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# Impact of emerging technologies on the cell disruption and fractionation of microalgal biomass

Rui Zhang

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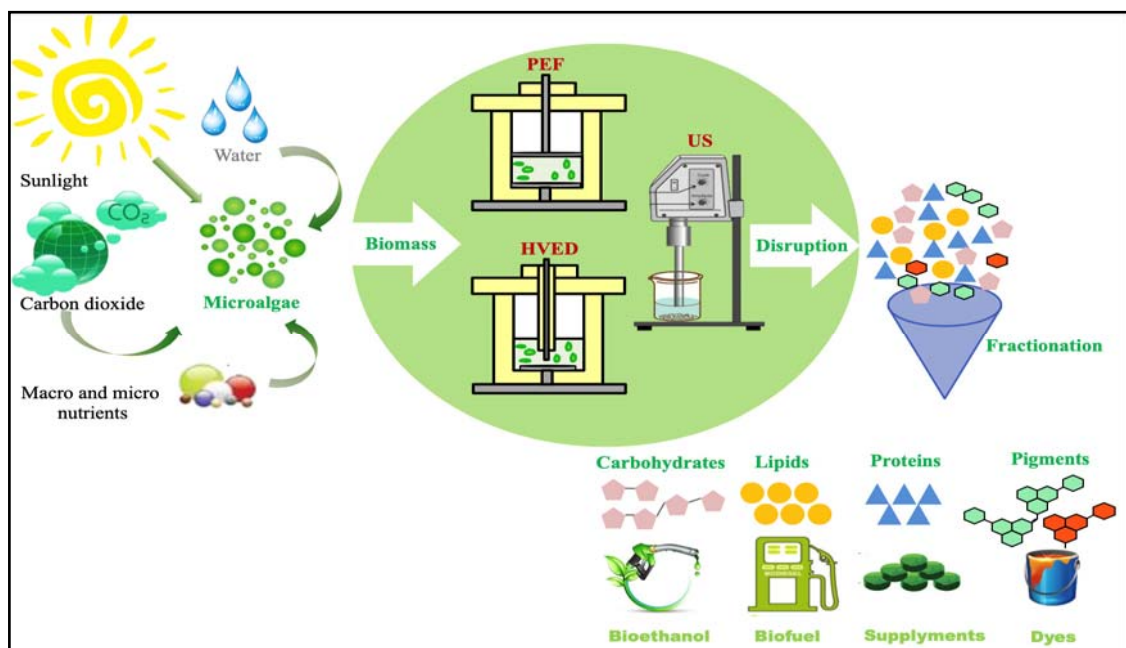
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Par Rui ZHANG

*Impact of emerging technologies on the cell disruption and fractionation of microalgal biomass*

Thèse présentée  
pour l'obtention du grade  
de Docteur de l'UTC



Soutenu le 8 juin 2020

**Spécialité :** Génie des Procédés Industriels et Bioprocédés :  
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Spécialité: Génie des Procédés Industriels et Bioprocédés

Par **Rui ZHANG**

<p style="text-align: center;"><b>IMPACT OF EMERGING TECHNOLOGIES ON THE CELL DISRUPTION AND FRACTIONATION OF MICROALGAL BIOMASS</b></p>
--

Soutenue le 08 June 2020

Devant la commission d'examen formée de:

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*... the memory of my grandmother who has passed away  
and was not able to see me graduate*

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## Abstract

This research work focuses on extraction and fractionation of bio-molecules from microalgae using physical treatments: pulsed electric fields (PEF), high voltage electrical discharges (HVED) and ultrasonication (US) techniques. In this study, three microalgae species *Nannochloropsis* sp., *Phaeodactylum tricornutum* (*P. tricornutum*) and *Parachlorella kessleri* (*P. kessleri*) were investigated. These species have different cell shapes, structure and intracellular contents. The effects of tested techniques on extraction of bio-molecules have been highlighted in a quantitative and qualitative analysis by evaluating the ionic components, carbohydrates, proteins, pigments and lipids.

A comparative study of physical treatments (PEF, HVED and US) at the equivalent energy input for release of intracellular bio-molecules from three microalgal species allowed us to better understand the different disintegration mechanisms. For each microalga at the same energy consumption, the HVED treatment proved to be the most efficient for extraction of carbohydrates, while the US treatment for extraction of proteins and pigments. In general, the smallest efficiency was observed for the PEF treatment. However, the highest selectivity towards carbohydrates can be obtained using the mild PEF or HVED technique.

