Pulsed microwave pretreatment of fresh microalgae for enhanced lipid extraction

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Abstract

Pulsed microwave (PMW) is considered as an energy-saving pretreatment for microalgae. The efficiency of PMW was studied using a generator delivering square-pulsed modulated microwave in continuous flow on fresh *Auxenochlorella protothecoides* suspension. The efficiency was evaluated by measuring the increase of the suspension's conductivity, the liberation of carbohydrates, the percentage of permeabilized microalgae cells and the lipid yield after solvent extraction. The properties of the pulses i.e. pulse duration, repetition rate and pulse

power had little effect on the efficiency and especially on lipid extraction performance. Lipid yield was positively correlated with the energy input and increased from 3.81% to 38.42% with microwave energy input increasing from 1.4 to 2.8 MJ/kg $_{DW}$ (Dry Weight). At a given PMW absorbed energy, the lipid yield decreased with the increase of algal concentration, whereas it increased with the suspension flow rate. Based on comparison with water-bath heating i.e. a pure thermal treatment, results suggest that both the microwave induced heating and non-thermal effects impact the efficiency of PMW treatment. An energy consumption of 2.53 MJ/kg$_{DW}$ achieved 37.29% lipid yield, which confirms that PMW is a potentially competitive, highly efficient and easy to implement method that could benefit downstream processing of microalgae.

Keywords: pulsed microwave, microalgae cells, lipid extraction, energy assessment

1. Introduction

Microalgae are considered as a promising source for the sustainable production of various biofuels, such as hydrogen, biogas, ethanol, biodiesel and bio-jet fuels [1]. Among these biofuels, biodiesel from microalgae has attracted a widespread interest for its potential to substitute conventional transportation fuels [2]. The advantages of biodiesel over petro-diesel include carbon neutrality, the renewable character and the lack of harmful impact on the environment and ecology [3]. In addition, microalgae have a wide range of industrial applications in aquaculture feed, drugs, cosmetics, functional food and others, since the lipids in microalgae cells contain many interesting hydrophobic or amphipathic small molecules, e.g., fatty acids, polyketides, carotenoids, sterols, terpernoids and others [4, 5]. Some of these metabolites are already commercialized on the market with a constant growing demand. For example, it is expected that the market demand for omega 3 fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), will increase from 21900 tons in 2012 to more than 135500 tons in 2025 with an annual growth rate of 16% [6]. Microalgae can be continuously cultivated throughout the year and therefore

ensure permanent supply of the various products. Other microalgae benefits include the high biomass productivity (up to 263 tons/ha/year), the high lipid content (up to 70% dry weight of microalgae) and the absence of need for arable land [7]. However, the main obstacles to the production of algal biofuels and high value metabolites are the economic and environmental costs such as large quantities of solvent required, especially in the processes of biodiesel production.

The rigid and resistant algal cell walls, composed of mainly neutral sugars, uronic acids, protein, glucosamine, and other monomers, hinder the extraction of lipid from intracellular space [8]. The tensile strength of microalgae cells can reach 9.5 MPa, which is about the same as the tensile strength of bacteria or yeast but three times stronger than that of other plant cells [9]. Conventional methods to disrupt microalgae cells such as bead milling, high pressure homogenizer, autoclaving and acid hydrolysis can affect the quality of lipids, generate hazardous substances, and are often energy intensive [5, 10]. A common result is the formation of emulsions which are then difficult to further process [11]. Therefore, it is urgent to develop a green, sustainable and efficient method to disrupt the microalgae cells and further perform lipid extraction.

Microwave (MW) is considered as a promising technology for cell disruption in the scientific and industrial communities [12, 13]. Microwave is commonly used either as a pretreatment method when applied on the raw biomass materials or as an assisted extraction method when biomass is already re-suspended in an appropriate solvent [14]. Both approaches have been already partially tested on microalgae [15, 16]. When microwave is applied directly as a pre-treatment on wet biomass, high energy absorption are reached because of the high water content [17]. This approach however, requires an extra step for lipid extraction which can increase processing time and solvent requirements [12]. On the contrary, microwave assisted extraction saves time and solvent by applying the microwave directly to

69 the system of microalgae and extraction solvent although lipid yields are still too low in the case of
70 microalgae [18]. Therefore, the different processes using microwave should be optimized to guarantee
71 good performances of lipid extraction from microalgae.

72 Previous studies focusing on continuous microwave for pretreatment of microalgae biomass using
73 commercial closed-vessel systems [19, 20], have evaluated the effect of microwave power, temperature,
74 residence time and pressure [15]. In most setups, microwave pretreatment is performed in batch mode,
75 durations are in a range of 3-40 min and the common power of microwave in the range of 30-1400 watts
76 [5, 21]. However, this way of applying microwave pretreatment is generally associated with high energy
77 consumption, typically in the range of 5.4-420 MJ/kg dry weight of algae biomass [21-24]. The minimum
78 reported value of energy consumption, 5.4 MJ/kg_{DW}, was only that low since sulfuric acid was in the
79 solution and the role of MW was only to improve the efficiency of the hydrolysis of the biomass and not
80 to act as a pre-treatment alone [25]. Higher energy input was required for microwave pretreatment of the
81 microalgae *Scenedesmus obliquus* with energy around 9.6 MJ/kg_{DW} in a resonant continuous microwave
82 processing system [26]. Most of the previously mentioned studies were performed on dry or at least
83 frozen microalgae, implying an already large energy consumption prior to the MW treatment [24].
84 Therefore, it is necessary to test microwave pre-treatment on wet biomass and to assess whether or not
85 it can be attractive to the industry for the down-processing of microalgae.

86 Pulsed microwave has been proposed as an alternative method to continuous microwave radiation for
87 several applications including biodiesel production from microalgae [27, 28]. To the best of our
88 knowledge, PMW has never been studied and compared to continuous MW as a pre-treatment method
89 of the microalgae biomass. In order to address this question, we have recently developed in our laboratory
90 a pulsed microwave generator and a microwave cavity applicator, operating at TM_{010} mode, both custom-

designed, for the evaluation of microwave disruption of fresh microalgae cells [29]. The fresh concentrated microalgae biomass is continuously flowing through microwave applicator, where it is exposed to the microwave field. The setup enables the pulse length in the range of 1 to 999 μs, with a peak power in the range of 2-4 kW. Repetition rate of the PMW can be chosen between 1 and 1000 Hz, resulting to different mean absorbed microwave energies for a given flow rate of the biomass suspension. Experiments were performed on *A. Protothecoides*, a microalgae notorious for its extremely robust cell wall [30]. The aim of this study was to investigate the effect of PMW pretreatment on the release of the water soluble content from microalgae and on the efficiency of lipid extraction using organic solvents. The comparative experiments using water bath heating as pre-treatment method i.e. a pure thermal method were performed on the same microalgae in order to evaluate the role of thermal effects in PMW pretreatment. Based on these experiments, an evaluation of the energy required for PMW pretreatment was performed and compared to other existing methods.

2. Materials and methods

2.1. Microalgae culture and harvest

A. Protothecoides (algae strain: No.211-7a), obtained from Culture Collection of Algae at Göttingen university (SAG), was grown in plastic cultivation flasks with modified Wu medium [31]. After ten days of cultivation, the concentration of microalgae *A. Protothecoides* was about 10 g/L. Microalgae were then harvested by centrifugation at 3000 *g* with a centrifuge. The fresh microalgae pellet obtained after centrifugation was re-suspended in the appropriate amount of the cultivation medium (i.e. the supernatant obtained after centrifugation) to obtain the desired concentration i.e.100 g/L unless stated otherwise. For all the experiments, the accurate final concentration was measured. Some of the concentrated suspension was freeze-dried and stored in a vacuumed sealed bag at -20 °C for further analysis.

