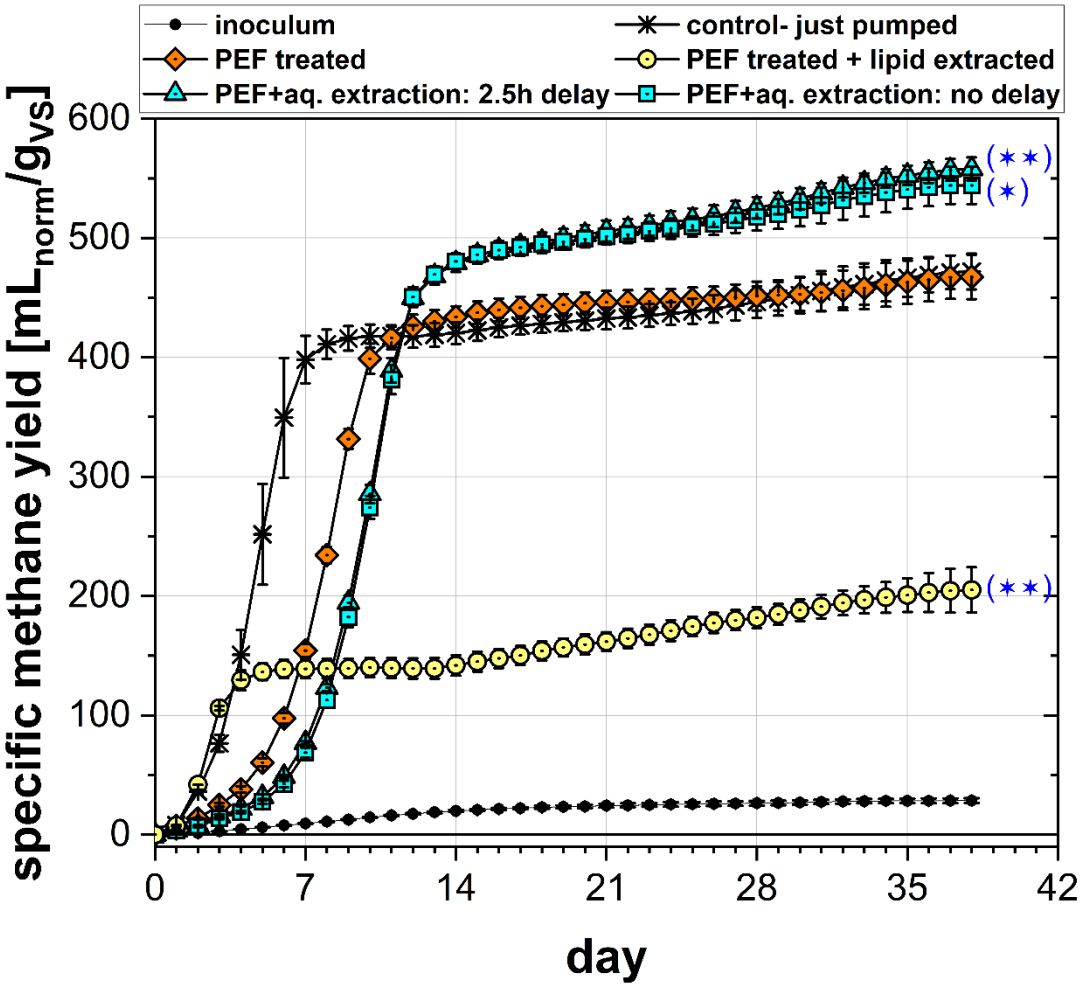



*Figure 7: First experiment: Methane yield from microalgae A. protothecoides. Data determined in duplicate. Lower remaining lipid inventory after lipid extraction causes lower biomethane yield. The results displayed are the average ± SD of the duplicates. Statistical significances are indicated, compared to control.*

