Evaluating microalgal cell disruption upon ultra high pressure homogenization

Tom M.M. ***Bernaerts*** [a], *Lore* ***Gheysen*** [b], *Imogen* ***Foubert*** [b], *Marc E.* ***Hendrickx*** [a], *Ann M.* ***Van Loey*** [a,*]

KU Leuven, Laboratory of Food Technology (member of Leuven Food Science and Nutrition Research Centre, LFoRCe), Department of Microbial and Molecular Systems (M^2S), Kasteelpark Arenberg 22 box 2457, 3001 Leuven, Belgium

Authors are affiliated to:

[a] KU Leuven, Laboratory of Food Technology (member of Leuven Food Science and Nutrition Research Center, LFoRCe), Department of Microbial and Molecular Systems (M^2S), Kasteelpark Arenberg 22 box 2457, 3001 Leuven, Belgium

[b] KU Leuven KULAK, Laboratory Food and Lipids (member of Leuven Food Science and Nutrition Research Center, LFoRCe), E. Sabbelaan 53, 8500 Kortrijk, Belgium

Corresponding author during revision process:

Tom M.M. **Bernaerts**

Telephone: +32 16 379 871

E-mail: tom.bernaerts@kuleuven.be

* Corresponding author for post-publication:

Ann M. **Van Loey**

Telephone: +32 16 321 567

E-mail: ann.vanloey@kuleuven.be

Abstract

The impact of (ultra) high pressure homogenization on the degree of cell disruption was investigated for *Nannochloropsis* sp. suspensions. The degree of cell disruption was studied by combining four evaluation methods: turbidity measurement, scanning electron microscopy, hexane:isopropanol extraction efficiency, and fluorescence microscopy using the viability stain SYTOX green. Applying an ultra high pressure of 250 MPa obviously reduced the number of homogenization passes required to obtain a specific degree of cell disruption compared to 100 MPa. However, heating of the sample occurred at 250 MPa, resulting in extensive aggregate formation of the released intracellular material after multiple homogenization passes. Furthermore, cell wall integrity was not necessarily linked to membrane integrity, implying that moderate (U)HPH conditions are possibly sufficient for certain applications by damaging the cell membrane, without achieving full rupture of the cell wall. Once again it was proven that different methods for evaluation of cell disruption should be combined to get comprehensive insight into the disruption of microalgae.

Keywords

Nannochloropsis; Chlorella; cell rupture; turbidity; scanning electron microscopy; SYTOX green

Highlights

- Some (U)HPH-treated cells seem visually intact, but have damaged cell membranes
- Ultra high pressures drastically reduce the number of homogenization passes
- Heating occurred at ultra high pressures, leading to aggregate formation

Abbreviations

CM	chloroform:methanol
HI	hexane:isopropanol
HPH	high pressure homogenization
SEM	scanning electron microscopy
UHPH	ultra high pressure homogenization

1 Introduction

Microalgae are of interest for a wide range of applications, including biofuels, nutraceuticals, and food and feed ingredients. For many of these applications, recovery of the intracellular metabolites is required, which is limited in untreated biomass due to the presence of an intact cellular structure. As some microalgae are characterized by a very rigid cell wall, research has in recent decades been focusing on the optimization of cell disruption processes. In this context, high pressure homogenization (HPH) is one of the most promising cell disruption techniques, mainly due to its scalability, its applicability on highly concentrated algal slurries, and its effectiveness for disruption of rigid cell walled microalgae species [1]. To date, most studies on HPH for microalgae disruption have been performed with homogenization pressures up to 100 – 150 MPa, requiring multiple homogenization passes for rigid microalgae [2]. Higher homogenization pressures might actually be applied (up to 300 MPa), designated as ultra high pressure homogenization (UHPH), probably resulting in a more efficient disruption process by reducing the number of homogenization passes. Recent studies showed promising results for disrupting microalgal cells by UHPH, although the disruption efficiency was indirectly deduced from the extractability of proteins, without mechanistic insight into the effect of different pressures and/or number of passes [3–6]. ~~However, little evidence has been found in literature in this range of ultra high homogenization pressures.~~

Critical to understanding the cell disruption process by (U)HPH is the ability to accurately evaluate and quantify the degree of disruption. Cell disruption is actually considered a continuous process, ranging from cell damage or cell lysis, over the release of intracellular metabolites, to complete cell fragmentation [7]. Whereas various methods have been proposed for quantitative assessment of the extent of cell disruption, it is recognized that different quantification techniques are related to different stages of the disruption process, and therefore give different measures of cell disruption. Common methods are generally categorized as cell counting, particle sizing, or measurement of metabolite release. Cell counting is commonly seen as the most reliable method, but is time consuming and does not provide any information on the integrity of the cell membrane. Measuring changes in particle size distribution is restricted when aggregation of cells and/or cell fragments occurs. Measurements of metabolite release (e.g. assays for protein release or UV absorbance for total metabolite release) should be used with caution, since these indirect methods were shown to over- or underestimate the

degree of cell disruption of microalgae [8]. Hence, it has been suggested to combine different quantification methods for a comprehensive characterization of the cell disruption process [7].

Despite the numerous studies evaluating microalgal cell disruption, there is a lack of clarity on the role of the cell membrane during disruption processes. Whereas many authors presume cell disruption to be solely related to the integrity of the cell wall, others do not exclude the importance of the cell membrane, as it is known that some processes such as pulsed electric field treatment mainly affect the cell membrane without disintegrating the cell wall layer [2,9]. Even though membrane integrity has received little attention in the context of microalgal cell disruption, it might be of great importance in the selection of disruption processes towards certain applications [9]. Moreover, cell counting is often appointed as the most reliable method for evaluation of microalgal cell disruption. However, this might be questioned if proven that cell disruption also depends on the integrity of the cell membrane, since the cell counting technique does not allow to differentiate cells with damaged membranes.

The microalga *Nannochloropsis* sp. was selected for the current study as an interesting source for various applications due to its high lipid content rich in eicosapentaenoic acid, being known as one of the most resistant microalgae species to several cell disruption techniques [10]. *Nannochloropsis* sp. belongs to the Eustigmatophyta, and is characterized by spherical cells with a diameter between 1 and 5 µm [10,11]. The cell wall is composed of an inner cell wall layer of cellulosic polymers (representing 75% of the cell wall) and an outer cell wall layer of algaenans. The latter are characterized as highly resistant long-chain aliphatic hydrocarbons cross-linked by ether bonds, and are most likely responsible for the strong resistance of the *Nannochloropsis* sp. cell wall against mechanical rupture [12]. To gain insight into the impact of (U)HPH on the extent of cell disruption, four techniques for evaluation of cell rupture will be combined. Turbidity measurement has been proposed by Spiden et al. [7,8] as a simple and fast technique to monitor microalgal cell disruption. In addition, scanning electron microscopy (SEM) will be used to visualize the microstructure of the homogenized suspensions. Even though it cannot be considered a quantitative technique, SEM imaging provides direct insight into the stage of the disruption process [13]. A third method for evaluation of the disruption degree is the lipid extractability with the different solvent mixtures hexane:isopropanol (HI) and chloroform:methanol (CM), as proposed by Balduyck et al. [14] and Lemahieu et al. [15]. Finally, a

microscopic technique with the viability stain SYTOX green will be applied to investigate the integrity of the cell membrane [16]. By combining these four methods, this approach provides comprehensive insights into the disruption process of *Nannochloropsis* sp. cells upon (U)HPH.

2 Materials and Methods

2.1 Microalgal biomass

Lyophilized biomass of *Nannochloropsis* sp. was purchased from Proviron Industries nv (Hemiksem, Belgium). The microalgae were cultivated as described by Fret et al. [17], with slight modifications. In short, the microalgae were cultivated in ProviAPT flat panel photobioreactors illuminated with LED-lighting in a semi-continuous mode. About one third of the culture was harvested daily and substituted by fresh culture medium. The microalgae were harvested by centrifugation, lyophilized, and stored in closed containers at -80 °C until use.