2.2. Water bath heating experiment

A volume of 10 mL of 100 g/L concentrated microalgae suspension was heated using a hot water bath with stirring at 300 rpm. The microalgae suspension was heated to temperatures of 50°C, 60°C or 70°C for 30 min, sampled at different time points and further processed for measurement of conductivity, evaluation of carbohydrates release and lipid extraction. A digital thermometer was used for real-time measurement of the temperature.

2.3. Pulsed microwave pretreatment experiment

The overall setup is depicted in **Fig. 1**. A custom magnetron-based generator (output frequency 2.46 GHz) operating in pulse mode was designed and constructed in our institute [29]. The generator can deliver pulses of several kilowatts with a duration ranging from 1 to 999 μs and repetition frequency ranging from 1 to 1000 Hz. The microwave power from the generator was guided through a standard WR340 waveguide to the microwave applicator consisting of a resonance cavity, a frequency tuning element and a quartz tube. The setup enabled the exposure of the microalgae suspension in a continuous flow mode. Additionally, the microwave circulator which redirected the reflected power to a water load, was installed in the feeding line. Finally, for precise control of incident and reflected microwave power, the microwave bidirectional coupler was installed between circulator and microwave applicator. Details on the hardware can be found in our previous publication [29].

Fresh concentrated microalgae suspension was continuously pumped through a quartz tube inside the resonant microwave cavity. When loaded with microalgae suspension, the cavity resonance frequency had to be matched with the magnetron frequency of 2.46 *GHz* in order to have the maximum efficiency. The matching was performed with a frequency tuning element which is a part of the reactor. Simulations had shown that all the energy absorbed in the cavity was well focused inside the microalgae

suspension in a homogeneous manner. PMW experiments were performed with pulses with a duration T [µs] ranging from 100 to 300 µs, a repetition rate frep [Hz] ranging from 1 to 10 Hz, and a generator power adjusted between 2 and 4 kW (see figure 1 b for notations). In the PMW experiments, the algal concentration was ranging from 10 to 200 g/L and the flow rate from 0.01 to 0.03 mL/s. After exposure to PMW, the microalgae suspension was further processed for measurement of its conductivity, measurement of cell permeabilisation with the impermeable nuclear dye YO-PRO, evaluation of intracellular carbohydrates release and lipid extraction. Temperature sensors (TD 242, VWR) were installed about 4 cm before and 4 cm after the cavity and provided T_{in} and T_{out}, respectively.

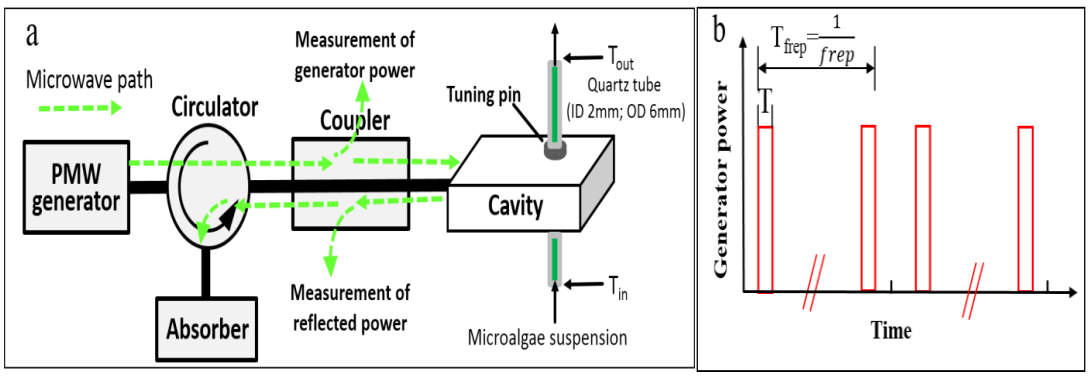


Fig 1. (a) Diagram of the pulsed microwave setup, and (b) description of the applied MW pulses.

2.4 Lipid extraction

The method for lipid extraction from microalgae was adapted from a previous paper [32] with slight modifications. Briefly, 3 mL of the fresh microalgae suspension concentrated at 100 g/L were centrifuged for 10 min at the acceleration of 10^4 *g*, the supernatant disregarded and the wet microalgae pellet was re-suspended in a solvent system of 16.1 mL ethanol and 6.6 mL hexane. Extraction was performed overnight with agitation and in the dark. Afterwards, the mixture was centrifuged at the acceleration of 10^4 *g* for 10 min. In order to accomplish phase separation, 10 mL of the upper organic phase containing the raw extract were supplemented with 30 mL hexane and 5 mL distilled water. The mixture was shaken

and then centrifuged to obtain neat phase separation. The upper hexane phase was collected and the hexane was evaporated under nitrogen flow. The lipid yield could be then determined gravimetrically with a precision balance.

2.5. Conductivity measurement

Conductivity measurement of the microalgae suspension is an indicator of the amount of ions and charged molecules present in the suspension. It is and therefore a good indicator of the integrity of the cell membrane and a routine diagnostic to detect the efficiency of pulsed electric field treatment [32, 33]. The conductivity of microalgae suspension σ_T [µS/cm] was measured with a conductivity meter (Endress+Hauser, CLM 381). The value of conductivity was recorded as well as the temperature. The conductivity was converted to the equivalent conductivity value at 25ºC, σ_{25} [µS/cm], using the equation below [34]. The value of coefficient α_{25} was 2.8%/°C, which was determined experimentally in previous studies by measuring conductivity of a microalgae suspension at different temperatures [32].

$$\sigma_{25} = \sigma_T/[1 + \alpha_{25}(\mathrm{T} - 25°\mathrm{C})]$$

2.6. Carbohydrates measurement

The amount of carbohydrates released into the supernatant after the pretreatment of the microalgae suspension was analyzed using the Anthrone Sulfuric Acid assay. The carbohydrates measurement protocol was fully described in a previous work [32].

2.7. Fraction of permeabilised cells measurement

The percentage of permeabilised cells was detected using Yo-Pro staining and flow cytometry detection. The initial microalgae samples were diluted to approximately 0.1 g/L with their own medium previously filtered at 0.2 µm. One mL of the diluted microalgae suspension was supplemented with 10 µL of Yo-Pro dye at 0.1 mM. The sample was left 10 min in the dark at room-temperature and then

diluted 1:5 with filtered medium. Flow cytometer measurements were conducted on an Attune NxT (Thermo Fisher Scientific) with a 488 nm laser as excitation source.

2.8. Total lipid determination

Total lipid content was determined using the Kochert method [35]. In brief, 0.1 g of the freeze-dried microalgae was bead-milled and resuspended in 2 mL of chloroform: methanol (v:v=2:1) in a glass tube. After centrifuging at 1700 *g* for 4 min, the supernatant was collected in a separate glass tube. This step was repeated twice on the microalgae biomass with the addition of 2 mL of fresh solvent each time and once more with the addition of only 1 mL for the last step. In order to remove the proteins in the supernatant, 3 mL of 0.1 M HCl and 0.3 mL of 0.5% (w/v) $MgCl_2$ were added to the collected supernatant. After centrifuging at 1700 *g* for 4 min, the lower phase was collected and evaporated under nitrogen flow. The total lipid content was determined gravimetrically using a precision balance. The total lipid content of microalgae harvested in the experiments was 42.47±1.89% dry weight.