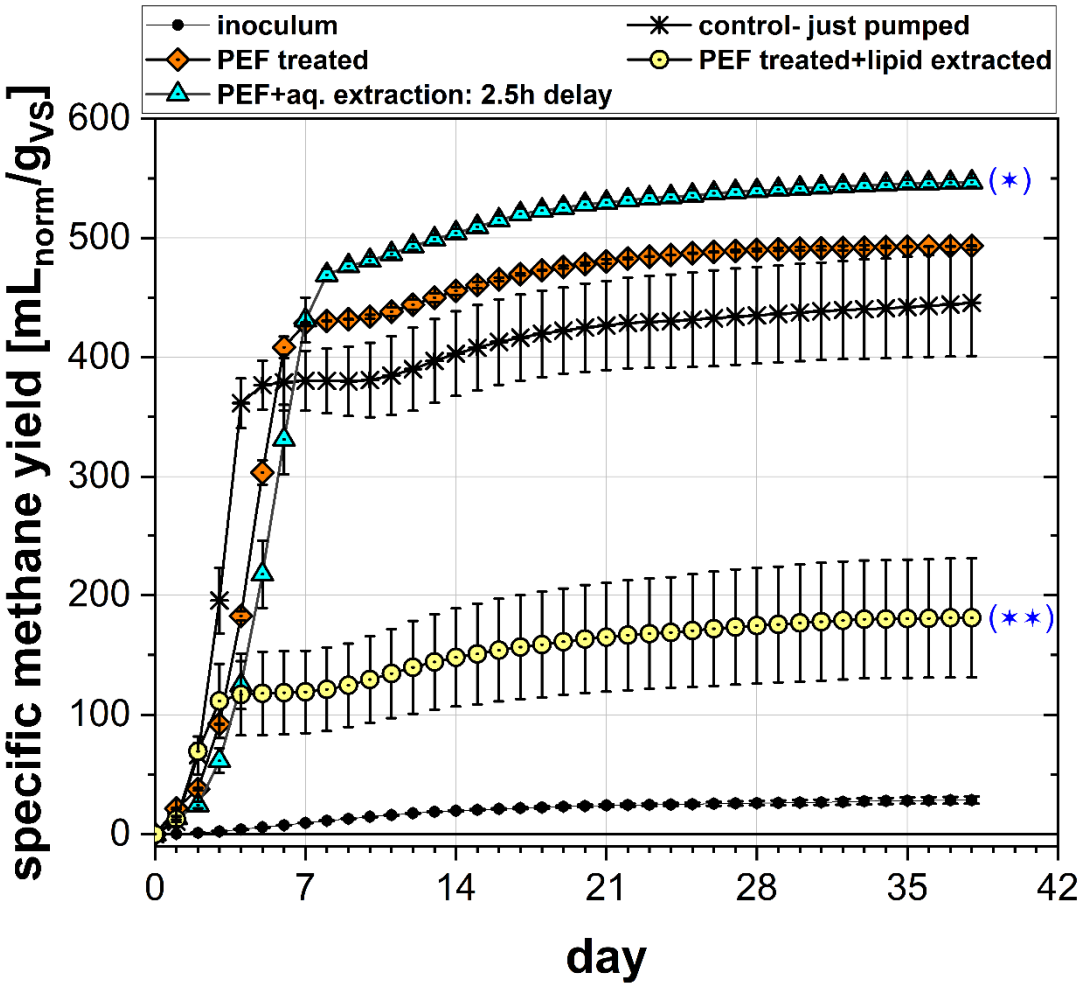



*Figure 8: Second experiment: Methane yield from microalgae A. protothecoides. Data determined in duplicate. Lower remaining lipid inventory after lipid extraction causes lower biomethane yield. The results displayed are the average ± SD of the duplicates. Statistical significances are indicated, compared to control.*

Using the methane yield obtained, it is possible to calculate the energy gained from the AD process. Results expressed in MJ/kg$_{VS}$ are presented in Table 2. The results were also normalized to one kilogram of initial cell dry weight (kg$_{CDW}$) obtained from microalgal biomass (second line of the table). Control and PEF samples enable to produce 16.20 MJ/kg$_{CDW}$ and 17.82 MJ/kg$_{CDW}$, respectively. There is a 10 % increased specific methane output, accompanied by a slight acceleration of the process, as previously mentioned. The rest biomass obtained after PEF-treatment and aqueous extraction enables to produce 17.82 MJ/kg$_{CDW}$ and thus the same energy as the solely PEF-treated sample. This implies that the aqueous extracted molecules do not contribute to the methane yield in the AD process. In case the samples were further submitted to lipid extraction, the energy gain from AD is reduced to

4.10 $MJ/kg_{CDW}$. This reduction is expected since lipid molecules, which were extracted, normally contribute considerably to energy production in AD processes due to their high calorific value. In both cases, the energy produced from the rest biomass after various extractions is not negligible and represents more energy (11,9 and 2,7 times respectively) than consumed by the PEF-process itself. Therefore, the integration of AD in a cascade process to valorize the rest biomass seems to be a promising approach. More detailed calculations are beyond the scope of this publication.