The preliminary physical treatments (PEF, HVED or US) of more concentrated suspensions followed by high pressure homogenization (HPH) of diluted suspensions allowed improving the extraction efficiency and decreasing the total energy consumption. The physical pretreatments permit to reduce the mechanical pressure of the HPH and number of passes, to reach the same extraction yield. For the maximum valorisation of microalgal biomass, extraction procedure assisted by HVED treatment (40 kV/cm, 1-8 ms) followed by aqueous and non-aqueous extraction steps seems to be useful for selective extraction and fractionation of different bio-molecules from microalgae. The significant effects of HVED pre-treatment on organic solvent extraction of pigments (chlorophylls, carotenoids) and lipids were also observed.

**Keywords:** Microalgae; Pulsed electric field; High voltage electrical discharges; Ultrasonication; High pressure homogenization; Selective extraction; Bio-molecules; Energy consumption

## Résumé

Ce travail de recherche se concentre sur l'extraction et le fractionnement des biomolécules à partir de microalgues par des traitements physiques: les champs électriques pulsés (CEP), les décharges électriques de hautes tensions (DEHT) et les ultrasons (US). Dans cette étude, trois espèces de microalgues *Nannochloropsis* sp., *Phaeodactylum tricornutum* (*P. tricornutum*) et *Parachlorella kessleri* (*P. kessleri*) ont été étudiées. Les espèces ont différentes formes cellulaires, structure et contenu intracellulaire. L'effet des techniques testées sur l'extraction des biomolécules a été mis en évidence à travers une analyse quantitative et qualitative: suivi du rendement des composés ioniques, des glucides, des protéines, des pigments et des lipides.

Une étude comparative des traitements physiques (CEP, DEHT et US), à la même énergie, pour la libération des biomolécules intracellulaires à partir des trois espèces de microalgues, a permis de mieux comprendre les différents mécanismes de désintégration. Pour chaque microalgue, à la même énergie consommée, le traitement par DEHT s'est révélé le plus efficace en terme d'extraction des glucides, tandis que les US sont plus efficaces pour l'extraction des protéines et des pigments. Le traitement par CEP a été moins efficace en terme du rendement d'extraction. Cependant, la meilleure sélectivité (extraction des glucides) a été obtenue en utilisant les CEP ou les DEHT.

Les prétraitements physiques (CEP, DEHT ou US) des suspensions plus concentrées suivis d'une homogénéisation haute pression (HHP) de suspensions diluées ont permis d'améliorer l'efficacité de l'extraction et de diminuer la consommation énergétique totale et le nombre de passages. Le prétraitement physique permet de réduire la pression mécanique de l'HHP, pour atteindre le même rendement d'extraction. Pour la valorisation maximale de la biomasse de microalgues, une procédure d'extraction assistée par DEHT (40 kV/cm, 1-8 ms) suivie de plusieurs étapes d'extraction aqueuses et non aqueuses semble être utile pour l'extraction sélective et le fractionnement de différentes biomolécules à partir de microalgues. Des effets significatifs du prétraitement HVED sur l'extraction par solvant organique des pigments (chlorophylles, caroténoïdes) et des lipides ont été observés.

**Mots-clés:** Microalgues; Champ électrique pulsé; Décharges électriques de haute tension; Ultrason; Homogénéisation haute pression; Extraction sélective; Biomolécules; Énergie consommée

## List of publications

### I. Journals:

- (1) Zhang, R., Lebovka, N., Marchal, L., Vorobiev, E., & Grimi, N. (2020). Multistage aqueous and non-aqueous extraction of bio-molecules from microalga *Phaeodactylum tricornutum*. *Innovative Food Science and Emerging Technologies*, 102367.
- (2) Zhang, R., Lebovka, N., Marchal, L., Vorobiev, E., & Grimi, N. (2020). Pulsed electric energy and ultrasonication assisted green solvent extraction of bio-molecules from different microalgal species, *Innovative Food Science and Emerging Technologies*, 102358.
- (3) Zhang, R., Lebovka, N., Marchal, L., Vorobiev, E., & Grimi, N. (2020). Comparison of aqueous extraction assisted by pulsed electric energy and ultrasonication: Efficiencies for different microalgal species. *Algal Research*, 101857.
- (4) Zhang, R., Marchal, L., Lebovka, N., Vorobiev, E., & Grimi, N. (2020). Two-step procedure for selective recovery of bio-molecules from microalga *Nannochloropsis oculata* assisted by high voltage electrical discharges. *Bioresource Technology*, 302, 122893
- (5) Zhang, R., Grimi, N., Marchal, L., Lebovka, N., & Vorobiev, E. (2019). Effect of ultrasonication, high pressure homogenization and their combination on efficiency of extraction of bio-molecules from microalgae *Parachlorella kessleri*. *Algal Research*, 40, 101524.
- (6) Zhang, R., Parniakov, O., Grimi, N., Lebovka, N., Marchal, L., & Vorobiev, E. (2019). Emerging techniques for cell disruption and extraction of valuable bio-molecules of microalgae *Nannochloropsis* sp. *Bioprocess and Biosystems Engineering*, 42(2), 173-186.
- (7) Zhang, R., Grimi, N., Marchal, L., & Vorobiev, E. (2019). Application of high-voltage electrical discharges and high-pressure homogenization for recovery of intracellular compounds from microalgae *Parachlorella kessleri*. *Bioprocess and Biosystems Engineering*, 42(1), 29–36.
- (8) Zhang, R., Marchal, L., Vorobiev, E., & Grimi, N. Effect of combined pulsed electric energy and high pressure homogenization on selective and energy efficient extraction of bio-molecules from microalga *Parachlorella kessleri*, submitted to *LWT*