2.9. Energy analysis

The energy density E_V (kJ/L suspension) consumed in the water bath heating experiment was calculated by the formula $E = c*\rho*\Delta T$. where c is the specific heat capacity of the suspension (taken equal to the one of water i.e. ~4.18 kJ kg^{-1} $^oC^{-1}$), ρ is the density of suspension (also taken equal to the one of water i.e. 1 kg/L) and ΔT is the temperature change of the suspension (°C) assuming a starting temperature of 25°C. This calculation corresponds to the minimum energy required to heat up the suspension in case of a system with no losses.

The microwave setup was equipped with a bidirectional coupler which enabled to measure both $Q_{generated}$ [W] and $Q_{reflected}$ [W] which were the mean incident and reflected microwave power, respectively. The mean microwave energy density E_V [kJ/L] absorbed in the suspension was calculated

using the equation: $E_V = f_{rep}.t_{pulse}.Q_{absorbed}/\dot{V}$, where f_{rep} is the pulse repetition rate [Hz], t_{pulse} is the duration of MW pulses [s], $Q_{absorbed}$ is the difference of incident and reflected microwave power [W], and $\dot{V}$ is the flow rate of the microalgae suspension [L/s].

For both water bath and PMW experiments, $E=E_V/C$ where E represents the energy consumption per unit of dry weight biomass [kJ/kg], E_V is the energy absorbed per unit of liter suspension [kJ/L], and C is the concentration of microalgae suspension [kg/L].

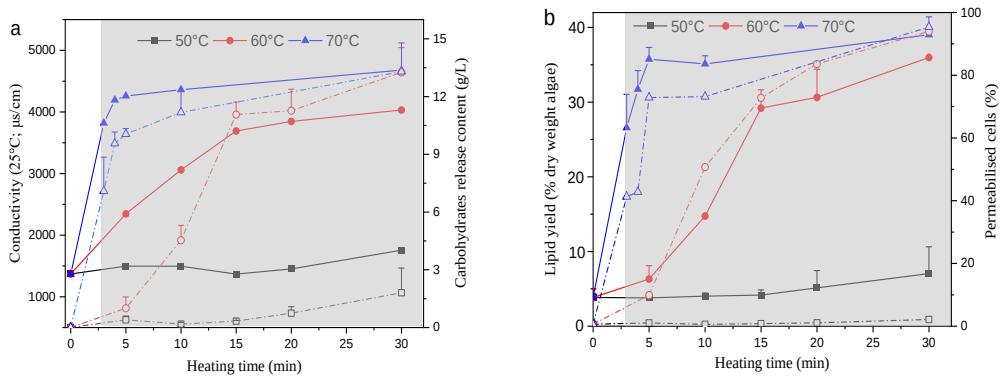
During PMW experiments, the specific measured temperature change (SMΔT, ºC), defined as the temperature difference between the input T_{in} and the output T_{out} of the PMW setup, was measured during all experiments. The theoretical temperature increase (TCΔT, ºC) was calculated using the formula $TC\Delta T = E_V/c_p/\rho$, where E_V represents the energy of microwave absorbed by the algal suspension [kJ/L], c_p is the specific heat capacity of microalgae suspension [4.18 kJ/kg/ ºC], and ρ is the density of suspension (also taken to be equal to the one of water i.e. 1 kg/L).

3. Results and discussion

3.1. Effect of water bath heating on the release of water-soluble contents and lipid extraction from microalgae

Since heating is one of the major consequences of exposure to MW, the first experiments were conducted to evaluate the impact of sole heating on the microalgae that were used in our study i.e. *A. Protothecoides*. For that matter, the fresh concentrated biomass was heated in a water bath. **Fig.2a** shows the effect of different temperatures on the suspension conductivity and on carbohydrates release as a function of the duration of the heat-treatment. Note that the microalgae suspension reached the specified temperature after heating for 3 min as assessed using a real-time monitoring with a digital thermometer and as indicated on **Fig. 2**. The "heating durations" which are further mentioned include those first 3 min

during which temperature is increasing to the targeted value. At treatment temperatures of 60ºC and 70ºC, the conductivity of the microalgae suspension and the carbohydrates concentration in the supernatant both increased significantly and rapidly. For example, the conductivity increased from 1370 μS/cm to 4190 μS/cm in 4 min at 70°C, and the corresponding carbohydrates released in the supernatant increased from 0 to 9.56 g/L. However, a heating temperature of only 50°C was much less efficient and even after 30 min of treatment, the conductivity of the suspension and amount of carbohydrates released remained low. Similar trends were observed in the yields of lipid after solvent extraction as displayed in **Fig. 2b**. The lipid yield after heating to 70°C increased sharply from 3.83% DW to 35.75% DW after only 5 min of heating, whereas after dwell time of 30 min at 50°C, it only slightly increased to 7.03%. The results show that relatively mild temperatures, i.e. around 60 °C, can effectively promote the release of some intracellular molecules in the extracellular medium and facilitate the lipid solvent extraction from microalgae in case the heating is applied long enough. The effect of water bath heating on the fraction of permeabilised cells, as assessed using Yo-pro staining, moreover shows that most of microalgae (about 90%) could not sustain temperatures above 60°C, whereas the microalgae cells could keep their membrane integrity at 50°C for at least 30 min (Fig 2b- right axis). As mentioned in other studies, high temperature can weaken the algal cell wall strength or destroy the structure of cellulose crystalline in biomass [36, 37]. In our study, according to observations under the microscope, the microalgae still possessed a relatively unaffected external cell structure after treatment in the water bath, although a few empty cell walls were observed after the treatment at 70°C (images are shown in the Fig. S1 of the supplementary data file). These algal cell shells, devoid of all the cell contents, are similar to the results reported by McMillan and colleagues with water bath treatment of *Nannochloropsis oculata* at 90°C for 20 min and more than 90% cell disruption percentage [17].



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242 **Fig.2.** The effect of different temperatures and different heating durations on the microalgae *A.*
243 *Protothecoides* using water bath as a heating source. (a) Conductivity of suspension (solid line) and
244 concentration of carbohydrates in the supernatant (dotted line), (b) lipid extraction yields (solid line) and
245 permeabilised cells (dotted line). At time zero, the biomass is placed in the water bath and heating of the
246 suspension occurs during the first three minutes. In the area marked in grey, the set temperature was
247 achieved and remained stable. The results were expressed as the average + standard deviation of three
248 independent experiments with internal duplicates, which were performed on fresh microalgae from
249 completely independent cultivation.

250 **3.2 Effect of PMW operating conditions on the release of water-soluble contents and lipid**
251 **extraction from microalgae**

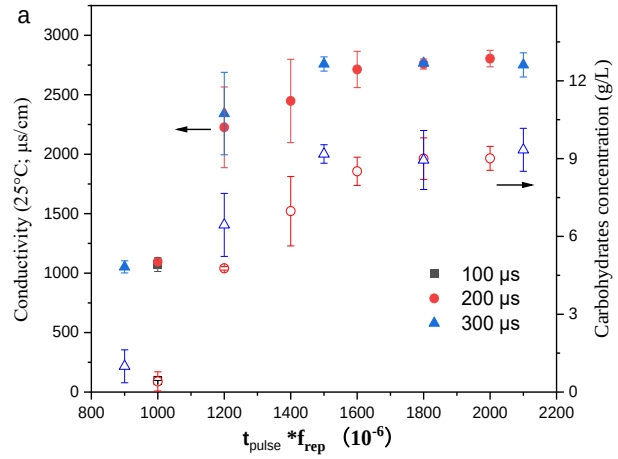
252 A first experiment was performed using microwave pulses with a duration t_{pulse} ranging from 100 to
253 300 µs and repetition rate f_{rep} ranging from 1 to 10 Hz. The experiment was performed in continuous
254 flow with a constant flow rate of Q=0.02 mL/s so that on average, the residence time of microalgae
255 suspension inside the MW cavity was 3.14 seconds. The generator peak power was fixed at 3.5±0.3 kW
256 and in all conditions, 86.5±1.5% of the power was absorbed i.e. the absorbed peak power was on average
257 3.0±0.3 kW. The exact measured values of generated and absorbed power are given in table S1 in the
258 supplementary materials. The increase of suspension conductivity, the release of carbohydrates into the

supernatant and the lipid extraction yields under different microwave operating conditions are shown in **Fig 3a** and **Fig 3b** as a function of t_{pulse}*f_{rep}. All three data sets display a similar trend. The conductivity of suspension and the concentration of carbohydrates increased with the value of t_{pulse}*f_{rep} increasing from $900*10^{-6}$ to $1500*10^{-6}$ while the corresponding lipid yield increased from 5.55% DW to 26.30% DW. For t_{pulse}*f_{rep} above $1500*10^{-6}$, a saturation of the conductivity and carbohydrate release is observed. In addition, no significant difference was observed in the lipid yield when t_{pulse}*f_{rep} was above $1800*10^{-6}$.