Spray-dried biomass of *Chlorella vulgaris* obtained from Allmicroalgae Natural Products (Lisbon, Portugal) was used for SEM imaging, for comparing the disruption process to *Nannochloropsis* sp. biomass.

2.2 (Ultra) high pressure homogenization

Microalgal biomass was suspended overnight in demineralized water in a concentration of 0.1% (w/w) to ensure complete hydration of the microalgal cells. Additional suspensions were prepared in a concentration of 0.025% (w/w) for evaluation of turbidity according to Spiden et al. [7]. The disruption efficiency of (U)HPH is expected to be independent of the biomass concentration, as previously shown for *Nannochloropsis* sp. by Yap et al. [18]. All suspensions were treated by (U)HPH using a pressure cell homogenizer (Stansted Fluid Power SPCH-10, Harlow, United Kingdom), operating at 100 MPa or 250 MPa. The suspensions were at room temperature when fed into the homogenizer and the pressure cell was cooled with an external cooling unit connected to a cryostat at 4 °C to minimize temperature increases. However, this could not prevent sample heating at the highest pressures, as temperatures up to ~70 °C were measured immediately after UHPH at 250 MPa. The homogenized samples were collected and immediately cooled to room temperature in an ice water bath between different passes and before conducting analyses. Part of the homogenized suspensions were lyophilized (Christ Alpha 2-4 LSCplus, Osterode, Germany) for determination of the HI extraction efficiency. All other analyses were performed on the freshly homogenized

suspensions, within the same day of the (U)HPH-treatment. Suspensions at each concentration were prepared in twofold and (U)HPH-treated independently from its duplicate.

2.3 Evaluation of degree of cell disruption

2.3.1 Turbidity

Turbidity of the 0.025% (w/w) suspensions was determined according to Spiden et al. [7]. The turbidity was determined by measuring the optical density of the samples at 750 nm using a UV-VIS spectrophotometer (Ultrospec 2100 pro, Biochrom, Cambridge, United Kingdom). Turbidity measurements were performed in duplicate.

Turbidity values were normalized to be expressed as relative turbidity, according to **Eq. 1**:

$$RT_i = 1 - \frac{T_i - T_0}{T_9 - T_0} \quad \textbf{(Eq. 1)}$$

with RT_i the relative turbidity after i passes, T_i the measured transmission for a suspension homogenized for i passes, T_9 the measured transmission for the suspension homogenized by UHPH at 250 MPa for 9 passes (i.e. the maximum transmission), and T_0 the measured transmission for the untreated suspension (corresponding to 0 passes).

2.3.2 Scanning electron microscopy

Visualization of cell disruption by SEM was performed as described by Spiden et al. [19], with minor modifications. Glass coverslips were coated with a 0.1% solution of polyethyleneimine and dried by heating under a flame. Freshly homogenized suspensions were incubated on the glass coverslips for 1 h. Then, the coverslips were immersed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 1 h and subsequently rinsed three times in PBS for 10 min. The samples were subsequently dehydrated using increasing concentrations of ethanol, by immersing for 10 min in 10%, 30%, 50%, and 70% ethanol in water. The coverslips were transferred to a mixture of 70% ethanol and dimethoxymethane (1:1 v/v) for 5 min, followed by immersing in pure dimethoxymethane for 20 min. The coverslips were then dried in a critical point dryer (CPD 030, Balzers, Liechtenstein), in which the solvent was gradually replaced with liquid CO_2 (8 °C, 50 bar), followed by bringing the liquid CO_2 to the gaseous phase by trespassing the critical point of CO_2 (45 °C, 100 bar). The dried coverslips were then mounted onto aluminum stubs with double-sided carbon tabs and coated with gold using a sputter coater (SPI-Module, SPI Supplies, West-

Chester, PA, USA). Finally, images were obtained with a scanning electron microscope (JSM-6360, Jeol, Tokyo, Japan) using a spot size of 15 kV.

2.3.3 Hexane:isopropanol extraction efficiency

The HI extraction efficiency was determined as the ratio of the extraction yield with hexane:isopropanol (3:2 v/v) compared to the extraction yield with chloroform:methanol (1:1 v/v). The latter solvent mixture is known to extract the total amount of lipids, whereas HI does not easily penetrate intact rigid microalgal cells, resulting in a lower extraction yield [14,20].

The HI extraction was performed in duplicate as described by Balduyck et al. [14]. Hereto, 6 mL of hexane:isopropanol (3:2 v/v) was added to 100 mg of microalgal biomass and the mixtures were vortexed for 30 s. The samples were centrifuged (10 min, 750g, 25°C) and the solvent layer was transferred to a weighed flask. In total, these extraction steps were performed 4 times. All solvent layers were combined, the solvent was removed by rotary evaporation, and lipids were quantified gravimetrically.

The CM extraction was performed according to Ryckebosch, Muylaert, and Foubert [21]. Briefly, 4 mL methanol, 2 mL chloroform, and 0.4 mL demineralized water were added to 100 mg of microalgal biomass and the samples were vortexed for 30 s. Subsequently, 2 mL chloroform and 2 mL demineralized water were added and the samples were again vortexed for 30 s. The mixtures were centrifuged (10 min, 750g, 25 °C) and the upper aqueous layer was discarded, while the lower solvent layer was transferred to a clean tube. The remaining pellet was re-extracted with 4 mL of chloroform:methanol (1:1 v/v) and vortexed for 30 s. After centrifugation (10 min, 750g, 25 °C), the solvent phase was collected. This extraction procedure was repeated on the remaining pellet. All solvent layers were combined and filtered through a filter paper (Whatman n°1, Sigma-Aldrich) with a layer of sodium sulphate to remove remaining water. The solvent was finally removed by rotary evaporation and lipids were quantified gravimetrically. The CM extraction procedure was performed in triplicate.

2.3.4 Fluorescence microscopy using a viability stain

The viability of the cells was investigated by a dual-fluorescence procedure of Sato et al. [16], using the fluorescent dye SYTOX green. In short, 0.5 µL of SYTOX green solution (supplied as a 5 mM stock solution in DMSO, ThermoFisher Scientific) was added to 1 mL cell suspension, and the mixture was incubated for at least 5 min in the dark. The samples were visualized by epifluorescence microscopy, using

an Olympus BX-51 light microscope equipped with a XC-50 digital camera (Olympus, Optical Co.Ltd., Tokyo, Japan), and an excitation filter between 460 and 495 nm (X-Cite® 120Q, EXFO Europe, Hants, United Kingdom).

The fraction of cells stained with SYTOX green was quantified using ImageJ 1.52k software. To identify stained cells (i.e. green-colored cells), the color threshold was adjusted using L*a*b* values ($0 \leq L^* \leq 255$; $0 \leq a^* \leq 110$; $0 \leq b^* \leq 255$). The identified cells were automatically counted by software's 'Find maxima' tool, using a noise tolerance of 20 and excluding edge maxima. Cells with intact membranes (i.e. red-colored cells) were identified with different color thresholds ($0 \leq L^* \leq 255$; $140 \leq a^* \leq 255$; $0 \leq b^* \leq 255$) and counted using a noise tolerance of 15 and excluding edge maxima. The fraction of cells with damaged membranes (x) was finally calculated using **Eq. 2**:

$$x(\%) = \frac{N_g}{N_g + N_r} \times 100 \quad \textbf{(Eq. 2)}$$

with N_g the number of green-colored cells (i.e. with damaged membranes, stained by SYTOX green) and N_r the number of red-colored cells (i.e. with intact membranes, colored by autofluorescence). For each sample 20 microscopic images were analyzed, and only images displaying more than 10 cells were used.