## II. Conferences

### ➤ Oral presentation:

(1) Zhang R., Grimi N., Lebovka N., Marchal L., Vorobiev E. High voltage electrical discharges and vacuum drying assisted selective extraction of bio-molecules from microalga *Nannochloropsis oculata*. *3<sup>rd</sup> World Congress on Electroporation & Pulsed Electric Fields in Biology, Medicine, Food and Environmental Technologies*, September 3-6, 2019, Toulouse, France.

### ➤ Poster presentation:

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(2) Zhang R., Grimi N., Lebovka N., Marchal L., & Vorobiev E. Ultrasound and high pressure homogenization assisted extraction of bio-molecules from microalga *Parachlorella kessleri*: Process and specific energy requirements. *3<sup>rd</sup> World Congress on Electroporation & Pulsed Electric Fields in Biology, Medicine, Food and Environmental Technologies*, September 3-6, 2019, Toulouse, France.

(3) Zhang R., Grimi N., Lebovka N., Marchal L., Vorobiev E. Extraction of bio-molecules from the microalga *Parachlorella kessleri* by pulsed electric technologies and high pressure homogenization. *Journée Scientifique Et Technique: Champs électriques pulsés et autres technologies innovantes pour la valorisation des agro-ressources: de la recherche à l'industrie*. Février 6, 2018, Compiègne, France.

(4) Zhang R., Grimi N., Lebovka N., Marchal L., & Vorobiev E. Extraction of intracellular components from the microalga *Parachlorella kessleri* by combining pulsed electric technologies and high pressure homogenization. *2<sup>nd</sup> World Congress on Electroporation & Pulsed Electric Fields in Biology Medicine, Food and Environmental Technologies*, September 24-28, 2017 Norfolk (VA), USA.



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## General Introduction

Nowadays, there is an increasing demand for exploration and exploitation of sustainable food, feed, cosmetic, pharmaceutical and bio-fuel feedstocks as an alternative for traditional agricultural crops (Postma et al., 2017). Microalgae have been so far identified as a promising source due to their rapid growth rate, ability to live in all existing earth ecosystems, such as marine, freshwater (ponds, puddles, canals, and lakes) and terrestrial habitats (Khili, 2013; Mata et al., 2010). They are able to efficiently produce valuable bio-molecules (such as proteins, carbohydrates, lipids, pigments and polyphenols, etc), over short periods of time by the photosynthesis (Khili, 2013). For example, some microalgal species contain high levels of lipids (up to 75 wt%) and they have been considered as most promising feedstocks to produce biodiesel (Hernández-Pérez et al., 2019; Veillette et al., 2017). Microalgal proteins can be used instead of conventional food supplements due to their nutritional values and amino acid profiles (Becker, 2007), and polysaccharides can be hydrolyzed to reduced sugars which have potential for the production of bioethanol (Fu et al., 2010).

In dependence of cultivation conditions different microalgal species may have rather different biomass compositions (Alhatab et al., 2019). For example, *Nannochloropsis* sp., *Phaeodactylum tricornutum* (*P. tricornutum*), and *Parachlorella kessleri* (*P. kessleri*) are promising microalgae source, that can rapidly accumulate biomass, starchs, proteins and lipids. Under unfavourable growth conditions (lack of light, nutrient stress, nitrogen starvation), these cultures can accumulate large amounts of energy-rich compounds such as triglycerides (TAG) and starchs (Taleb et al., 2018). However, these microalgae have different cell shapes and structures. The green marina microalgae *Nannochloropsis* sp. belongs to family Eustigmataceae, which present collection of six species of *Nannochloropsis* (*gaditana*, *granulate*, *limnetica*, *oceanica*, *oculata*, *salina*) (Zhang et al., 2018). The cells of *Nannochloropsis* sp. are spherical or slightly ovoid (2–4 µm in diameter) (Alhatab et al., 2019). *P. tricornutum* is a typical unicellular diatom, was found throughout marine and freshwater environments (Xu et al., 2010). *P. tricornutum* is also the only species of microalgae that can exist in three morphotypes (fusiform, triradiate, and oval) (Flori et al., 2016). The cells of *P. tricornutum* are fusiform with a length of 20–30 µm and a diameter of 1–3 µm (Alhatab et al., 2019). The green microalga *P. kessleri* is a unicellular fresh organism (Chlorophyta), their cells are near spherical (3–4 µm in diameter) (Alhatab et al., 2019). The *Nannochloropsis* sp. and *P. kessleri* cells have the rigid cell walls mainly composed of