| | **control** | **PEF-treated** | **PEF-treated + aqueous extracted** | **PEF-treated + aqueous- and lipid extracted** |
|---|---|---|---|---|
| **thermal energy gain by methane (MJ/kg $_{VS}$)** | 16.20 | 17.82 | 19.80 | 6.84 |
| | complete biomass ↓ | complete biomass ↓ | ≈10% mass reduction (extracted salts) ↓ | ≈40% mass reduction (extracted salts + lipids) ↓ |
| **thermal energy gain by methane (MJ/kg $_{CDW}$)** | 16.20 | 17.82 | 17.82 | 4.10 |

*Table 2: Values in the first line are energies obtained from the methane produced during AD. In the second line of the table results were normalized to one kilogram of initial cell dry weight of microalgae.*

## 4 Conclusion

This investigation showed the positive impact of PEF-pretreatment for the AD of microalgal substrates. PEF-treatment alone, tended to slow down the methane production during the early stage of AD. It can be assumed that PEF-treatment facilitates the marginal release of LCFAs from microalgae, which are known to be inhibitors and therefore hamper the beginning of the AD-process. Additionally, it has been demonstrated that PEF-treated microalgae, which were submitted to aqueous extraction, are the most performant for AD, providing the highest methane yield of all investigated microalgae samples. This is remarkable, because substantial amounts of minerals, useful for future cultivation procedures, can be separated, before usage of the microalgae in the final AD-procedure. This can help to reduce total cost in microalgae biorefinery concepts. Finally, it was shown that even PEF-treated microalgae which underwent additional lipid extraction, contribute to an enhanced positive energy balance. The study therefore demonstrates several ways in which PEF-treatment can be used for a better incorporation of AD into a cascade processing of microalgae biomass, fitting better to circular economy.

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**Bibliography**


[1] IEA, Oil – World Energy Outlook 2018 – Analysis - IEA, n.d. https://www.iea.org/reports/world-energy-outlook-2018/oil#abstract (accessed June 20, 2020).
[2] Govindjee, R. Govindjee, What is Photosynthesis, (n.d.). http://www.life.illinois.edu/govindjee/whatisit.htm (accessed June 20, 2020).
[3] E. Kendir, A. Ugurlu, A comprehensive review on pretreatment of microalgae for biogas production, Int. J. Energy Res. 42 (2018) 3711–3731. https://doi.org/10.1002/er.4100.
[4] H.M. Amaro, A.C. Guedes, F.X. Malcata, Advances and perspectives in using microalgae to produce biodiesel, Appl. Energy. 88 (2011) 3402–3410. https://doi.org/10.1016/j.apenergy.2010.12.014.
[5] M. Faried, M. Samer, E. Abdelsalam, R.S. Yousef, Y.A. Attia, A.S. Ali, Biodiesel production from microalgae: Processes, technologies and recent advancements, Renew. Sustain. Energy Rev. 79 (2017) 893–913. https://doi.org/10.1016/j.rser.2017.05.199.