3 Results and discussion

3.1 Turbidity

The impact of (U)HPH on the cell disruption of *Nannochloropsis* sp. was evaluated by different analyses. Although measuring the turbidity is an indirect quantification technique, it has been proposed as a fast and reproducible analysis to monitor cell disruption of *Nannochloropsis* sp., only giving slight underestimation of the degree of cell disruption compared to cell counting [8]. The observed changes in turbidity upon (U)HPH are presented in **Fig. 1**. A decrease in turbidity as a function of homogenization passes was in agreement with studies of Spiden et al. [7,8], corresponding to a reduction in the effective solid biomass concentration and the subsequent light scattering. A similar decay was observed for HPH at 100 MPa as in the study of Spiden et al. [8], and the relative turbidity of 0.33 ± 0.03 obtained after 9 passes coincides well with cell count data obtained by the same authors. The same relative turbidity (0.32 ± 0.08) was however obtained after a single pass when

applying a higher homogenization pressure of 250 MPa. As a matter of fact, these observations correspond well with the predictions of the exponential decay model for *Nannochloropsis* sp. constructed by Spiden et al. [8]. Similarly, the use of a homogenization pressure of 270 MPa led to disruption of 58% of *Nannochloropsis* sp. cells in the study of Angles et al. [22]. It might however still be an underestimation of the disruption degree, as Montalescot et al. [5] even reported > 95% of *Nannochloropsis oculata* cells to be disrupted after a single pass at 250 MPa based on cell counting. Moreover, the current study shows that a plateau value seems to be reached after 2 passes at 250 MPa, indicating that further UHPH caused little changes in turbidity. Hence, increasing the homogenization pressure obviously reduces the number of passes required to obtain a certain degree of cell disruption.

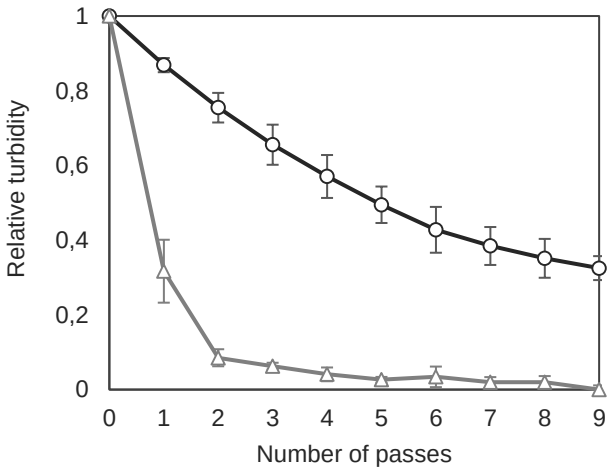


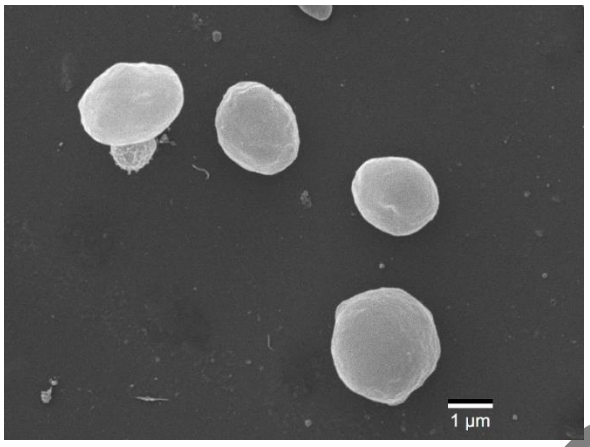
Fig. 1 Relative turbidity of *Nannochloropsis* sp. suspensions as a function of passes of (ultra) high pressure homogenization at 100 MPa (circles) and 250 MPa (triangles). Turbidity values were normalized to the value for untreated suspensions (0 passes). Error bars represent the standard error from duplicate sample preparation and duplicate measurements (n = 4).

It is worth noting that the applicability of a processing treatment (e.g. combination of passes and pressure level of (U)HPH) and/or quantification parameter (e.g. indirect versus direct technique) is largely depending on the depicted definition of cell disruption. In fact, cell disruption generally covers the whole range from damaged cells to completely fragmented cell compounds, as clearly illustrated by Spiden et al. [7]. In case of turbidity, the indirect nature of this evaluation technique might complicate the interpretation of the results obtained, since turbidity values are also affected by further fragmentation of released intracellular components, even after complete cell breakage. Therefore, turbidity curves were combined with SEM images in the present study.

3.2 Scanning electron microscopy

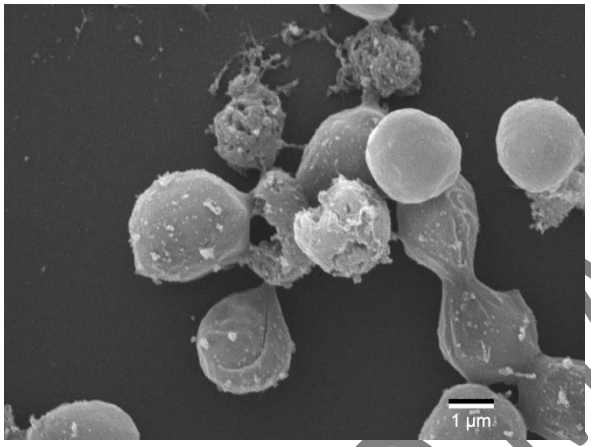
Intact cells of *Nannochloropsis* sp. can be described as spherical cells with a smooth cell surface and a diameter of approximately 2 µm [10,11], corresponding to the visual observations in **Fig. 2**. In addition, **Fig. 2** illustrates the impact of (U)HPH on the microstructure of *Nannochloropsis* sp. suspensions, showing SEM images after a different number of passes at different pressure levels (100 MPa and 250 MPa). To assess the effect of cell disruption in SEM images, intact cells were distinguished from damaged ones based on the smoothness of their cell surface. Hence, a single pass of HPH at 100 MPa resulted in the disruption of some microalgal cells, while the majority of the cells seemed unaffected. This corresponds to the turbidity curves (**Fig. 1**), indicating that less than 20% of the cells were disrupted under these HPH conditions. A low degree of cell disruption after a single pass at 100 MPa was also observed in our previous study, irrespective of the pH of the *Nannochloropsis* sp. suspensions [23]. The more passes of HPH at 100 MPa were applied, the lesser the number of undamaged cells were observed, and the larger the aggregates of released compounds. However, even after 4 passes at 100 MPa undamaged cells were still observed, indicating incomplete cell disruption at these conditions. This is in agreement with the turbidity results, since no plateau was reached after 4 passes of HPH at 100 MPa. Applying a higher pressure of 250 MPa obviously enhanced cell disruption, since aggregated material was observed together with a minority of undamaged cells, even after a single pass. A more efficient cell disruption by UHPH was also concluded from the turbidity curves, although it should be noted that temperature increases during UHPH at 250 MPa could also have contributed to larger changes in relative turbidity, e.g. by inducing heat-dependent reactions causing modified light scattering properties of the suspensions. Furthermore, intact cells were only sporadically encountered after 2 passes of UHPH at 250 MPa. In fact, the larger degree of cell disruption by UHPH is obvious from comparing the abundance of intact cells in SEM micrographs at lower magnification in contrast to HPH for 2 passes at 100 MPa (**Fig. 3**). Finally, no intact cells were observed after 4 passes of UHPH at 250 MPa. Hence, the latter UHPH conditions evidently lead to complete cell disruption, generating large aggregates of released cell material in which interactions seem to occur between proteins (globular structures) and cell wall fragments (smooth layers), amongst others. The visual appearance of the aggregates observed after 4 passes of UHPH at 250 MPa suggests a larger extent of network formation compared to milder homogenization conditions (i.e. lower number of passes and/or lower pressure level), probably related to the temperature increases that occurred during UHPH at 250 MPa (up to ~70 °C).