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cellulose and hemicelluloses (Payne and Rippingale, 2000), and cell wall of *P. tricornutum* is very poor in silica and composed of different organic compounds, particularly sulfated glucomannan (Francius et al., 2008).

For maximum valorisation of microalgal biomass, the extraction of high purity of intracellular bio-molecules from microalgal biomass is the crucial step. However, these bio-molecules are usually enclosed in intracellular vacuoles and chloroplasts, protected by the rigid cell walls and membranes, thus greatly limiting their recovery during the process of extraction. For the recovery of both hydrophilic and hydrophobic microalgal bio-molecules, the wet route processing (with no preliminary drying) is the possible most adopted and low-energy demand strategy due to reduces the process cost of dewatering, and it starts with the hydrophilic compounds (e.g. carbohydrates and proteins) release in the aqueous phase (Orr et al., 2015; Zinkoné et al., 2018). By contrast, the recovery of bio-molecules from dry microalgae requires a large amount of energy for drying process, and may lead to losses in valuable compounds through oxidation caused by high temperature (Luengo et al., 2015). In this line, different cell disruption/extraction techniques have been applied in the last decades. The most commonly used techniques are depending on the chemical/mechanical methods, such as chemical treatments (solvent, acids), supercritical fluids, high pressure homogenization (HPH), bead milling, etc (Grimi et al., 2014). However, they suffer from some disadvantages like high temperature, high pressure and long treatment time.

In this line, ultrasonication (US) has been used to assist extraction of bio-molecules from microalgal species (Parniakov et al., 2015a). This technology can disrupt microalgal cell walls based on the cavitation phenomena, favored improve the extraction efficiency and decrease solvent consumption and extraction time. Moreover, compared to other emerging methods, it is a well-known technology with low capital cost and can be easily implemented in the field of industry (Barba et al., 2015b). Recently, the application of pulsed electric energy (pulsed electric field (PEF) and high voltage electric discharges (HVED)) technologies were shown to be promising for recovery of bio-molecules from bio-suspensions (Vorobiev et al., 2012). The PEF treatment appeared to be useful for extraction of pigments, proteins, polyphenol, lipids from microalgal species (*Nannochloropsis* sp., *Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Dunaliella salina*) (Foltz, 2012; Parniakov et al., 2015b, 2015c). Moreover, the PEF treatment allowed selective extraction of small weight molecules from microalgae (Carullo et al., 2018). More efficient for extraction of intracellular bio-

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molecules from electrically resistant strain requires more powerful mechanical disintegration of the cell walls, which is provided by high voltage electrical discharges (HVED) (Grimi et al., 2014). A pulsed streamer discharge in water is usually accompanied with the phenomenon of electrical breakdown leads to the liquid turbulence and intense mixing, the emission of high-intensity UV light, the generation of hydrogen peroxide, the production of shock waves, and bubble cavitation. These secondary phenomena cause cell structure damage and particle fragmentation, consequently facilitating the release of intracellular bio-molecules (Zhang et al., 2019a).

Therefore, the objective of this thesis is to find or to develop an alternative process for extraction of fractionation of bio-molecules with high efficiency, selectivity and low energy consumption. This subject of thesis covers two main aspects of microalgae biorefineries: cell disintegration and extraction. The thesis is dressed on five chapters accessorised with six publications (published or submitted by the time of writing) that reflects the principal results obtained:

**Chapter I** presents an overview on the introduction of microalgal properties (i.e. biodiversity and classification, cell structure and chemical composition) followed by a summary of microalgae biorefineries processing (i.e. upstream and downstream processing) and concludes on the objective of this work;

**Chapter II** describes methodology and protocols used in this thesis;

**Chapter III** is composed of two publications related to the comparison of extraction of hydrophilic and hydrophobic bio-molecules assisted by different physical technologies (i.e. pulsed electric fields (PEF), high voltage electrical discharges (HVED) and ultrasounds (US)). This chapter discussed and compared the impact of physical treatments on extraction of bio-molecules from different microalgal species (*Nannochloropsis* sp., *P. tricornutum*, and *P. kessleri*).