By measuring the absorbed power in a pulse (peak power), the duration of pulse and the repetition rate, it is possible to calculate for each exposure condition the mean energy input. A positive relationship was observed between the absorbed PMW energy and permeabilised cells (**Fig 3c)**. The percentage of permeabilised cells increased from 1% to 95% with the absorbed PMW energy density increasing from 138 kJ/L to 259 kJ/L. The permeabilisation of microalgae was associated with facilitated carbohydrates release and lipid extraction, as can be seen by the similar increasing trend observed between the lipid yield and absorbed PMW energy in Fig 3 and Fig 4. The results suggest that the performance of PMW on microalgae cells permeabilisation and lipid extraction is only dependent on the total energy input. Indeed, the efficiency of the PMW treatment seems to be independent on the duration of the pulse at a given energy input. For example, no significant difference was observed in the lipid yields for the two exposure conditions with the same t_{pulse}*f_{rep}=$1200*10^{-6}$ but different pulse duration and repetition frequency: 200 µs-6 Hz and 300 µs-4 Hz, which corresponds to mean absorbed energy of about 190 kJ/L. However, the duration of pulses in experiment were all quite close which makes it difficult to recognize its effect on the algae disruption. Therefore, further work should be considered to test other pulses

durations such as a few microseconds with the same power and the same energy input. It was however, not yet possible with the current setup.

Regarding thermal aspects, the theoretical temperature increase based on adiabatic consideration, TCΔT [°C], was expected to be between 33.0°C and 81.4°C, but the effective measured temperature increase of microalgae suspension, SMΔT [°C], was much lower and remained between 9.0°C to 23.1°C with the absorbed MW energy density increasing from 138 kJ/L to 339 kJ/L. These large differences between expected and measured temperature are due to the intense and continuous cooling, happened on the way between heating zone in the reactor and temperature sensor (distant by about 60 mm when accounting the cavity thickness of 20 mm) and resulted from the shape and the material of the quartz tube and of the cavity, which are not thermally isolated. The fast cooling in the after-reactor region can be considered as an advantage to preserve thermal sensitive molecules, but from another hand, the scenarios and set-ups where algae can be still disrupted behind the reactor zone, in a "warm" after-reactor region, might be more energy efficient. In any case, it should be noted, that in present experiment the temperature at the output of the cavity was always lower that 50°C i.e. lower than the minimal effective temperature according to the water-bath experiments. Nevertheless, higher local temperature inside the suspension when flowing through the cavity cannot be excluded. Indeed, it was already reported that some hot spots could be formed within the biomass in the process of continuous microwave heating, such that the high heat and vibrational molecular motion resulted in local instantaneous high temperature [21]. Similar behavior can also be expected for PMW exposure.



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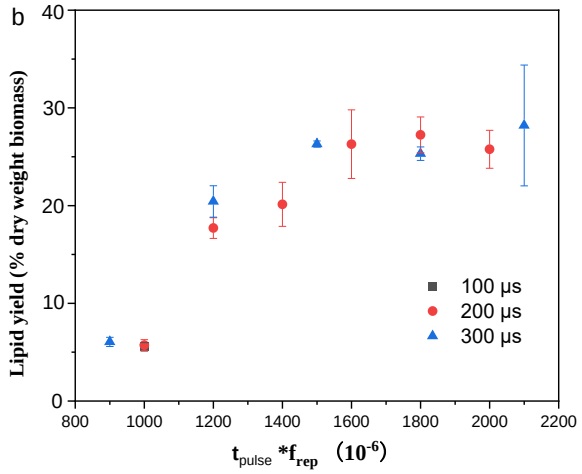

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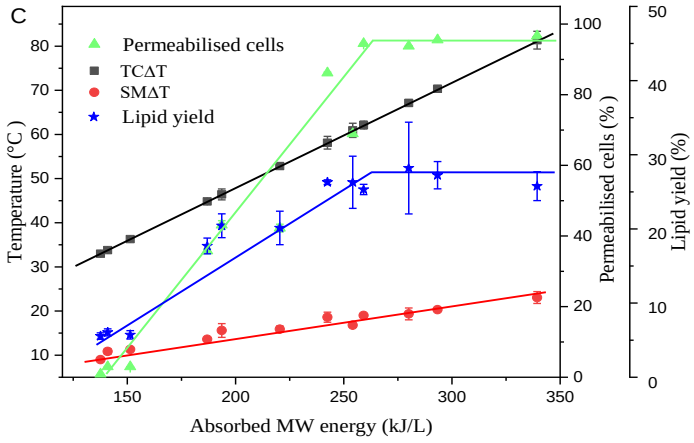

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302 **Fig. 3.** Impact of PMW exposure on (a) the conductivity of suspension (filled symbols) and the

303 carbohydrate spontaneous release in the supernatant (open symbols), on (b) lipid extraction yields and

(c) impact of PMW absorbed energy density on the TCΔT, SMΔT of microalgae suspension, and the fraction of permeabilised cells. The x-axis is graduated as a function of t_{pulse}*frep (vaule*10^{-6}) which is the product of pulses duration t_{pulse} (μs) by the pulse repetition rate (Hz). Black squares, red circles and blue triangle correspond respectively to samples treated with 100 μs, 200 μs and 300 μs pulses in Fig 3a and Fig 3b. The results were expressed as the average + standard deviation of three independent experiments with internal duplicates, which were performed on fresh microalgae from completely independent cultivation.

3.3 Effect of absorbed microwave power on the release of water-soluble contents and lipid extraction from microalgae

The impact of the absorbed microwave power was tested by adjusting the peak power from 1800 to 2800 W. The experiment was performed using a constant flow rate of 0.02 mL/s and with a microalgae concentration of 100 g/L. The applied MW pulses had a constant duration of 200 μs and the repetition rate was varied from 5 to 10 Hz in order to test different energy input. **Fig. 4** shows the effect of absorbed microwave power on the release of water fraction from microalgae and lipid extraction yields for different powers and different repetition rates. The results displayed in Fig. 4a and 4c are plotted versus repetition frequency multiplied the pulse length (which in fact is the duty cycle), show that for a given power, the release of water-soluble contents (the conductivity and carbohydrates release) and the lipid yield increase with the repetition rate i.e. with a mean applied energy. However, when the same results are displayed as a function of the mean absorbed energy density (Fig. 4b and 4d) it appears that none of the three diagnostics depends on the applied power but rather on the mean absorbed energy. For example, the suspension conductivity increased from 1020 to 2770 μS/cm with the absorbed MW energy density increasing from 140 to 250 kJ/L, and the corresponding carbohydrates release in supernatant increased

from 1.50 to 10.72 g/L. The lipid yield increased from 3.81% to 38.42% with the absorbed microwave energy density increasing from 140 kJ/L to 280 kJ/L while once again, at a given absorbed energy density, no impact of peak power is observed on lipid yield (**Fig. 4d)**. A positive correlation was observed between the MW energy input and the lipid yield, a result similar to what was obtained on *Nannochloropsis oculata* [22]. In order to exclude an influence of the peak power level, it would be necessary to perform experiments with a larger range of MW powers.