[6] T. Heredia-Arroyo, W. Wei, B. Hu, Oil Accumulation via Heterotrophic/Mixotrophic Chlorella protothecoides, Appl. Biochem. Biotechnol. 162 (2010) 1978–1995. https://doi.org/10.1007/s12010-010-8974-4.
[7] Y.-T. Huang, C.-W. Lai, B.-W. Wu, K.-S. Lin, J.C.S. Wu, M.S.A. Hossain, Y. Yamauchi, K.C.-W. Wu, Advances in bioconversion of microalgae with high biomass and lipid productivity, J. Taiwan Inst. Chem. Eng. 79 (2017) 37–42. https://doi.org/10.1016/j.jtice.2017.05.026.
[8] J. Liu, Y. Song, W. Qiu, Oleaginous microalgae Nannochloropsis as a new model for biofuel production: Review & analysis, Renew. Sustain. Energy Rev. 72 (2017) 154–162. https://doi.org/10.1016/j.rser.2016.12.120.
[9] S. Srinuanpan, B. Cheirsilp, W. Kitcha, P. Prasertsan, Strategies to improve methane content in biogas by cultivation of oleaginous microalgae and the evaluation of fuel properties of the microalgal lipids, Renew. Energy. 113 (2017) 1229–1241. https://doi.org/10.1016/j.renene.2017.06.108.
[10] G. Brownbridge, P. Azadi, A. Smallbone, A. Bhave, B. Taylor, M. Kraft, The future viability of algae-derived biodiesel under economic and technical uncertainties, Bioresour. Technol. 151 (2014) 166–173. https://doi.org/10.1016/j.biortech.2013.10.062.
[11] J. Milano, H.C. Ong, H.H. Masjuki, W.T. Chong, M.K. Lam, P.K. Loh, V. Vellayan, Microalgae biofuels as an alternative to fossil fuel for power generation, Renew. Sustain. Energy Rev. 58 (2016) 180–197. https://doi.org/10.1016/j.rser.2015.12.150.
[12] S. Khanra, M. Mondal, G. Halder, O.N. Tiwari, K. Gayena, T.K. Bhowmick, Downstream processing of microalgae for pigments, protein and carbohydrate in industrial application: A review, Food Bioprod. Process. Trans. Inst. Chem. Eng. Part C. 110 (2018) 60–84. https://doi.org/10.1016/j.fbp.2018.02.002.
[13] N.M. U, J.G. Mehar, S.N. Mudliar, A.Y. Shekh, Recent Advances in Microalgal Bioactives for Food, Feed, and Healthcare Products: Commercial Potential, Market Space, and Sustainability, Compr. Rev. Food Sci. Food Saf. 18 (2019) 1882–1897. https://doi.org/10.1111/1541-4337.12500.
[14] C.G. Golueke, W.J. Oswald, H.B. Gotaas, Anaerobic Digestion of Algae, Appl Env. Microbiol. 5 (1957) 47–55.
[15] O. Córdova, J. Santis, G. Ruiz-Fillipi, M.E. Zuñiga, F.G. Fermoso, R. Chamy, Microalgae digestive pretreatment for increasing biogas production, Renew. Sustain. Energy Rev. 82 (2018) 2806–2813. https://doi.org/10.1016/j.rser.2017.10.005.
[16] E. Jankowska, A.K. Sahu, P. Oleskowicz-Popiel, Biogas from microalgae: review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion., Renew. Sustain. Energy Rev. 75 (2017) 692–709.
[17] F. Passos, M. Solé, J. García, I. Ferrer, Biogas production from microalgae grown in wastewater: Effect of microwave pretreatment, Appl. Energy. 108 (2013) 168–175. https://doi.org/10.1016/j.apenergy.2013.02.042.