*The first article* highlights the aqueous extraction of carbohydrates (relatively small molecules) and proteins (larger molecules) assisted by PEF, HVED and US techniques. The extraction efficiency in dependence of specific energy consumption for tested techniques, extraction selectivity and correlations between extraction of carbohydrates and proteins were discussed. *The second article* is devoted to explore the feasibility of physical pre-treatments (PEF, HVED and US) assisted enthal extraction of chlorophyll *a* (hydrophobic) from

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different microalgal species. Attention was also focused on the effects of physical treatments on extraction kinetics of chlorophyll *a*.

**Chapter IV** compiles two publications focus on the extraction of bio-molecules from *P. kessleri* using advanced protocols based on preliminary physical treatments (by US, PEF, HVED) combined mechanical treatment (by high pressure homogenization (HPH)). This chapter provides insights into the effects of combined protocols for extraction of bio-molecules from microalgae in terms of extraction efficiency, selectivity and energy efficient.

The third article develops a combination of US and HPH on extraction of ionic components, proteins, carbohydrates, and pigments from *P. kessleri*. The extraction efficiency in dependence of specific energy consumption and concentration of suspension were discussed. The fourth article proposes a combination of pulsed electrical energy (PEF/HVED) and HPH on selective and energy efficient extraction of bio-molecules from *P. kessleri*. The dependence of recovery behaviors of bio-molecules on the different extraction protocols was discussed.

**Chapter V** includes two publications, and it is concentrated on extraction and fractionation of bio-molecules by using a multistage process, in order to evidence the HVED pre-treatment allow optimization of integrated biorefinery with defined selectivity and maximum valorisation of microalgal biomass. The multistage processes included the application of HVED pre-treatment in combination of aqueous and non-aqueous extractions.

The fifth article investigates the efficiency of HVED pre-treatment on the selective recovery of bio-molecules from *P. tricornutum* during a multi-step extraction process. The efficiency of recovery of ionic components, proteins, carbohydrates, pigments and lipids at different stages of extraction procedures were estimated. The results were compared to the pretreatment with HPH. The sixth article proposes a multi-step procedure based on the initial aqueous extraction assisted by HVED from *Nannochloropsis oculata* and secondary organic solvent extraction from vacuum dried (VD) microalgae. The washed and unwashed slurries were compared. The impact of HVED treatment and washing on vacuum drying kinetics were also studied.

Finally, summarizing conclusion of the discussed papers and presents some suggestions for further work.

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## Chapter I Literature Review

### I.1 Microalgae

#### I.1.1 Introduction

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to unicellular or simple multi-cellular structure (Mata et al., 2010). Most of microalgae are autotrophic organisms, which require only inorganic compounds such as sunlight, atmospheric CO<sub>2</sub>, water, N, P and K for growth (Brennan and Owende, 2010a). Throughout the process of photosynthesis CO<sub>2</sub> absorbed from the atmosphere is converted into valuable bio-molecules like lipids, proteins, pigments and carbohydrates in large amounts over short periods of time (Khili, 2013). These bio-molecules can be further processed into bio-products and energy feedstock.

Microalgae have a wide range of cell size from nanometre to millimetre, they exist as independent organisms or in chains/groups (Saharan et al., 2013). Moreover, microalgae are recognised as one of the oldest life-forms without roots, stems and leaves and have no sterile covering of cells around the reproductive cells. They are present in all existing earth ecosystems, such as marine, freshwater (ponds, puddles, canals, and lakes) and terrestrial habitats (Khili, 2013; Mata et al., 2010). They are estimated that more than 50,000 species exist, but only about 35,000 species have been characterized and studied so far (Mata et al., 2010).

#### I.1.2 Biodiversity and classification

Algae can be classified into “microalgae” and “macroalgae”. Macrophytic algae, typically Rhodophyta (red algae), Chlorophyta (green algae), and Phaeophyta (brown algae), are referred to as macroalgae (i.e. seaweeds), while the unicellular forms are called microalgae (Beetul et al., 2016). The majority of microalgae exist as small cells (3-20 μm) representing both photoauto- and hetero-trophic eukaryotes, such as cyanophyta (blue-green algae), pyrrhophyta (dinoflagellates), chrysophyta (golden, green and yellow-brown flagellates), chlorophyta (microscopic green algae), bacillariophyta (diatoms), raphidophyta, haptophyta, prasinophyta, prymnesiophyta and cryptophyta, as well as photoautotrophic prokaryotic such as cyanobacteria (Ejike et al., 2017; El Gamal, 2010). For the classification of algae, pigments



are one of the most important criteria used in the differentiation of classes. Figure I.1 shows different phyla of algae are known to have different pigments present in their cells.