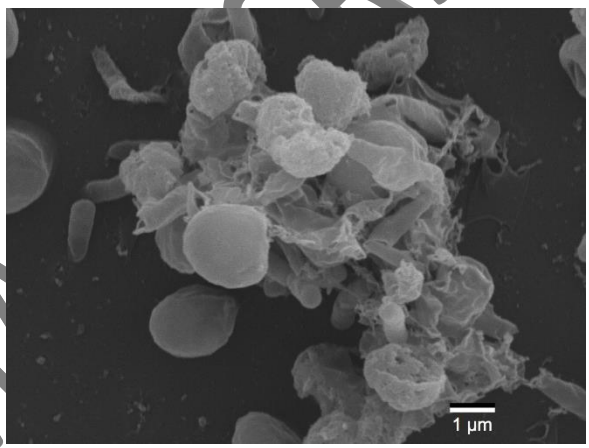
Untreated



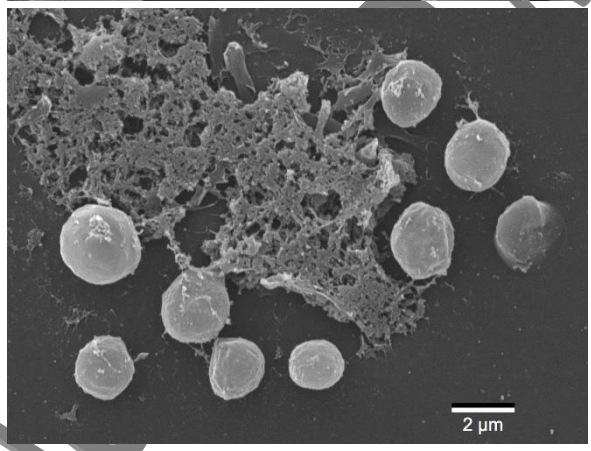
HPH 100 MPa **UHPH 250 MPa**

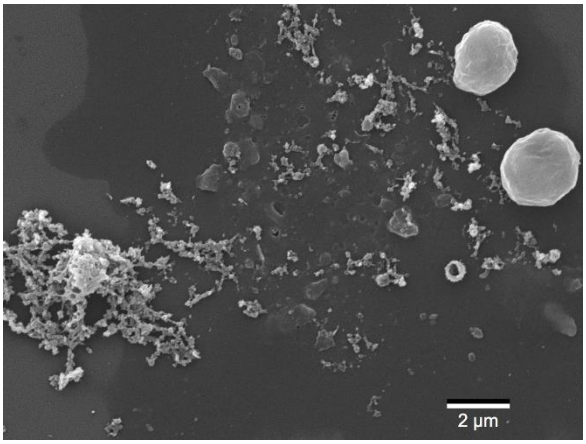
1 pass



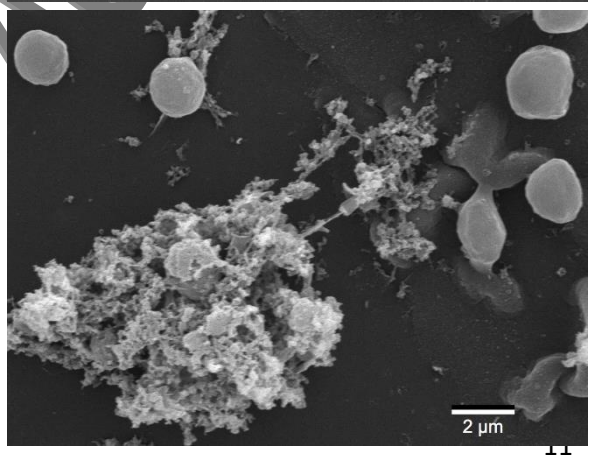


2 passes





4 passes



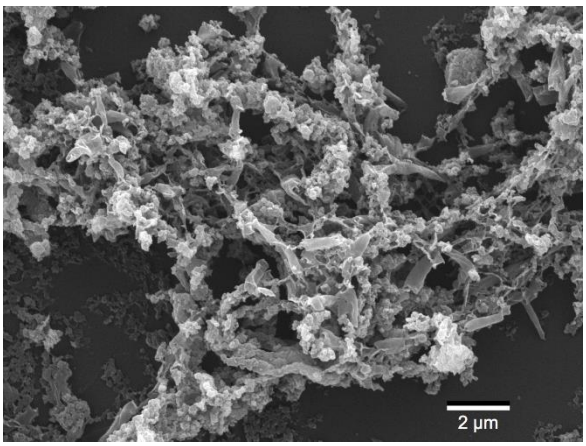


Fig. 2 Representative scanning electron microscopy images of *Nannochloropsis* sp. suspensions before (Untreated) and after different passes of high pressure homogenization (HPH 100 MPa) and ultra high pressure homogenization (UHPH 250 MPa).

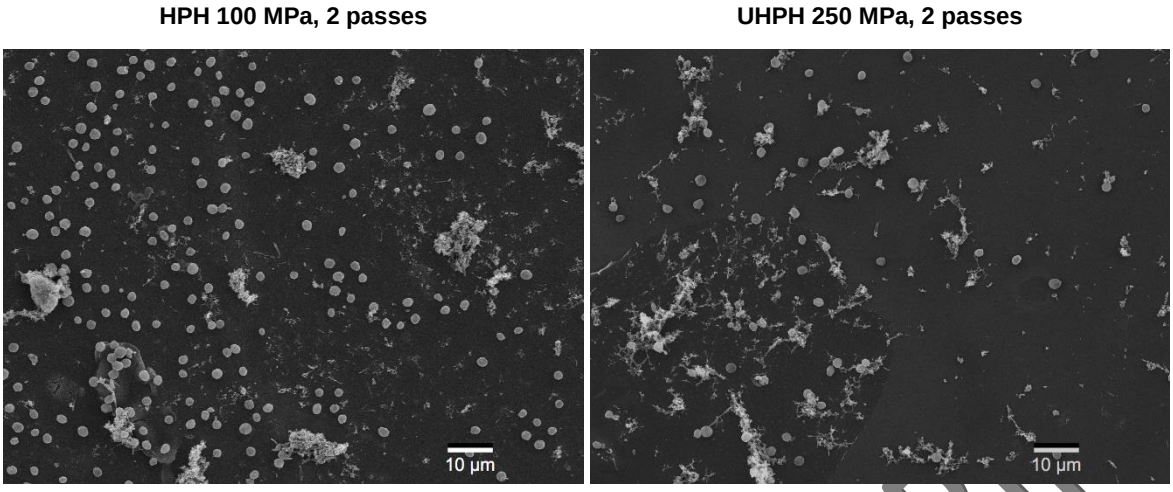


Fig. 3 Representative scanning electron microscopy images of *Nannochloropsis* sp. suspensions treated by two passes of high pressure homogenization (HPH 100 MPa) and ultra high pressure homogenization (UHPH 250 MPa).

The SEM images illustrated that *Nannochloropsis* sp. cells rather experience cell damaging by (U)HPH than disintegration of the cell wall. This was evidenced by the fact that (U)HPH mainly resulted in sphere-like structures with a roughened surface, and no cells were encountered with a partially broken cell wall layer under any of the (U)HPH conditions. In contrast, when applying a single pass of HPH at 100 MPa on *Chlorella vulgaris* suspensions, disintegration of the cell wall was clearly observed together with the release of intracellular material, as shown in **Fig. 4**. Similar cell breakage has been previously visualized for *Chlorella* sp. by Yap et al. [13] after HPH at 75 MPa. The difference in cell disruption mechanism between the two microalgae species is probably related to the composition of the cell wall, suggesting a higher rigidity and lower flexibility of the algaenan-based cell wall of *Nannochloropsis* species.