[18] F. Passos, M. Hernández-Mariné, J. García, I. Ferrer, Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment, Water Res. 49 (2014) 351–359. https://doi.org/10.1016/j.watres.2013.10.013.
[19] D.U. Santos-Ballardo, S. Rossi, C. Reyes-Moreno, A. Valdez-Ortiz, Microalgae potential as a biogas source: current status, restraints and future trends, Rev. Environ. Sci. Biotechnol. 15 (2016) 243–264. https://doi.org/10.1007/s11157-016-9392-z.
[20] M.R. Gruber-Brunhumer, J. Jerney, E. Zohar, M. Nussbaumer, C. Hieger, G. Bochmann, M. Schagerl, J.P. Obbard, W. Fuchs, B. Drosg, Acutodesmus obliquus as a benchmark strain for evaluating methane production from microalgae: Influence of different storage and pretreatment methods on biogas yield, Algal Res. 12 (2015) 230–238. https://doi.org/10.1016/j.algal.2015.08.022.
[21] M.E. Alzate, R. Muñoz, F. Rogalla, F. Fdz-Polanco, S.I. Pérez-Elvira, Biochemical methane potential of microalgae: Influence of substrate to inoculum ratio, biomass concentration and pretreatment, Bioresour. Technol. 123 (2012) 488–494. https://doi.org/10.1016/j.biortech.2012.06.113.
[22] E. Uggetti, F. Passos, M. Solé, J. García, I. Ferrer, Biogas from Algae via Anaerobic Digestion, in: Algae Biotechnol., Springer, Cham, 2016: pp. 195–216. https://doi.org/10.1007/978-3-319-12334-9_11.
[23] R. Cardeña, G. Moreno, P. Bakonyi, G. Buitrón, Enhancement of methane production from various microalgae cultures via novel ozonation pretreatment, Chem. Eng. J. 307 (2017) 948–954. https://doi.org/10.1016/j.cej.2016.09.016.
[24] L. Mendez, A. Mahdy, R.A. Timmers, M. Ballesteros, C. González-Fernández, Enhancing methane production of Chlorella vulgaris via thermochemical pretreatments, Bioresour. Technol. 149 (2013) 136–141. https://doi.org/10.1016/j.biortech.2013.08.136.
[25] P. Bohutskyi, M.J. Betenbaugh, E.J. Bouwer, The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains, Bioresour. Technol. 155 (2014) 366–372. https://doi.org/10.1016/j.biortech.2013.12.095.
[26] F. Ometto, G. Quiroga, P. Pšenička, R. Whitton, B. Jefferson, R. Villa, Impacts of microalgae pre-treatments for improved anaerobic digestion: Thermal treatment, thermal hydrolysis, ultrasound and enzymatic hydrolysis, Water Res. 65 (2014) 350–361. https://doi.org/10.1016/j.watres.2014.07.040.
[27] A. Mahdy, L. Mendez, M. Ballesteros, C. González-Fernández, Enhanced methane production of Chlorella vulgaris and Chlamydomonas reinhardtii by hydrolytic enzymes addition, Energy Convers. Manag. 85 (2014) 551–557. https://doi.org/10.1016/j.enconman.2014.04.097.
[28] C. González-Fernández, B. Sialve, N. Bernet, J.-P. Steyer, Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production, Biofuels Bioprod. Biorefining. 6 (2012) 205–218. https://doi.org/10.1002/bbb.337.
[29] J.J. Milledge, S. Heaven, Methods of energy extraction from microalgal biomass: a review, Rev. Environ. Sci. Biotechnol. 13 (2014) 301–320. https://doi.org/10.1007/s11157-014-9339-1.
[30] J.R. Miranda, P.C. Passarinho, L. Gouveia, Pre-treatment optimization of Scenedesmus obliquus microalga for bioethanol production, Bioresour. Technol. 104 (2012) 342–348. https://doi.org/10.1016/j.biortech.2011.10.059.
[31] J. Carrillo-Reyes, M. Barragán-Trinidad, G. Buitrón, Biological pretreatments of microalgal biomass for gaseous biofuel production and the potential use of rumen microorganisms: A review, Algal Res. 18 (2016) 341–351. https://doi.org/10.1016/j.algal.2016.07.004.
[32] M.E. Montingelli, S. Tedesco, A.G. Olabi, Biogas production from algal biomass: A review, Renew. Sustain. Energy Rev. 43 (2015) 961–972. https://doi.org/10.1016/j.rser.2014.11.052.
[33] F. Passos, E. Uggetti, H. Carrère, I. Ferrer, Pretreatment of microalgae to improve biogas production: A review, Bioresour. Technol. 172 (2014) 403–412. https://doi.org/10.1016/j.biortech.2014.08.114.
[34] S. Schwede, Z.-U. Rehman, M. Gerber, C. Theiss, R. Span, Effects of thermal pretreatment on anaerobic digestion of Nannochloropsis salina biomass, Bioresour. Technol. 143 (2013) 505–511. https://doi.org/10.1016/j.biortech.2013.06.043.
[35] S. Schwede, A. Kowalczyk, M. Gerber, R. Span, Influence of different cell disruption techniques on mono digestion of algal biomass, in: Linköping, 2011: p. 7.