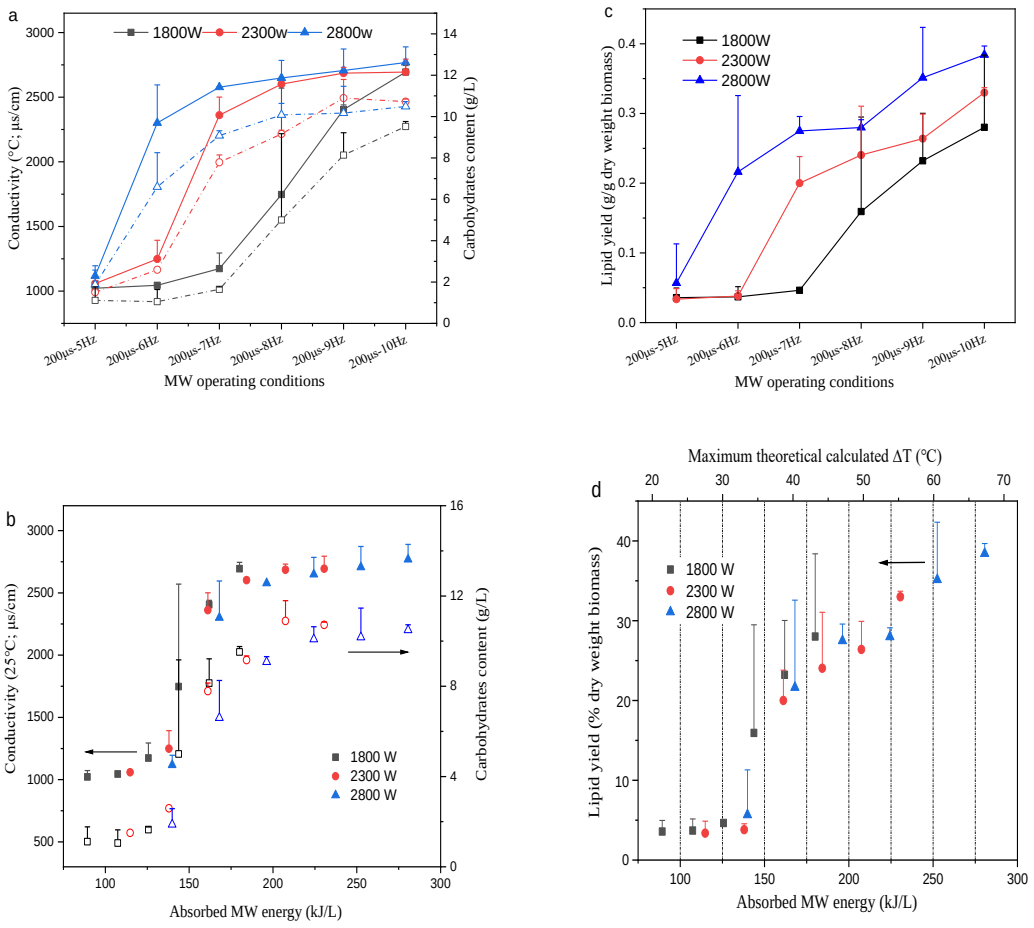


Fig. 4. Impact of microwave peak power on the efficiency of PMW treatment for different pulse duration and repetition rate for a constant flow rate of 0.02 mL/s and a microalgae concentration of 100 g/L. (a) Conductivity of suspension (solid line) and concentration of carbohydrates in the supernatant (dash-dotted line) as a function of the PMW operating conditions, (b) Conductivity of suspension (solid

symbol) and concentration of carbohydrates (hollow symbol) as a function of absorbed microwave energy, (c) lipid yield as a function of the PMW operating conditions and (d) lipid yield as a function of the absorbed microwave energy. The data in graph a, respectively c, are the same as the data reported in graphs b and d respectively. For each condition, the maximum theoretical temperature increase was calculated based on the absorbed microwave energy in the top axis of the graph d. The results were expressed as the average + standard deviation of three independent experiments with internal duplicates, which were performed on fresh microalgae from completely independent cultivation.

3.3 Effect of flow rate of microalgae suspension on efficiency of PMW treatment

The effect of microwave pretreatment on microalgae is commonly tested at laboratory scale in batch mode. In this study, the setup enables to process the feedstock in continuous flow and therefore, it offers the possibility to test the impact of the flow rate on the efficiency of the treatment. Three different flow rates of microalgae suspension were tested: 0.01, 0.02 and 0.03 mL/s. The absorbed peak power of PMW was fixed at 2.3 kW. The pulse duration (100-300 μs) was chosen accordingly, in order to keep a mean energy delivered per volume of suspension constant. The repetition rate was varied between 5 and 8 Hz in order to test different energy input. Results show a significant role of the flow rate of algal suspension on the increase of conductivity of the suspension, on the release of carbohydrate and on lipid extraction performance at constant absorbed microwave energy as displayed in **Fig. 5**. All three quantities increased with the flow rate increasing from 0.01 to 0.03 mL/s at any given absorbed microwave energy. For example, the concentration of carbohydrates increased from 6.71 g/L to 9.09 g/L with the flow rate increasing from 0.01 mL/s to 0.03 mL/s at the MW energy input of 185 kJ/L. Meanwhile, the lipid extraction yields also increased from 20.99% to 31.52%. In addition, the lipid yield at 0.03 mL/s increased from 2.31% to 31.52% with the absorbed MW energy increasing from 115 kJ/L to 185 kJ/L.

360 Measurements of the temperature increase of the algae suspension immediately after microwave
361 pretreatment are listed in the **Table 1**. The TCΔT of suspension is in the range of 27.6-44.2°C which is
362 much higher than the SMΔT since the setup enables important cooling. More specifically, the values of
363 SMΔT increased from 3.3-5.6°C to 13.9-18.2°C with the flow rate increasing from 0.01 mL/s to 0.03
364 mL/s i.e. SMΔT was lower at low flow rate than at high flow rate. This can be simply explained by the
365 fact that at a low flow rate more time is available for heat to diffuse from a sample to surroundings. The
366 different diagnostics that were used to test PMW combined with temperature measurements suggest that
367 global temperature increase might play a role in the efficiency of the MW pre-treatment and not only the
368 energy input. Indeed, at a given energy, the flow rate for which the highest global temperature is
369 achieved, induces the highest conductivity increase, the highest carbohydrate release and results in the
370 highest lipid yield.
371 In the above results of conventional heating experiments using water bath, it was shown that for
372 permeabilizing half of microalgae cell population, heating to at least 60°C is required i.e. a temperature
373 increase of about 35°C when starting from 25°C. However, the specific temperature increase of algae
374 suspension at different flow rates it this experiment were all below 20°C which implies a final maximum
375 temperature below 50°C. Therefore, the results demonstrate that the thermal increase induced by MW is
376 not the only factor responsible for the observed results and especially for the enhanced lipid extraction
377 from microwave-treated microalgae cells. Several publications have proposed mechanisms that could
378 explain why MW is more efficient than conventional heating [21, 22]. For example, it was recently
379 suggested that the formation of hot spots by microwave radiation caused the relocation of crystalline
380 structures within switchgrass [38]. Additionally, Alejandra et al. [21] reported that microwave heat and
381 vibrational motion in the MW pretreatment process could result in the rupture of some components of

lignocellulosic materials. Another theory proposed that the generation of resultant stress in the wheat starch led to the collapse or rupture of the structure during microwave pretreatment [39]. And another one, that the O-H bonds of biopolymer absorbed the microwave efficiently, which caused the expansion of components in wood due to the heat accumulation and the pressure increase [40]. However, according to optical microscopy observations, a collapse or rupture of microalgae cells induced by PMW treatment has never been observed during our experiments. Some rupture at a microscopic level may have been induced in microalgae cells, but observing it would require specific diagnostics such as scanning electron microscope. Other modification at molecular scale are also plausible but could not be investigated during this study. This can be done in the future and will help to clarify the mode of action of microwave pretreatment.