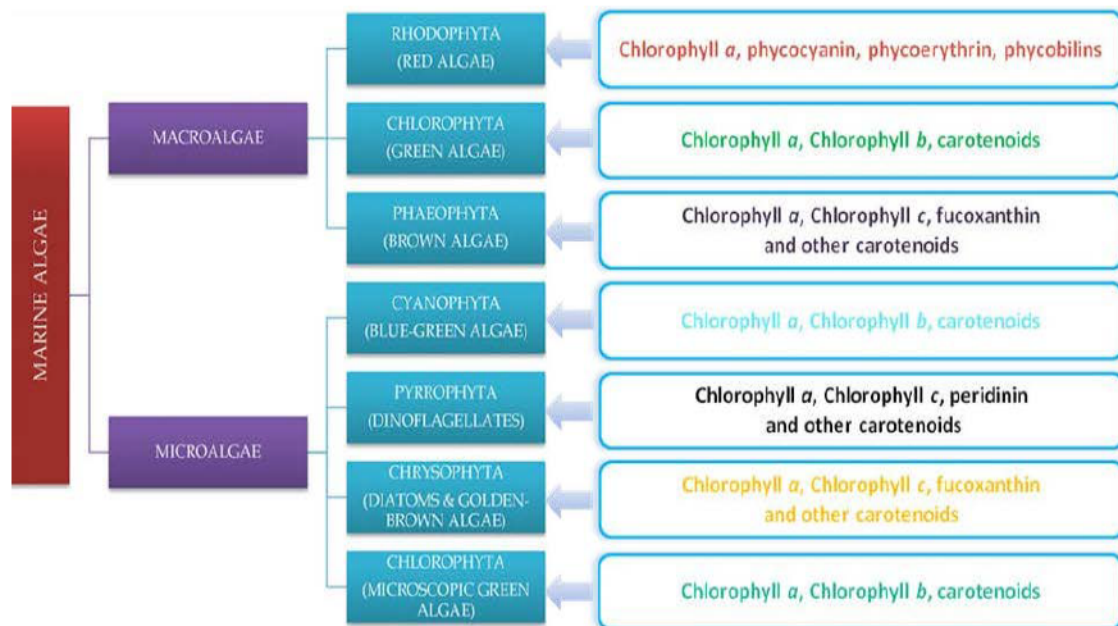


Figure I.1: Different phyla of algae are known to have different pigments present in their cells (Beetel et al., 2016; Deglint et al., 2018).

### I.1.3 Cell structure

Microalgal strains may differ in size and shape but they possess similar organelles with specific functions in the cellular metabolism and enclosed in the polar lipid membrane. A plasma membrane separates the interior of the cell from the external environment (Bodenes, 2017a). Like terrestrial plants, most of microalgae also possess a cell wall which provides a good mechanical resistance to the cell. A typical microalgal eukaryotic cell structure is presented in Figure I.2. Some organelles might be absent or differently organized in certain microalgal species (Bernaerts et al., 2019a). The nucleus is a membrane-enclosed organelle found in eukaryotic cells which contains most of the cell genetic material organized as chromosomes. The cytoplasm comprises the cytosol and organelles, the internal sub-structures. Cytosol represents up about 70% of the cell volume and is a complex mixture of cytoskeleton filaments (e.g. actin filaments and microtubules), dissolved molecules, and water. Vacuoles allow the cell to control turgor pressure (Becker, 2007), associated with the gradient of osmotic pressure between the interior and exterior of the cell. The Golgi apparatus has a major role in protein glycosylation and sorting, but is also a major biosynthetic organelle that synthesizes large quantities of cell wall polysaccharides (Dupree and Sherrier, 1998). The



lipid body are made up of neutral lipids (mainly triacylglycerols, TAGs) stored in the cytoplasm as energy sinks for future use.

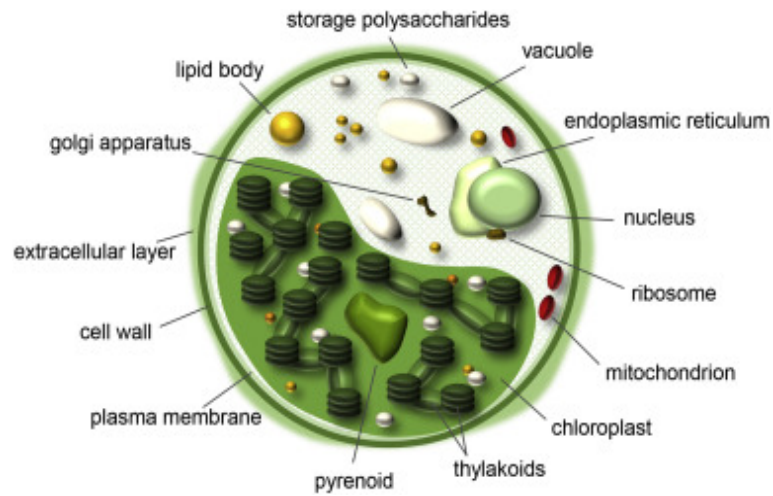


Figure I.2: Schematic representation of a eukaryotic microalgal cell structure (Bernaerts et al., 2019a).