[36] D.S. Domozych, M. Ciancia, J.U. Fangel, M.D. Mikkelsen, P. Ulvskov, W.G.T. Willats, The Cell Walls of Green Algae: A Journey through Evolution and Diversity, Front. Plant Sci. 3 (2012). https://doi.org/10.3389/fpls.2012.00082.
[37] F. Passos, A. Hom-Diaz, P. Blanquez, T. Vicent, I. Ferrer, Improving biogas production from microalgae by enzymatic pretreatment, Bioresour. Technol. 199 (2016) 347–351. https://doi.org/10.1016/j.biortech.2015.08.084.
[38] M.A. Rodrigues, E.P. da S. Bon, Evaluation of *Chlorella* (Chlorophyta) as Source of Fermentable Sugars via Cell Wall Enzymatic Hydrolysis, Enzyme Res. 2011 (2011). https://doi.org/10.4061/2011/405603.
[39] M. Coustets, N. Al-Karablieh, C. Thomsen, J. Teissié, Flow Process for Electroextraction of Total Proteins from Microalgae, J. Membr. Biol. 246 (2013) 751–760. https://doi.org/10.1007/s00232-013-9542-y.
[40] E. Luengo, J.M. Martínez, M. Coustets, I. Álvarez, J. Teissié, M.-P. Rols, J. Raso, A Comparative Study on the Effects of Millisecond- and Microsecond-Pulsed Electric Field Treatments on the Permeabilization and Extraction of Pigments from Chlorella vulgaris, J. Membr. Biol. 248 (2015) 883–891. https://doi.org/10.1007/s00232-015-9796-7.
[41] J. Teissie, M. Golzio, M.P. Rols, Mechanisms of cell membrane electropermeabilization: A minireview of our present (lack of ?) knowledge, Biochim. Biophys. Acta BBA - Gen. Subj. 1724 (2005) 270–280. https://doi.org/10.1016/j.bbagen.2005.05.006.
[42] M. Goettel, C. Eing, C. Gusbeth, R. Straessner, W. Frey, Pulsed electric field assisted extraction of intracellular valuables from microalgae, Algal Res. 2 (2013) 401–408. https://doi.org/10.1016/j.algal.2013.07.004.
[43] A. Silve, I. Papachristou, R. Wüstner, R. Sträßner, M. Schirmer, K. Leber, B. Guo, L. Interrante, C. Posten, W. Frey, Extraction of lipids from wet microalga Auxenochlorella protothecoides using pulsed electric field treatment and ethanol-hexane blends, Algal Res. 29 (2018) 212–222. https://doi.org/10.1016/j.algal.2017.11.016.
[44] M. Sack, J. Sigler, C. Eing, L. Stukenbrock, R. Stangle, A. Wolf, G. Muller, Operation of an Electroporation Device for Grape Mash, IEEE Trans. Plasma Sci. 38 (2010) 1928–1934. https://doi.org/10.1109/TPS.2010.2050073.
[45] G. Pataro, G.M.J. Barca, G. Donsì, G. Ferrari, On the modelling of the electrochemical phenomena at the electrode-solution interface of a PEF treatment chamber: Effect of electrical parameters and chemical composition of model liquid food, J. Food Eng. 165 (2015) 45–51. https://doi.org/10.1016/j.jfoodeng.2015.05.010.
[46] D. Carullo, B.D. Abera, A.A. Casazza, F. Donsì, P. Perego, G. Ferrari, G. Pataro, Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from the microalgae Chlorella vulgaris, Algal Res. 31 (2018) 60–69. https://doi.org/10.1016/j.algal.2018.01.017.
[47] P.R. Postma, G. Pataro, M. Capitoli, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, G. Olivieri, G. Ferrari, Selective extraction of intracellular components from the microalga Chlorella vulgaris by combined pulsed electric field–temperature treatment, Bioresour. Technol. 203 (2016) 80–88. https://doi.org/10.1016/j.biortech.2015.12.012.
[48] H.R. Azencott, G.F. Peter, M.R. Prausnitz, Influence of the Cell Wall on Intracellular Delivery to Algal Cells by Electroporation and Sonication, Ultrasound Med. Biol. 33 (2007) 1805–1817. https://doi.org/10.1016/j.ultrasmedbio.2007.05.008.
[49] A. Silve, C.B. Kian, I. Papachristou, C. Kubisch, N. Nazarova, R. Wüstner, K. Leber, R. Strässner, W. Frey, Incubation time after pulsed electric field treatment of microalgae enhances the efficiency of extraction processes and enables the reduction of specific treatment energy, Bioresour. Technol. (2018). https://doi.org/10.1016/j.biortech.2018.08.060.
[50] D. Scherer, D. Krust, W. Frey, G. Mueller, P. Nick, C. Gusbeth, Pulsed electric field (PEF)-assisted protein recovery from Chlorella vulgaris is mediated by an enzymatic process after cell death, Algal Res. 41 (2019) 101536. https://doi.org/10.1016/j.algal.2019.101536.
[51] S. Akaberi, D. Krust, G. Müller, W. Frey, C. Gusbeth, Impact of incubation conditions on protein and C-Phycocyanin recovery from Arthrospira platensis post- pulsed electric field treatment, Bioresour. Technol. 306 (2020) 123099. https://doi.org/10.1016/j.biortech.2020.123099.
[52] B. Guo, B. Yang, A. Silve, S. Akaberi, D. Scherer, I. Papachristou, W. Frey, U. Hornung, N. Dahmen, Hydrothermal liquefaction of residual microalgae biomass after pulsed electric field-assisted valuables extraction, Algal Res. 43 (2019) 101650. https://doi.org/10.1016/j.algal.2019.101650.