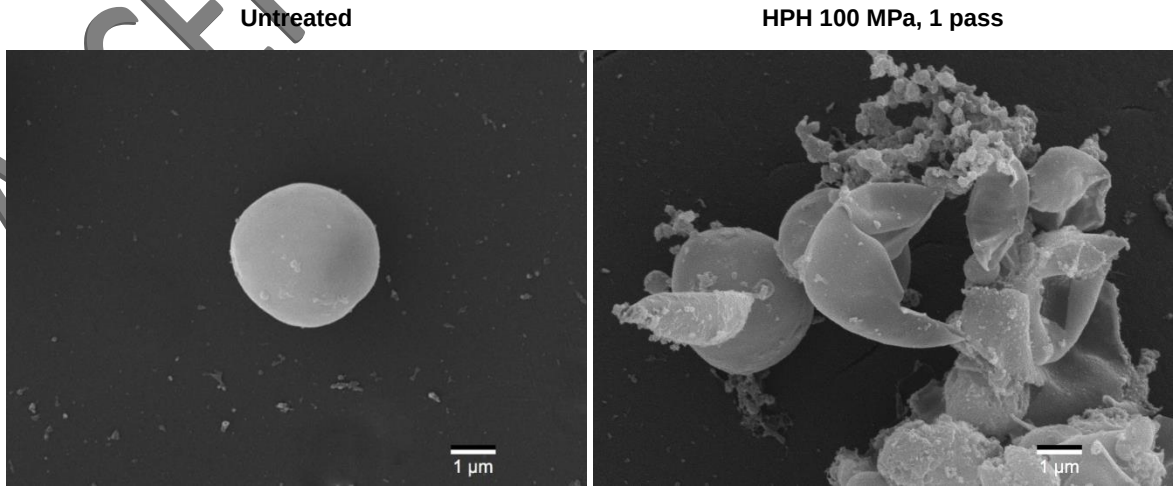


Fig. 4 Representative scanning electron microscopy images of *Chlorella vulgaris* cells before and after 1 pass of high pressure homogenization (HPH, 100 MPa).

Even though SEM imaging proves useful to get more insight into the impact of (U)HPH-treatments on the microstructure, the use of this technique for evaluation of the cell disruption degree has some drawbacks. First, assessment of the cell integrity is solely based on the visual appearance of the cells, and an irregular cell surface might also be induced by experimental defects such as the collapse during (incorrect) drying of the sample. Secondly, SEM imaging is a non-quantitative technique, complicating the interpretation at intermediate levels of cell disruption. Hence, combination of SEM imaging with a quantitative technique for evaluation of cell disruption is recommended, as previously suggested by other authors [13,19].

3.3 Hexane:isopropanol extraction efficiency

Comparison of lipid extraction yields obtained by different solvents has recently been suggested as a quantitative measure for the degree of cell disruption [14,15,24]. This is based on the fact that the non-halogenated solvent mixture HI is not able to penetrate into intact cells, whereas the halogenated solvent mixture CM has been demonstrated to extract the total amount of lipids regardless of the cell integrity [14,20,21]. Even though not specified by the authors, it is hypothesized that the lipid extractability with different solvent mixtures is not only related to the integrity of the cell wall, but also to the integrity of the cell membrane. In fact, Lee et al. [9] described that specific extraction solvents dissolve hydrophobic components of the cell membrane, and therefore only indirectly affect disruption of the cell wall by reducing its wall strength.

The HI extraction efficiency, given as the ratio of HI extraction yield to CM extraction yield, is shown in **Fig. 5** for the (U)HPH-treated *Nannochloropsis* sp. suspensions. HI extraction efficiency as a function of the number of homogenization passes follows a similar trend as observed in the turbidity curves (**Fig. 1**). In addition, the use of a higher homogenization pressure requires a reduced number of passes to obtain complete lipid extraction, since the plateau value seems to be reached after 2 passes for UHPH at 250 MPa, similarly as concluded from the turbidity curves. However, a less pronounced difference between the different pressures was observed from the HI extraction efficiency as compared to turbidity values. Moreover, suspensions homogenized at these different pressure levels tend to reach the same plateau value close to 100%, implying extraction of the total lipid content regardless of the homogenization pressure. A possible explanation for the discrepancy between turbidity and HI extraction efficiency might be the fact that absolute turbidity values were

largely influenced by heating of the sample at ultra high pressures (due to heat-induced reactions resulting in distinct light scattering properties of the sample), while HI extraction efficiency is assumed to be less affected by short-time temperatures increases during (U)HPH (except for possible microstructural changes facilitating or obstructing lipid extraction). In addition, it is likely that partial disruption of a microalgal cell might be sufficient for extraction of the lipids with HI, whereas turbidity measurements are sensitive to further fragmentation of the cell material.

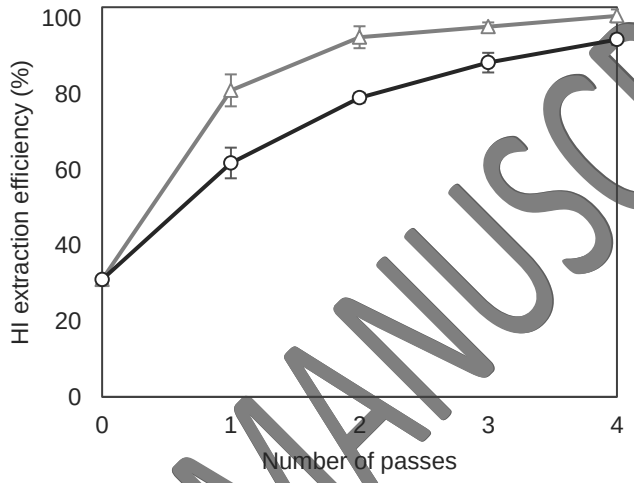


Fig. 5 Hexane:isopropanol (HI) extraction efficiency of *Nannochloropsis* sp. suspensions as a function of passes of (ultra) high pressure homogenization at 100 MPa (circles) and 250 MPa (triangles). HI extraction efficiency is defined as the ratio between the extraction yield with hexane:isopropanol (3:2 v/v) and the extraction yield with chloroform:methanol (1:1 v/v). Error bars represent the standard error from duplicate sample preparation and duplicate measurements (n = 4).

It is worth noting that a HI extraction efficiency of 31.0 ± 1.3% was observed for the untreated *Nannochloropsis* sp. biomass, even though the cells appeared to be intact from the SEM image based on their smooth cell surface (**Fig. 2**). HI extraction efficiencies between 17.3% and ~45% have actually been reported for untreated *Nannochloropsis* sp. biomasses in previous studies [14,15,24]. Since the dry biomass was suspended in demineralized water in the current study, membrane permeabilization might have occurred due to osmotic stress, possibly resulting in a higher extraction yield with HI. However, Balduyck et al. [24] reported an even higher HI extraction efficiency (~45%) for microalgal paste of *Nannochloropsis* sp., suggesting that the enhanced lipid extractability possibly results from the cultivation and/or harvesting to obtain the concentrated algal paste, rather than from resuspending dried biomass in demineralized water. Nevertheless, based on the SEM images, the HI extractability for the untreated suspension could not be related to any microstructural characteristics of the surface cell wall layer.

3.4 SYTOX green staining

In order to investigate the integrity of the cell membrane before and after (U)HPH, a dual-fluorescence microscopy method with SYTOX green fluorescent dye was used. SYTOX green is a nucleic acid stain which can only penetrate damaged cell membranes, thereby functioning as a viability stain [16]. This is illustrated in **Fig. 6**, representing a micrograph of the untreated *Nannochloropsis* sp. suspension. Cells with intact membranes can be observed as red-colored cells, resulting from autofluorescence, whereas cells with damaged membranes are recognized by the green fluorescence color of the SYTOX green dye that was able to penetrate into the cells. It is clearly shown that before applying any (U)HPH-treatment, about one third of the *Nannochloropsis* sp. cells showed damaged cell membranes. This microscopic observation corresponds well with the HI extraction efficiency of the untreated suspension (31.0 ± 1.3%), suggesting that the integrity of the cell membrane is presumably the limiting factor for lipid extraction with the HI solvent mixture.