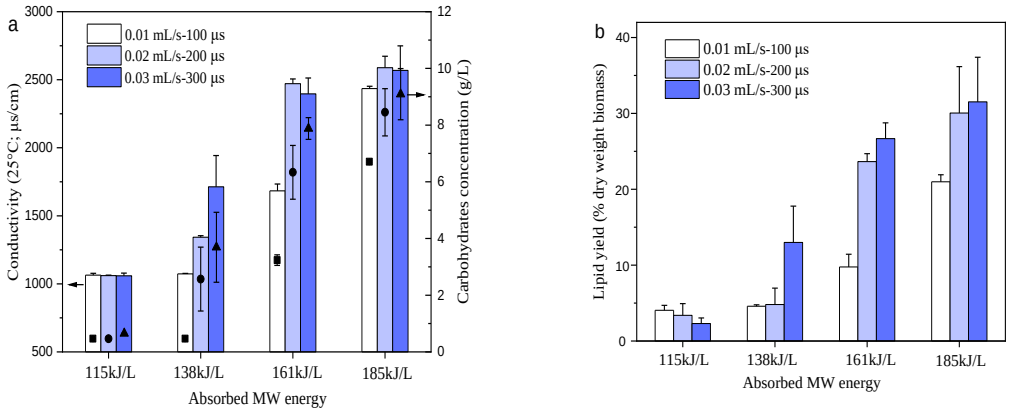


Fig. 5. Impact of flow rate on the efficiency of PMW treatment for different energy density input. The absorbed peak power was fixed at 2.3 kW. (a) Conductivity of algae suspension (bars) and carbohydrates release in the supernatant (markers) and (b) lipid extraction yields. The results were expressed as the average + standard deviation of three independent experiments with internal duplicates, which were performed on fresh microalgae from completely independent cultivation.

Table 1. Theoretical calculated temperature increase (TCΔT) and specific measured temperature increase (SMΔT) of microalgae suspension after pulsed microwave pretreatment

Microwave energy input (kJ/L)	TCΔT (°C)	SMΔT (°C)		
		0.01 mL/s	0.02 mL/s	0.03 mL/s
115	27.6	3.3±0.1	8.3±0.0	13.9±0.1
138	33.1	4.2±0.1	10.1±0.2	15.9±0.3
161	38.7	4.7±0.1	11.1±0.1	17.8±1.1
185	44.2	5.6±0.1	12.8±0.6	18.2±0.7

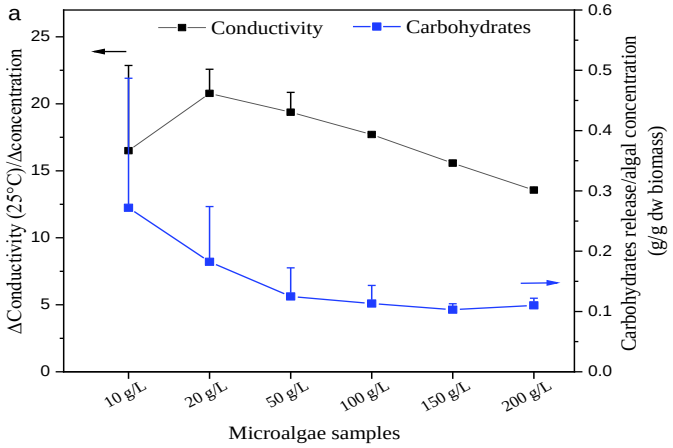
3.5 Effect of microalgae suspension concentration on PMW treatment

Finally, the impact of the concentration of the microalgae suspension was tested since it can affect a) the absorption of microwave due to change of dielectric properties [21] and b) the temperature distribution inside the sample at a local level. For this experiment, the MW pulses had a duration of 300 μs and were applied with a repetition rate of 5 Hz on the microalgae suspension flowing through the cavity with a flow rate of 0.02 mL/sec. Note that the increase of microalgae concentration did not impact the flow rate of the suspension which stayed the same at all concentrations for a given setting of the peristaltic pump. The generator power was fixed at 3.65 kW and the measured absorbed power was 3.17±0.02 kW which corresponds to a specific applied energy density of 240 kJ/L. Under those experimental conditions, the percentage of permeabilised algal cells, as measured by Yo-pro uptake, was higher than 80% for all concentration, indicating an efficient pre-treatment (data not shown). The effect of microalgae concentration on the conductivity of the suspension, on the release of carbohydrate and on lipid yields are shown in **Fig. 6**. The conductivity and carbohydrate results have been normalized to the microalgae concentration in order to correct for this bias. Once normalized to the average microalgae concentration, both quantities decrease with the increase of algal concentration. Similarly, the lipid yield

after solvent extraction decreased from 32.62% to 25.71% when the algal concentration increased from 20 g/L to 200 g/L. The lipid yield of 10 g/L was only 27.43% but for this specific condition, some biomass loss was observed after the centrifugation step at the start of the lipid extraction procedure. The results therefore indicate that a higher concentration of feedstock tends to reduce slightly the effectiveness of microwave pretreatment

The reason why PMW is less efficient at high concentrations is not currently understood. In the case of conductivity (which is a measurement of how much ions are released from intracellular space) and of carbohydrates release, it can be argued that since those two diagnostics rely on the diffusion of particles, it is logical that it is more efficient when more empty extracellular environment is available for free diffusion. In that case, this would imply that not the PMW in itself is less efficient at high concentrations but the externalization of the molecules from the damaged cells. However, this cannot directly explain why also lipid yield are reduced at higher concentration. Since for all concentrations, the same energy was absorbed, it can also be ensured that the global temperature increase of the microalgae suspensions was the same in all cases and therefore that global heating is not responsible for the differences observed. In a previous study we had shown that the dielectric losses in a microalgae suspension increased with the algal concentration. This suggests that microalgae themselves absorb MW better than the surrounding water and therefore that a temperature gradient across the microalgae cell wall can be obtained especially when working in pulsed mode. However, this temperature gradient, which often is suggested to be the cause of the destruction of cells by MW, rapidly vanishes as the temperature equilibrates [15]. Moreover, the temperature equilibrium will be reached more rapidly when cells are at high concentration. This last explanation could be a reason why treating at high concentration is less efficient in terms of yield.