[53] S.M. Safavi, R. Unnthorsson, Methane yield enhancement via electroporation of organic waste, Waste Manag. 66 (2017) 61–69. https://doi.org/10.1016/j.wasman.2017.02.032.
[54] M.B. Salerno, H.-S. Lee, P. Parameswaran, B.E. Rittmann, Using a pulsed electric field as a pretreatment for improved biosolids digestion and methanogenesis, Water Environ. Res. Res. Publ. Water Environ. Fed. 81 (2009) 831–839.
[55] I.-S. Lee, B.E. Rittmann, Effect of low solids retention time and focused pulsed pre-treatment on anaerobic digestion of waste activated sludge, Bioresour. Technol. 102 (2011) 2542–2548. https://doi.org/10.1016/j.biortech.2010.11.082.
[56] H. Choi, S.-W. Jeong, Y. Chung, Enhanced anaerobic gas production of waste activated sludge pretreated by pulse power technique, Bioresour. Technol. 97 (2006) 198–203. https://doi.org/10.1016/j.biortech.2005.02.023.
[57] D.S. Gorman, R.P. Levine, Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi., Proc. Natl. Acad. Sci. U. S. A. 54 (1965) 1665–1669.
[58] R. Sager, S. Granick, Nutritional Studies with Chlamydomonas Reinhardi, Ann. N. Y. Acad. Sci. 56 (1953) 831–838. https://doi.org/10.1111/j.1749-6632.1953.tb30261.x.
[59] R. Straessner, A. Silve, C. Eing, S. Rocke, R. Wuestner, K. Leber, G. Mueller, W. Frey, Microalgae precipitation in treatment chambers during pulsed electric field (PEF) processing, Innov. Food Sci. Emerg. Technol. 37 (2016) 391–399. https://doi.org/10.1016/j.ifset.2016.07.008.
[60] G. Sverre, O. Martinsen, Bioimpedance and Bioelectricity Basics | ScienceDirect, (n.d.). https://www.sciencedirect.com/book/9780123740045/bioimpedance-and-bioelectricity-basics (accessed June 16, 2020).
[61] T. Rausch, The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein, Hydrobiologia. 78 (1981) 237–251. https://doi.org/10.1007/BF00008520.
[62] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[63] DIN EN 1484:2019-04, Wasseranalytik_- Anleitungen zur Bestimmung des gesamten organischen Kohlenstoffs_(TOC) und des gelösten organischen Kohlenstoffs_(DOC); Deutsche Fassung EN_1484:1997, Beuth Verlag GmbH, n.d. https://doi.org/10.31030/3042067.
[64] DIN EN 15936:2012-11, Schlamm, behandelter Bioabfall, Boden und Abfall_- Bestimmung des gesamten organischen Kohlenstoffs (TOC) mittels trockener Verbrennung; Deutsche Fassung EN_15936:2012, Beuth Verlag GmbH, n.d. https://doi.org/10.31030/1866720.
[65] DIN EN 12260:2003-12, Wasserbeschaffenheit_- Bestimmung von Stickstoff_- Bestimmung von gebundenem Stickstoff_(TN<(Index)b>) nach Oxidation zu Stickstoffoxiden; Deutsche Fassung EN_12260:2003, Beuth Verlag GmbH, n.d. https://doi.org/10.31030/9490237.
[66] VDI 4630 - Vergärung organischer Stoffe - Substratcharakterisierung, Probenahme, Stoffdatenerhebung, Gärversuche, n.d. https://www.vdi.de/richtlinien/details/vdi-4630-vergaerung-organischer-stoffe-substratcharakterisierung-probenahme-stoffdatenerhebung-gaerversuche (accessed April 5, 2021).
[67] M.R. Mormile, J.M. Suflita, The Toxicity of Selected Gasoline Components to Glucose Methanogenesis by Aquifer Microorganisms, Anaerobe. 2 (1996) 299–303. https://doi.org/10.1006/anae.1996.0038.
[68] B. Zhao, J. Ma, Q. Zhao, L. Laurens, E. Jarvis, S. Chen, C. Frear, Efficient anaerobic digestion of whole microalgae and lipid-extracted microalgae residues for methane energy production, Bioresour. Technol. 161 (2014) 423–430. https://doi.org/10.1016/j.biortech.2014.03.079.
[69] Y. Chen, J.J. Cheng, K.S. Creamer, Inhibition of anaerobic digestion process: A review, Bioresour. Technol. 99 (2008) 4044–4064. https://doi.org/10.1016/j.biortech.2007.01.057.
[70] Zhao B., Ma J., Zhao Q., Frear C., Anaerobic Digestion of Algal Biomass Residues with Nutrient Recycle, (2013). http://www.tfrec.wsu.edu/pdfs/P2770.pdf (accessed May 3, 2018).
[71] X. He, J. Dai, Q. Wu, Identification of Sporopollenin as the Outer Layer of Cell Wall in Microalga Chlorella protothecoides, Front. Microbiol. 0 (2016). https://doi.org/10.3389/fmicb.2016.01047.
[72] A. Silve, C.B. Kian, I. Papachristou, C. Kubisch, N. Nazarova, R. Wüstner, K. Leber, R. Strässner, W. Frey, Incubation time after pulsed electric field treatment of microalgae enhances the efficiency of extraction processes and enables the reduction of specific treatment energy, Bioresour. Technol. (2018). https://doi.org/10.1016/j.biortech.2018.08.060.