This rather high amount of cells with damaged cell membranes in the untreated biomass suspensions demonstrates the impact of downstream processing of cultivated microalgal biomass (including harvesting and lyophilization) on the membrane integrity of *Nannochloropsis* sp. cells. Even though it is unclear whether the cell wall integrity was also affected by these processing steps, there are several reasons to believe that the impact of harvesting and lyophilization on the *Nannochloropsis* sp. cell wall might be limited. While some authors state that microalgal cell walls become more porous during the freezing step prior to lyophilization due to ice crystal formation in the intracellular environment, it is generally assumed that this only occurs when slow freezing is applied [9]. Moreover, cell rupture by freezing or freeze-drying processes has only been reported for fragile cell-walled species such as *Isochrysis galbana*, while this has not been observed for rigid cell-walled species such as *Nannochloropsis* sp. [25]. As a matter of fact, lyophilization is reported to preserve the cell constituents inside the cells and to not break cellulosic cell walls of microalgae [26]. The latter was confirmed in the current study, as cells with smooth surfaces were observed in SEM micrographs after fully rehydrating the lyophilized *Nannochloropsis* sp. biomass (**Fig. 2**). However, it cannot be excluded that the preceding lyophilization treatment caused some minor structural changes of the cell wall, which could have facilitated cell disruption during subsequent (U)HPH.

Microscopic images of (U)HPH-treated suspensions stained with SYTOX green are also shown in **Fig. 6**. When applying HPH at 100 MPa for multiple passes, the fraction of cells with intact membranes was noticeably reduced, with almost no intact cell membranes observed after 4 passes. This corresponds well with the HI extraction efficiency, as the plateau value for total lipid extraction seemed to be reached under these conditions (**Fig. 5**). Even though one might conclude that intact cells are still present after 4 passes of HPH at 100 MPa based on their visual appearance in SEM images, it is shown in **Fig. 6** that these remaining cells are damaged in terms of membrane integrity. This observation raises concerns about the use of cell counting as the reference method for evaluating microalgal cell disruption, as our results clearly show that the visual appearance of spherical smooth cells under light microscopy does not predict the integrity of the cell membrane, therefore presumably underestimating the degree of cell disruption in terms of membrane integrity. After UHPH at 250 MPa, cells with intact membranes were less abundant, in agreement with the higher HI extraction efficiency of UHPH-treated suspensions. Furthermore, the presence of the large aggregates can be observed from the micrographs. The different size and shapes of fluorescent spots after multiple passes of UHPH at 250 MPa suggests the staining of nucleic acids that have been released from the cells. This is also evidenced by a higher background fluorescence, indicating more nucleic acids being released into the aqueous phase of the *Nannochloropsis* sp. suspensions. It should be noted that the temperature increase during UHPH at 250 MPa might also have contributed to damaging the cell membranes. For instance, González-Fernández et al. [27] reported an enhanced SYTOX green staining of thermally pretreated cells of *Scenedesmus* sp. at 70 °C and 80 °C.

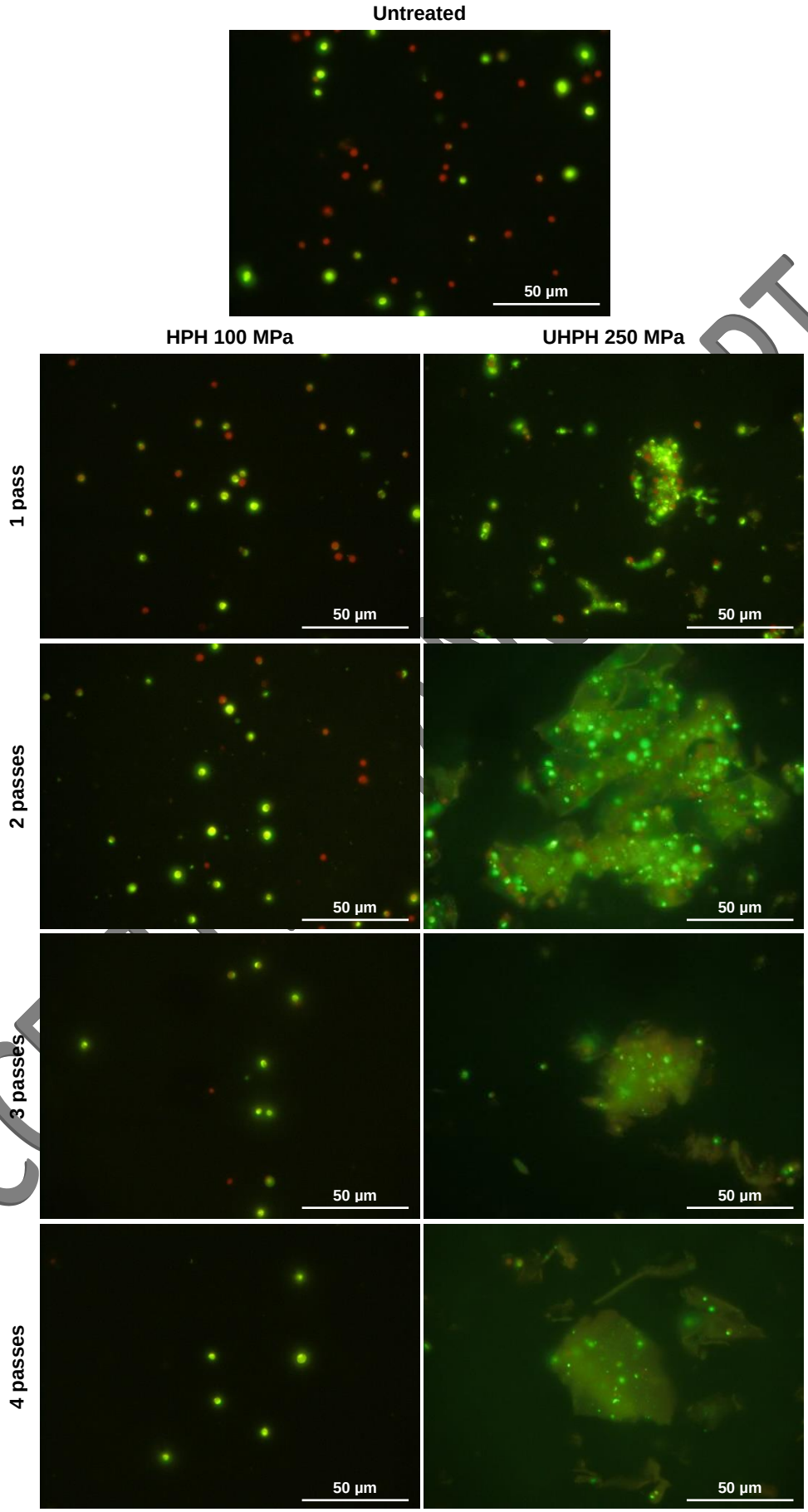


Fig. 6 Representative microscopic images of *Nannochloropsis* sp. suspensions before (Untreated) and after different passes of high pressure homogenization (HPH 100 MPa) and ultra high pressure homogenization (UHPH 250 MPa) stained with SYTOX green. Cells with intact cell membranes (only red-colored autofluorescence) can be distinguished from cells with damaged cell membranes (green fluorescence due to penetration of the SYTOX green dye).

The fraction of cells stained with SYTOX green was quantified using image analysis, and compared to the HI extraction efficiency in **Fig. 7**. The proportions of cells with damaged cell membranes estimated by these two techniques clearly coincide at all disruption stages, confirming the hypothesis that lipid extractability by these different solvent mixtures is related to the integrity of the cell membrane. Hence, both methods are considered reliable techniques for quantitative evaluation of the membrane integrity of microalgal cells, which might be relevant for screening microalgae species and/or processing conditions based on membrane disruption degrees as desired for specific applications. Even though the dual-fluorescence method with SYTOX green is the least time-consuming method, it should be noted that this technique is not applicable when substantial aggregate formation occurs. This was observed from the micrographs of UHPH-treated *Nannochloropsis* sp. suspensions in **Fig. 6**, in which cellular structures cannot be accurately identified due to the extensive disintegration and aggregate formation. In contrast, HI extraction efficiency can be performed regardless of the extent of cellular disintegration.