437 The energetic consumption of PMW treatment at different microalgae concentration was evaluated by
438 looking at the energy required per kg of microalgae dry biomass or per kg of lipid (**Fig. 6c)**. The energy
439 consumption decreased from 24 to 1.2 MJ/kg dry weight biomass with the microalgae concentration
440 increasing from 10 to 200 g/L, while the corresponding energy input per kg of lipid also decreased from
441 87.50 to 4.67 MJ/kg dry weight lipid. Therefore, despite the fact that the efficiency of PMW treatment
442 is lower at higher concentration, the energetic balance is much more favorable at high than that at low
443 concentration. In particular, in the case of lipids, the slight reduction of yield which is observed at high
444 concentration does not suppress the benefit of energy saving obtained by using higher concentration. In
445 addition, working at low concentration of microalgae would require a lot of time to process the samples
446 which is a drawback in practical applications. Therefore, based on the evaluation of energy consumption
447 and processing capacity, it appears that for industrial applications, microalgae should be processed at the
448 highest possible concentration for the PMW treatment. Note however that some microalgae species such
449 as *Chlorella* and *Nannochloropsis* are already in the form of paste at concentrations higher than 100 g/L
450 [41]. The high viscosity would in that case prevent to perform PMW pretreatment in a continuous mode
451 [42]. The highest possible concentration will therefore depend on the microalgae type.



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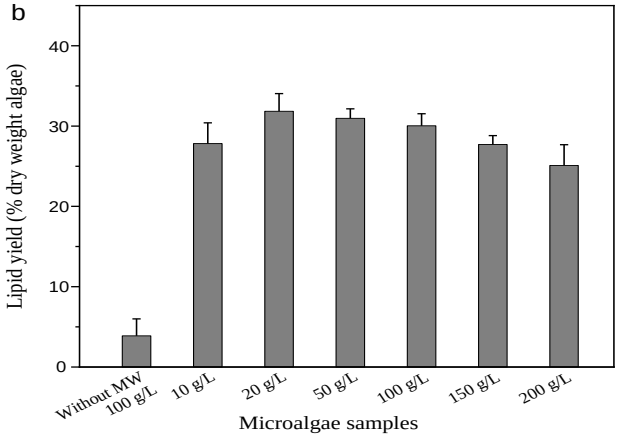

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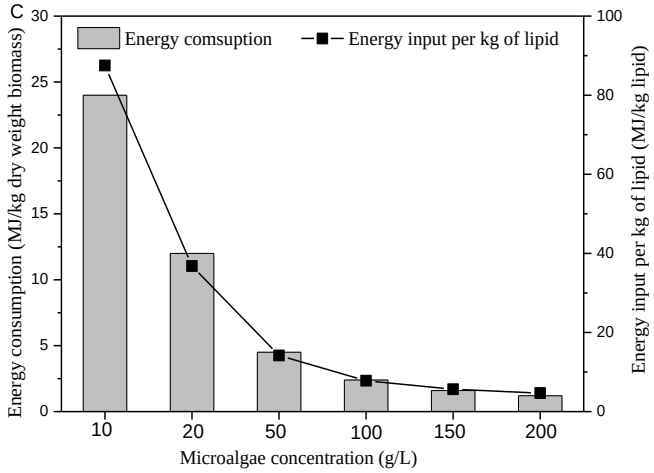

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455 **Fig. 6.** Impact of microalgae concentration on the efficiency of PMW treatment. Duration of microwave

456 pulse is of 300 μs, repetition frequency of 5 Hz, flow rate of 0.02 mL/sec, the absorbed power of

3.17±0.02 kW corresponds to energy density of 240 kJ/L. (a) Conductivity increase of suspension and carbohydrates in the supernatant after PMW pretreatment normalized to the microalgae concentration in the suspension, (b) lipid extraction yields, and (c) the energy consumption per kg of dry microalgae and per kg of extracted lipids for microalgae concentrations from 10 to 200 g/L. The results were expressed as the average + standard deviation of three independent experiments with internal duplicates, which were performed on fresh microalgae from completely independent cultivation.

3.6 Energy analysis and comparison with other pre-treatment method

Energy consumption is a critical factor for the sustainable development of microalgae energy industrialization. **Table** 3 summarized the energy consumption of various disruption methods of microalgae cells for lipid extraction as found in the literature. Only studies performed on microalgae possessing a resistant cell wall were included in this table and the energy consumptions which are reported for these different methods are the lowest values in the literature, to the best of our knowledge. In this current study, the pretreatment using water bath heating at 70°C for 5 min consumed 1.88 MJ/kg_{DW} and enabled to extract 84.2% of the total lipid content from fresh *A. Protothecoides* with a blend of ethanol and hexane. Traditional heating therefore seams to achieve good performances from an energetic point of view. However, prolonged traditional heating can reduce quality of lipid and deteriorate bioactive molecules such as pigments and vitamins [24] which can be critical depending on the application. For example, Mehmood et al. [22] reported that long heating time decreased the lipid yield due to the oxidation of unsaturated fatty acids and therefore the quality of the lipids should be controlled. In our study, pulsed MW with pulses of 200 µs duration and 2.8 kW peak power enabled to recover almost 90% of the total lipid content with an energy consumption of 2.53 MJ/kg_{DW}. Previous work had shown that for the same microalgae, i.e. *A. Protothecoides*, cultivated in our laboratory in the same

conditions and using similar extraction procedure, 95% of the total lipid content could be extracted after treatment with pulsed electric field (PEF) with an energy consumption of 1.5 MJ/kg_{DW} [30]. Additionally, PEF treatment combined with a 20 h incubation also enabled to extract 90% of the total lipid content, and the energy requirement was reduced to only 0.25 MJ/kg_{DW} [32]. Based solely on this study it seems that PEF treatment, i.e. a non-thermal method can surpass the performance of PMW. Additionally, PEF-treatment has also been shown to work at industrial scale in food application such as apple juice production [43, 44]. However, PEF-treatment implies direct contact of biomass with electrodes which can induce some chemical reactions and also some metal release [45, 46]. From that point of view, PMW has the advantage that biomass is only in contact with inert material and therefore will not be contaminated. This can be crucial in some application especially in the food sector and pharmaceutical industry. Moreover, PEF treatment is difficult to apply on sea-water microalgae since the high conductivity of such suspension induces high energy consumption. Table 3 also includes two studies using continuous microwave, either as a pre-treatment method or as an assisted extraction method. Cheng et al. [48] utilized continuous MW to pretreat fresh *Chlorella PY-ZU1* which also possess strong cell walls. Similar energy consumption i.e. 2.4 MJ/kg_{DW} is reported although one should note that this was only evaluated based on the temperature change of microalgae suspension. In that study however, the energy consumption calculated on the lipid yield was 12.83 MJ/kg lipid and strong aggressive solvents were used, i.e. chloroform and methanol. Compared to MW pretreatment of microalgae suspension, the method of microwave assisted extraction (MAE) consists in applying the MW treatment directly to the biomass already mixed with the extraction solvent [21]. When MAE is performed using continuous MW with an energy input of 5.76 MJ/kg_{DW}, it was shown that 40% of the lipid could be recovered from dry *Nannochloropsis gaditana* using a blend of water, chloroform and methanol [47]. Although the energy

level for MAE itself is relatively high in Menendez's study, MAE might be a good approach to reduce the energy consumption by reducing the unit operations and therefore the overall energy consumption in the whole processing chain. Future MAE should be tested on fresh microalgae and use less aggressive solvents such as hexane and ethanol. Tests with the PMW approach will therefore be performed in the near future. Regarding other established technologies such as ultrasonication assisted extraction, bead milling and high pressure homogenization, they possess effective cell wall disruption (>90% cell disintegration), and the associated energy consumptions are in the range of 2.92-6.12 MJ/kg $_{DW}$ (table 3). Based on the literature, the energy consumptions per kilogram lipid (19.49-23.69 MJ/kg lipid) are much higher than with the PMW (6.78 MJ/kg lipid) in our research, although this will highly depend on microalgae type and on lipid content. It therefore appears that PMW can compete with other more established pre-treatment methods from an energetic point of view. Moreover, the performances declared here are not yet optimum since experimental conditions were limited by the possibilities offered by the prototype exposure setup. Future study on pulse microwave assisted extraction as well as a comparison of PMW with continuous microwave technology should be conducted to assess the potential of PMW in industrial applications.