[73] C. González-Fernández, B. Sialve, N. Bernet, J.-P. Steyer, Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production, Biofuels Bioprod. Biorefining. 6 (2012) 205–218. https://doi.org/10.1002/bbb.337.
[74] Parkin Gene F., Owen William F., Fundamentals of Anaerobic Digestion of Wastewater Sludges, J. Environ. Eng. 112 (1986) 867–920. https://doi.org/10.1061/(ASCE)0733-9372(1986)112:5(867).
[75] H.-W. Yen, D.E. Brune, Anaerobic co-digestion of algal sludge and waste paper to produce methane, Bioresour. Technol. 98 (2007) 130–134. https://doi.org/10.1016/j.biortech.2005.11.010.
[76] B. Sialve, N. Bernet, O. Bernard, Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable, Biotechnol. Adv. 27 (2009) 409–416. https://doi.org/10.1016/j.biotechadv.2009.03.001.
[77] H. Wang, I.A. Fotidis, I. Angelidaki, Ammonia–LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process, Bioresour. Technol. 209 (2016) 282–289. https://doi.org/10.1016/j.biortech.2016.03.003.
[78] O. Yenigün, B. Demirel, Ammonia inhibition in anaerobic digestion: A review, Process Biochem. 48 (2013) 901–911. https://doi.org/10.1016/j.procbio.2013.04.012.
[79] S. Suzuki, M. Shintani, Z.K. Sanchez, K. Kimura, M. Numata, A. Yamazoe, K. Kimbara, Effects of phosphate addition on methane fermentation in the batch and upflow anaerobic sludge blanket (UASB) reactors, Appl. Microbiol. Biotechnol. 99 (2015) 10457–10466. https://doi.org/10.1007/s00253-015-6942-1.
[80] A.J. Ward, D.M. Lewis, F.B. Green, Anaerobic digestion of algae biomass: A review, Algal Res. 5 (2014) 204–214. https://doi.org/10.1016/j.algal.2014.02.001.
[81] H. Alexandre, M. Guilloux-Benatier, Yeast autolysis in sparkling wine – a review, Aust. J. Grape Wine Res. 12 (2006) 119–127. https://doi.org/10.1111/j.1755-0238.2006.tb00051.x.
[82] T.L. Babayan, M.G. Bezrukov, Autolysis in yeasts, Acta Biotechnol. 5 (1985) 129–136. https://doi.org/10.1002/abio.370050205.
[83] T. Hernawan, G. Fleet, Chemical and cytological changes during the autolysis of yeasts, J. Ind. Microbiol. 14 (1995) 440–450. https://doi.org/10.1007/BF01573955.
[84] J.M. Martínez, G. Cebrián, I. Álvarez, J. Raso, Release of Mannoproteins during Saccharomyces cerevisiae Autolysis Induced by Pulsed Electric Field, Front. Microbiol. 7 (2016). https://doi.org/10.3389/fmicb.2016.01435.
[85] R. Conrad, M. Klose, P. Claus, Phosphate inhibits acetotrophic methanogenesis on rice roots, Appl. Environ. Microbiol. 66 (2000) 828–831. https://doi.org/10.1128/aem.66.2.828-831.2000.
[86] T. Kobayashi, A Simple Method for the Detection of Long-Chain Fatty Acids in an Anaerobic Digestate Using a Quartz Crystal Sensor, (2016).