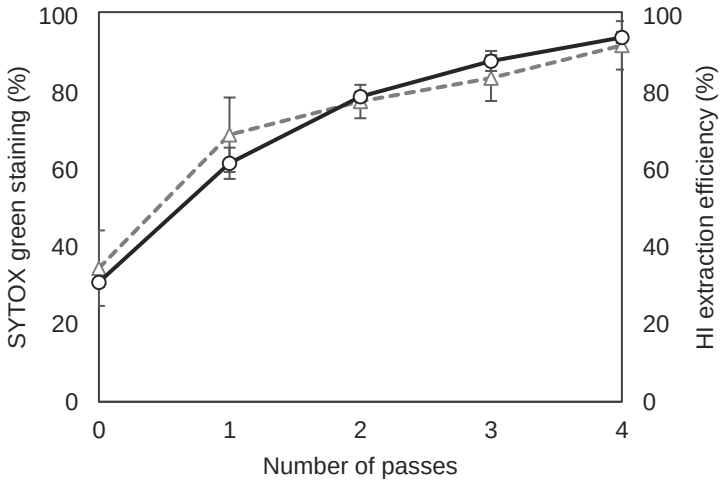


Fig. 7 Fraction of *Nannochloropsis* sp. cells with damaged cell membranes as a function of passes of high pressure homogenization at 100 MPa, determined by image analysis of microscopic images stained with SYTOX green (triangles, n = 20) and by hexane:isopropanol extraction efficiency (circles, n = 4).

Even though SYTOX green staining is directly related to the integrity of the cell membrane, it is worth noting that no conclusive information is gained on the integrity of the cell wall by use of this viability stain. In fact, it is generally assumed that membrane-impermeable fluorescent probes can passively diffuse through cell walls [28]. Contrary to this general statement, a limited staining in *Nannochloropsis* sp. has been observed for some microscopic dyes such as Nile Red, which has been attributed to a reduced penetration of the dye due to

the cell wall structure and/or cell wall thickness [29,30]. In case of SYTOX green, a limited penetration has been reported once for *Nannochloropsis* sp. [31], while other authors did not report any penetration issues for this dye [32,33]. Moreover, SYTOX green penetration was studied for PEF-treated cells of *Chlamydomonas reinhardtii* and *Scenedesmus* sp., i.e. for cells with electroporated membranes without distorted cell walls, showing successful staining with SYTOX green [34,35]. Hence, it is concluded that SYTOX green is a successful dye for nucleic acids in microalgal cells with damaged membranes, regardless of the cell wall integrity. Nevertheless, it should be kept in mind that changes in cell wall integrity might have occurred during (U)HPH (even for cells displaying a smooth surface in SEM images), which could not be investigated by use of SYTOX green staining.

4 Conclusions

The current study investigated the impact of (U)HPH on the disruption of the rigid *Nannochloropsis* sp. cells. It was obvious that a higher homogenization pressure drastically reduced the number of passes required to obtain a certain degree of cell disruption. However, heating of the sample occurred during UHPH at 250 MPa, which might not only lead to a different microstructure, but also to degradation of heat-labile compounds. In addition, it should be noted that the use of ultra high pressures also requires a higher energy input compared to moderate homogenization pressures. However, since the specific energy is not only proportional to the applied pressure level but also to the number of homogenization passes [36,37], the reduced number of passes required at ultra high pressures to obtain a specific degree of cell disruption might therefore even lead to a lower energy requirement, as previously shown for HPH of *Nannochloropsis* sp. between 100 and 150 MPa by Yap et al. [18]. Even though the specific energy can be further decreased by (U)HPH at high biomass concentrations (up to 25% w/w), (U)HPH of rigid cell walled microalgae is still considered an high energy-consuming disruption technique [2,18]. Finally, it was shown that (U)HPH not only affected the cell wall, but also the membrane integrity. While cells would be considered intact based on the smoothness of the cell surface in SEM images, epifluorescence microscopy with the viability stain SYTOX green revealed the damaged membranes of several cells after (U)HPH, which was confirmed by an improved lipid extractability

with HI. Hence, an accurate definition of cell disruption (i.e. membrane damaging vs. disintegration of the cell wall) for a specific application is crucial in order to select appropriate (U)HPH conditions.

Acknowledgements

Tom Bernaerts and Lore Gheysen are PhD fellows funded by the Research Foundation Flanders (FWO SB PhD fellowships 1S 099 18N and 1S 270 18N). In addition, financial support was obtained from the Research Fund KU Leuven (KP/14/004). We would like to thank Alexander Vrijdaghs (Plant Conservation and Population Biology, KU Leuven, Belgium) for access to and assistance with the scanning electron microscope.

Contributions of the authors

All authors made substantial contributions to this work: T.M.M.B., M.E.H. and A.M.V.L. designed the experiments; T.M.M.B. performed the experimental work; T.M.M.B., L.G., I.F. and A.M.V.L. interpreted and discussed the data; and T.M.M.B. wrote the manuscript. All authors critically revised the manuscript and gave their final approval for submission.

Conflict of interest

The authors have declared no conflict of interest.

References

[1] S.Y. Lee, J.M. Cho, Y.K. Chang, Y. Oh, Cell disruption and lipid extraction for microalgal biorefineries: A review, Bioresour. Technol. 244 (2017) 1317–1328. doi:10.1016/j.biortech.2017.06.038.

[2] E. Günerken, E. D'Hondt, M.H.M. Eppink, L. Garcia-Gonzalez, K. Elst, R.H. Wijffels, Cell disruption for microalgae biorefineries, Biotechnol. Adv. 33 (2015) 243–260. doi:10.1016/j.biotechadv.2015.01.008.

[3] C. Safi, C. Frances, A. V Ursu, C. Laroche, C. Pouzet, C. Vaca-Garcia, P. Pontalier, Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins and pigments in the aqueous phase, Algal Res. 8 (2015) 61–68. doi:10.1016/j.algal.2015.01.002.

[4] C. Safi, A. V Ursu, C. Laroche, B. Zebib, O. Merah, P. Pontalier, C. Vaca-Garcia, Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods, Algal Res. 3 (2014) 61–65. doi:10.1016/j.algal.2013.12.004.

[5] V. Montalescot, T. Rinaldi, R. Touchard, S. Jubeau, M. Frappart, P. Jaouen, P. Bourseau, L. Marchal, Optimization of bead milling parameters for the cell disruption of microalgae: Process modeling and application to Porphyridium cruentum and Nannochloropsis oculata, Bioresour. Technol. 196 (2015) 339–346. doi:10.1016/j.biortech.2015.07.075.

[6] S. Jubeau, L. Marchal, J. Pruvost, P. Jaouen, J. Legrand, J. Fleurence, High pressure disruption: a two-step treatment for selective extraction of intracellular components from the microalga Porphyridium cruentum, J. Appl. Phycol. 25 (2013) 983–989. doi:10.1007/s10811-012-9910-5.

[7] E.M. Spiden, P.J. Scales, S.E. Kentish, G.J.O. Martin, Critical analysis of quantitative indicators of cell disruption applied to Saccharomyces cerevisiae processed with an industrial high pressure homogenizer,

Biochem. Eng. J. 70 (2013) 120–126. doi:10.1016/j.bej.2012.10.008.

[8] E.M. Spiden, B.H.J. Yap, D.R.A. Hill, S.E. Kentish, P.J. Scales, G.J.O. Martin, Quantitative evaluation of the ease of rupture of industrially promising microalgae by high pressure homogenization, Bioresour. Technol. 140 (2013) 165–171. doi:10.1016/j.biortech.2013.04.074.

[9] A.K. Lee, D.M. Lewis, P.J. Ashman, Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements, Biomass and Bioenergy. 46 (2012) 89–101. doi:10.1016/j.biombioe.2012.06.034.

[10] M. Alhattab, A. Kermanshahi-Pour, M.S. Brooks, Microalgae disruption techniques for product recovery: influence of cell wall composition, J. Appl. Phycol. 31 (2019) 61–88. doi:10.1007/s10811-018-1560-9.

[11] K.P. Fawley, M.W. Fawley, Observations on the diversity and ecology of freshwater Nannochloropsis (Eustigmatophyceae), with descriptions of new taxa, Protist. 158 (2007) 325–336. doi:10.1016/j.protis.2007.03.003.