516 **Table 3.** Summary of cell disruption methods and their energy consumptions for lipid extraction from microalgae

Methods	Feedstock	Setting conditions	Solvents used	Yield	Energy consumption per kg of DW biomass (MJ/kg $_{DW}$)	Energy consumption per kg of DW lipid (MJ/kg lipid)	References
Water bath heating	Fresh *A. Protothecoides*	70°C for 5 min	Ethanol-Hexane	35.75% DW lipids	1.88	5.26	Present study
Pulse MW pretreatment	Fresh *A. Protothecoides*	2.46 GHz, 2800W, 200μs and 9Hz pulse	Ethanol-Hexane	37.29% DW lipids	2.53	6.78	Present study
Continuous MW pretreatment	Fresh *Chlorella PY-ZU1*	2.45 GHz at 80°C for 10 min	Chloroform-Methanol	18.70% DW lipids	2.4	12.83	[48]
Continuous MW assisted extraction	Dry *Nannochloropsis gaditana*	2.45 GHz, 30-35W at 90°C for 10 min	Water-Chloroform-Methanol	40.00% DW lipids	5.76	14.4	[47]
Pulse electric field	Fresh *A. Protothecoides*	4 MV/m, 1μs and 3Hz pulse	Ethanol-Hexane	36.00% DW lipids	1.50	4.17	[31]
PEF+Incubation	Fresh *A. protothecoides*	PEF pretreatment with 4 MV/m, 1μs and 3Hz pulse and then incubation at 25°C for 20 h	Ethanol-Hexane	35.00% DW lipids	0.25	0.71	[32]
Ultrasonication assisted extraction	Dry *Nannochloropsis gaditana*	100W, 50-60°C for 5 min	Water-Chloroform-Methanol	31.40% DW lipids	6.12	19.49	[47]
Bead milling	Fresh *Chlorella vulgaris*	ZrO_2 beads 1mm, agitator speed 6 m/s	n. a.	degree of disintegration 97.60%	2.92	n. a.	[49]
High pressure homogenization	Fresh *Nannochloropsis* sp.	1200 bar for single pass	Hexane	14.35% DW lipids	3.40	23.69	[50, 51]

517 dw: dry weigh; n. a.: not applicable

3.7 Application prospects

The microalgae *A. protothecoides* used in our study belongs to the *Chlorella* sp. and is known to be a very robust microalgae which is hard to breakdown at least using mechanical methods [30, 52, 53]. For example, Papachristou et al. [53] reported that 35% intact cells of the microalgae *A. protothecoides* were still observed after a pretreatment with high pressure homogenizer of 5 passes at 1500 bar. The observations made after PMW treatment which showed that the overall structure of the cell-wall was not affected are therefore in agreement with the literature which demonstrates the strength of the cell-wall. Despite the absence of visible damage on the cell-wall, the PMW treatment was very efficient as pre-treatment before lipid extraction and this in conditions that induced only a moderate rise in temperature. On the contrary, most references using continuous microwave pretreatment of microalgae for lipid extraction were efficient only when the temperatures achieved were above 80°C [21, 41, 54] which implies that the energy consumption was higher but also could be harmful for high value-added metabolites. Therefore, despite the high robustness of the microalgae used, the PMW used in this study performed well in comparison with other MW pretreatment studies.

In the present study, a water-bath experiment was conducted in order to have a comparison for the pulsed microwave experiments. This pre-treatment turned out to be very efficient. The PMW pretreatment only enhanced the lipid yield by 2% of cell dry weight. This is already an important result of the study. Since from a strict energetic point of view, the water bath approach might seem more interesting, especially since it is extremely easy to implement and can be advantageously combined with heat recovery. However, it should also be noted, that the pretreatment duration of pulsed MW is only of a few seconds in comparison with the water-bath heating which requires several minutes. This might be beneficial for some very sensitive molecules which can be damaged by prolonged heating. In addition, the highest temperature which is reached with the pulsed MW pretreatment (<50 °C) is lower than the temperature which was efficient during the water bath heating experiment (60°C-70°C), which is also beneficial to maintain the activity and quality of high value metabolites. The reason why PMW was efficient although lower temperature was achieved might rely on the different heating mechanisms. In conventional water bath heating, the diffusion rates of heat and mass were limited by the heat convection and conduction transfer [26]. In contrast, microwave heating as a pretreatment method resulted in near instantaneous temperature rise of the matrix which can also cause pressure effects on the microalgae structure of cell wall membrane [26]. Additionally, we cannot exclude that non-thermal effect of the microwave are acting synergistically with the heating and improving it. For example, Choi et al. [55] reported that electroporation-like effects were observed after pretreatment with continuous microwave which led to the release of microalgae intracellular contents i.e. similar observations as in the present study.

In the future, even though the energy consumption of pulse microwave is only 2.53 MJ/kg $_{DW}$, it might be possible to improve the PMW treatment in order to further reduce the energy consumption, enhance the efficiency of microalgae cell wall disruption and increase the lipid extraction yield. Moreover, the technology of pulsed microwave assisted lipid extraction from microalgae needs further research to simplify the operation process, shorten the processing time and reduce the organic solvents requirements. Overall, this study should be considered as the starting point of investigations in the field of microalgae biorefinery.

4. Conclusions

This study has demonstrated that PMW can be used as an efficient pre-treatment to extract water-soluble contents and additionally to perform solvent extraction of lipids from the microalgae *A. Protothecoides*. Experiments were performed on fresh microalgae suspension, directly after harvest which proves that this technology in principle does not require expensive drying. Regarding the performance, it was shown for example that pulses of 200 µs duration and 2.8 kW power enabled to achieve lipid yield of 37.29% dry weight, i.e. extraction of almost 90% of the total lipid content, with an energy consumption not exceeding 2.53 MJ/kg $_{DW}$ when PMW treatment is applied on a microalgae suspension concentrated at 100 g/L and with a flow rate of 0.02 mL/s. Experiments have suggested that the total energy delivered to the suspension was the main parameter determining the outcome of an experiment. Indeed, pulse duration, repetition rate and pulse power in this research seemed to have little effect at a given PMW energy input. However, the experiments which were conducted using water bath heating indicated that the global microwave heating might not be the only factor responsible for enhanced lipid extraction. The fact that microalgae concentration also influenced the outcome of experiments also speaks in favor of a process, which is not purely driven by global heating. Therefore, the study suggests that PMW are efficient either because of the combination of heating together with the effect of the electric field or only because of the thermal effect but with some spatial and temporal distribution of temperature which makes it more efficient than a standard slow and homogeneous heating which can be achieved with a water bath. Understanding the mode of action of PMW on microalgae remains an open challenge but already it can be affirmed that this technology of pulse microwave has potential in pre-treatment of microalgae. Apart from a good energy efficiency, which can probably be even further improved, other intrinsic advantages such as the possibility to work sterile, the absence of mechanical wear or the good social acceptance of such a technology can make it a great tool for microalgae processing.

Credit authorship contribution statement

Yi Zhang: Writing - original draft, Conceptualization, Methodology, Visualization, Investigation, Formal analysis. **Sergey Soldatov**: Resources, Methodology, Review & editing. **Ioannis Papachristou**: Methodology. **Natalja Nazarova**: Methodology. **Guido Link**: Methodology, Review & editing. **Wolfgang Frey**: Methodology. **Aude Silve**: Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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