### I.1.3.1 Plasma membrane

The plasma membrane (also known as cell membrane or cytoplasmic membrane) is common to all eukaryotic microalgal cells and separates the cytoplasm containing organelles from the extracellular fluid (Figure I.3). It is protected by a complex cell wall, and consists in a phospholipid bilayer with embedded proteins (Lee et al., 2017). The cell membrane is selectively permeable and able to regulate the entering and exiting of molar fluxes across itself, by transfer thanks to gradients of e.g ions, dissolved CO<sub>2</sub> and O<sub>2</sub> and other compounds (Bodenes, 2017a).

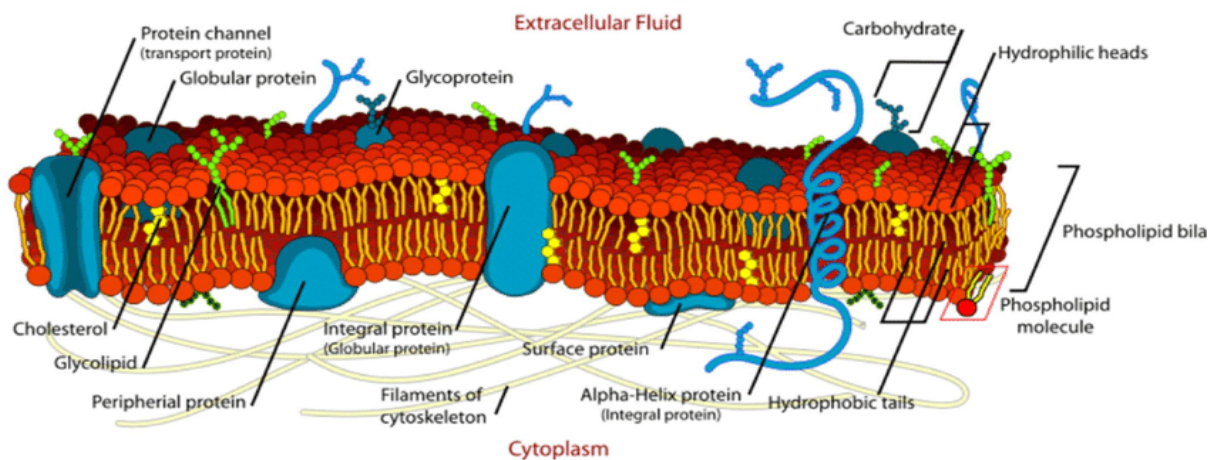


Figure I.3: Diagram of the cell membrane (Bodenes, 2017a).

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In addition, the transmembrane proteins enable the transport of nutrients such as sugars and amino acids into the cell and the excretion of metabolites by active pumping. The cytoskeleton underlying the cell membrane is a complex network of filaments and tubules that extends through the cytoplasm to the nucleus. It provides an internal mechanical resistance to the cell and helps to maintain its shape. The cell membrane also contains various proteins (around 50% of membrane volume) and carbohydrates (Bodenes, 2017a).

#### I.1.3.2 Cell wall

The cell wall of microalgae displays structural diversity and rigidity, complicating the development of efficient downstream processing for recovery intracellular bio-molecules. Therefore, an understanding of microalgal cell wall's structure and composition is important from the point of view cell disruption (Jankowska et al., 2017). The fundamental components of microalgal cell wall consisted of a microfibrillar network within a gel-like protein matrix (Yap et al., 2016). In general, the chemical composition of cell wall included celluloses, proteins, glycoproteins, polysaccharides and lipids. However, microalgal cell walls are complex, their thickness and chemical composition change significantly in response to the growth environment (Praveenkumar et al., 2015). Here we summarize the respective composition and structure of the cell walls of several microalgae (namely *Parachlorella kessleri* (*P. kessleri*), *Nannochloropsis* sp. and *Phaeodactylum tricornutum* (*P. tricornutum*)).