[12] M.J. Scholz, T.L. Weiss, R.E. Jinkerson, J. Jing, R. Roth, U. Goodenough, M.C. Posewitz, H.G. Gerken, Ultrastructure and composition of the Nannochloropsis gaditana cell wall, Eukaryot. Cell. 13 (2014) 1450–1464. doi:10.1128/EC.00183-14.

[13] B.H.J. Yap, S.A. Crawford, G.J. Dumsday, P.J. Scales, G.J.O. Martin, A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction, Algal Res. 5 (2014) 112–120. doi:10.1016/j.algal.2014.07.001.

[14] L. Balduyck, T. Stock, S. Bijttebier, C. Bruneel, G. Jacobs, S. Voorspoels, K. Muylaert, I. Foubert, Integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage, Algal Res. 25 (2017) 516–524. doi:10.1016/j.algal.2017.06.013.

[15] C. Lemahieu, C. Bruneel, C. Dejonghe, J. Buyse, I. Foubert, The cell wall of autotrophic microalgae influences the enrichment of long chain omega-3 fatty acids in the egg, Algal Res. 16 (2016) 209–215. doi:10.1016/j.algal.2016.03.015.

[16] M. Sato, Y. Murata, M. Mizusawa, H. Iwahashi, S. Oka, A simple and rapid dual-fluorescence viability assay for microalgae, Microbiol. Cult. Collect. 20 (2004) 53–59.

[17] J. Fret, L. Roef, R. Blust, L. Diels, S. Tavernier, W. Vyverman, M. Michiels, Reuse of rejuvenated media during laboratory and pilot scale cultivation of Nannochloropsis sp., Algal Res. 27 (2017) 265–273. doi:10.1016/j.algal.2017.09.018.

[18] B.H.J. Yap, G.J. Dumsday, P.J. Scales, G.J.O. Martin, Energy evaluation of algal cell disruption by high pressure homogenisation, Bioresour. Technol. 184 (2015) 280–285. doi:10.1016/j.biortech.2014.11.049.

[19] E.M. Spiden, P.J. Scales, B.H.J. Yap, S.E. Kentish, D.R.A. Hill, G.J.O. Martin, The effects of acidic and thermal pretreatment on the mechanical rupture of two industrially relevant microalgae: Chlorella sp. and Navicula sp., Algal Res. 7 (2015) 5–10. doi:10.1016/j.algal.2014.11.006.

[20] E. Ryckebosch, C. Bruneel, R. Termote-Verhalle, K. Muylaert, I. Foubert, Influence of extraction solvent system on extractability of lipid components from different microalgae species, Algal Res. 3 (2014) 36–43. doi:10.1016/j.algal.2013.11.001.

[21] E. Ryckebosch, K. Muylaert, I. Foubert, Optimization of an analytical procedure for extraction of lipids from microalgae, J. Am. Oil Chem. Soc. 89 (2012) 189–198. doi:10.1007/s11746-011-1903-z.

[22] E. Angles, P. Jaouen, J. Pruvost, L. Marchal, Wet lipid extraction from the microalga Nannochloropsis sp.: Disruption, physiological effects and solvent screening, Algal Res. 21 (2017) 27–34. doi:10.1016/j.algal.2016.11.005.

[23] T.M.M. Bernaerts, A. Panozzo, V. Doumen, I. Foubert, L. Gheysen, K. Goiris, P. Moldenaers, M.E. Hendrickx, A.M. Van Loey, Microalgal biomass as a (multi)functional ingredient in food products: Rheological properties of microalgal suspensions as affected by mechanical and thermal processing, Algal Res. 25 (2017) 452–463. doi:10.1016/j.algal.2017.05.014.

[24] L. Balduyck, C. Bruneel, K. Goiris, C. Dejonghe, I. Foubert, Influence of high pressure homogenization on free fatty acid formation in Nannochloropsis sp., Eur. J. Lipid Sci. Technol. 120 (2018) 1–6. doi:10.1002/ejlt.201700436.

[25] J.P. Cañavate, C. Fernández-Díaz, Pilot evalutation of freeze-dried microalgae in the mass rearing of gilthead seabream (Sparus aurata) larvae, Aquaculture. 193 (2001) 257–269. doi:10.1016/S0044-8486(00)00492-0.

[26] E.W. Becker, Microalgae: Biotechnology and Microbiology, Cambridge University Press, 1994.

[27] C. González-Fernández, B. Sialve, N. Bernet, J.P. Steyer, Comparison of ultrasound and thermal pretreatment of

Scenedesmus biomass on methane production, Bioresour. Technol. 110 (2012) 610–616. doi:10.1016/j.biortech.2012.01.043.

[28] P. Lebaron, P. Catala, N. Parthuisot, Effectiveness of SYTOX green stain for bacterial viability assessment, Appl. Environ. Microbiol. 64 (1998) 2697–2700.

[29] L. Balduyck, C. Veryser, K. Goiris, C. Bruneel, K. Muylaert, I. Foubert, Optimization of a Nile Red method for rapid lipid determination in autotrophic, marine microalgae is species dependent, J. Microbiol. Methods. 118 (2015) 152–158. doi:10.1016/j.mimet.2015.09.009.

[30] J. Rumin, H. Bonnefond, B. Saint-Jean, C. Rouxel, A. Sciandra, O. Bernard, J. Cadoret, G. Bougaran, The use of fluorescent Nile red and BODIPY for lipid measurement in microalgae, Biotechnol. Biofuels. 8 (2015) 42–57. doi:10.1186/s13068-015-0220-4.

[31] M.J.W. Veldhuis, T.L. Cucci, M.E. Sieracki, Cellular DNA content of marine phytoplankton using two new fluorochromes: Taxonomic and ecological implications, J. Phycol. 33 (1997) 527–541. doi:10.1111/j.0022-3646.1997.00527.x.

[32] R. Halim, D.R.A. Hill, E. Hanssen, P.A. Webley, S. Blackburn, A.R. Grossman, C. Posten, G.J.O. Martin, Towards sustainable microalgal biomass processing: anaerobic induction of autolytic cell-wall self-ingestion in lipid-rich Nannochloropsis slurries, Green Chem. 21 (2019) 2967–2982. doi:10.1039/c8gc03186j.

[33] S.W. Jeong, S.W. Nam, K. Hwangbo, W.J. Jeong, B. Jeong, Y.K. Chang, Y. Park, Transcriptional regulation of cellulose biosynthesis during the early phase of nitrogen deprivation in Nannochloropsis salina, Nat. Sci. Reports. 7 (2017) 5264–5274. doi:10.1038/s41598-017-05684-4.

[34] P. Bodénès, S. Bensalem, O. Français, D. Pareau, B. Le Pioufle, F. Lopes, Inducing reversible or irreversible pores in Chlamydomonas reinhardtii with electroporation: Impact of treatment parameters, Algal Res. 37 (2019) 124–132. doi:10.1016/j.algal.2018.11.016.

[35] Y.S. Lai, P. Parameswaran, A. Li, M. Baez, B.E. Rittmann, Effects of pulsed electric field treatment on enhancing lipid recovery from the microalga, Scenedesmus, Bioresour. Technol. 173 (2014) 457–461. doi:10.1016/j.biortech.2014.09.124.

[36] C. Safi, L. Cabas Rodriguez, W.J. Mulder, N. Engelen-Smit, W. Spekking, L.A.M. van den Broek, G. Olivieri, L. Sijtsma, Energy consumption and water-soluble protein release by cell wall disruption of Nannochloropsis gaditana, Bioresour. Technol. 239 (2017) 204–210. doi:10.1016/j.biortech.2017.05.012.

[37] N. Grimi, A. Dubois, L. Marchal, S. Jubeau, N.I. Lebovka, E. Vorobiev, Selective extraction from microalgae Nannochloropsis sp. using different methods of cell disruption, Bioresour. Technol. 153 (2014) 254–259. doi:10.1016/j.biortech.2013.12.011.