##### ➤ *Parachlorella kessleri*

The green microalga *P. kessleri* is a unicellular freshwater organism (Chlorophyta, Trebouxiophyceae). The cells of *P. kessleri* are spherical with a mean diameter ranging from 2.5 to 10  $\mu\text{m}$ . Transmission electron microscopy (TEM) micrographs of *P. kessleri* were presented in Figure I.4. The TEM studies revealed the presence of a unique excentric nucleus containing a low electron-dense nucleolus (Figure I.4a and b). A single parietal chloroplast was presented surrounding the entire cell and formed a small aperture (“mantel-shaped”) (Figure I.4c and d). One pyrenoid in the thickening of the chloroplast surrounded by two starch granules and bisected by two thylakoids was evident (Figure I.4c and e). Small starch grains and small lipid droplets also lay scattered in the chloroplast matrix and cytoplasm, respectively (Figure I.4c). The cell wall was electron-transparent homogeneous structure 60–80 nm in thickness (Figure I.4f), mainly consisted of  $\beta$ -1, 3-glucan and WGA specific N-acetyl- $\beta$ -D-glucosamine (Juarez et al., 2011). The cell wall hemicelluloses matrix contained

rhamnose, galactose, glucose and xylose together with minor quantities of arabinose, mannose and fucose (Yamamoto et al., 2005).

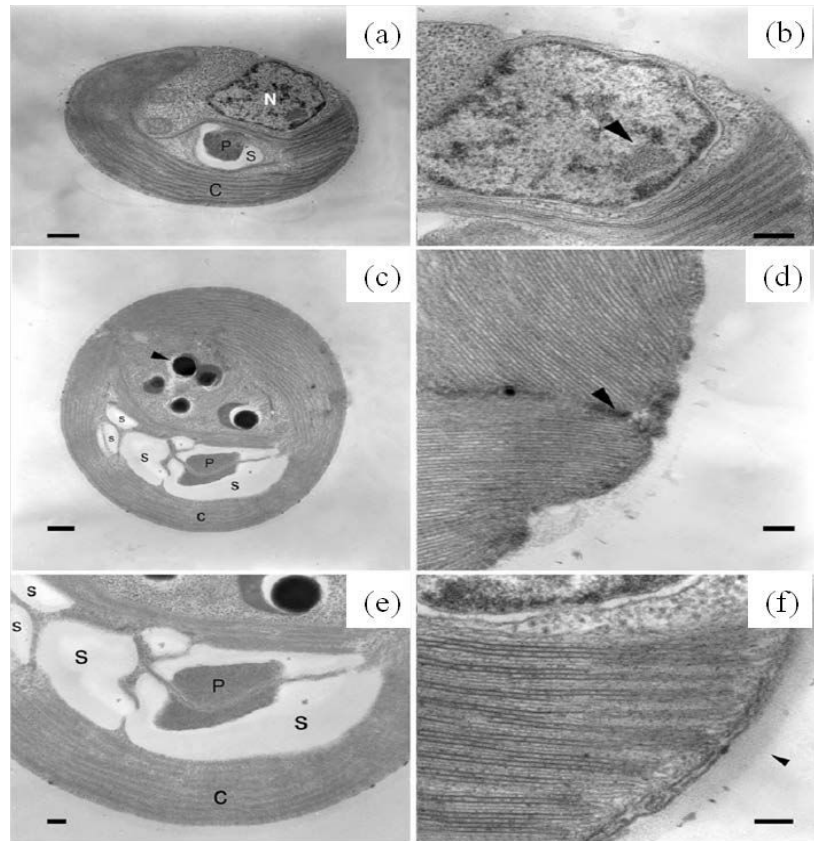


Figure I.4: Transmission electron micrographs of *P. kessleri*. An excentric nucleus (*N*) and a parietal chloroplast (*C*) with one pyrenoid (*P*) covered by starch granules (*S*) in vegetative cell (*a*); nucleus (*b*); the parietal chloroplast (*C*), starch granules (*S*) and lipid droplets (arrowhead) in vegetative cell (*c*); the small opening of the chloroplast (arrowhead) (*d*); the pyrenoid (*P*) bisected by two thylakoids and covered by starch granules (*S*) (*e*); the thick electron-transparent single-layer structure of typical cell wall (arrowhead) (Juarez et al., 2011).

➤ *Nannochloropsis* sp.

The green microalgae *Nannochloropsis* sp. are unicellular marina organism belonging family Eustigmataceae, which present collection of six species of *Nannochloropsis* (*gaditana*, *granulate*, *limnetica*, *oceanica*, *oculata*, *salina*) (Zhang et al., 2018). It has a complex bilayer cell wall structure with a cellulosic inner layer protected by an outer hydrophobic algaenan layer (Gerken et al., 2013; Scholz et al., 2014). Figure I.5 shows a representative TEM image from *Nannochloropsis* strain. The average cell size and cell wall thickness was also evaluated

(Beacham et al., 2014). The authors observed that all *Nannochloropsis* sp. cells are near spherical with relatively small size (2–4 µm in diameter) and a large chloroplast. However, cell wall thickness varied widely both between the 4 different species, range from 60 to 110 nm.

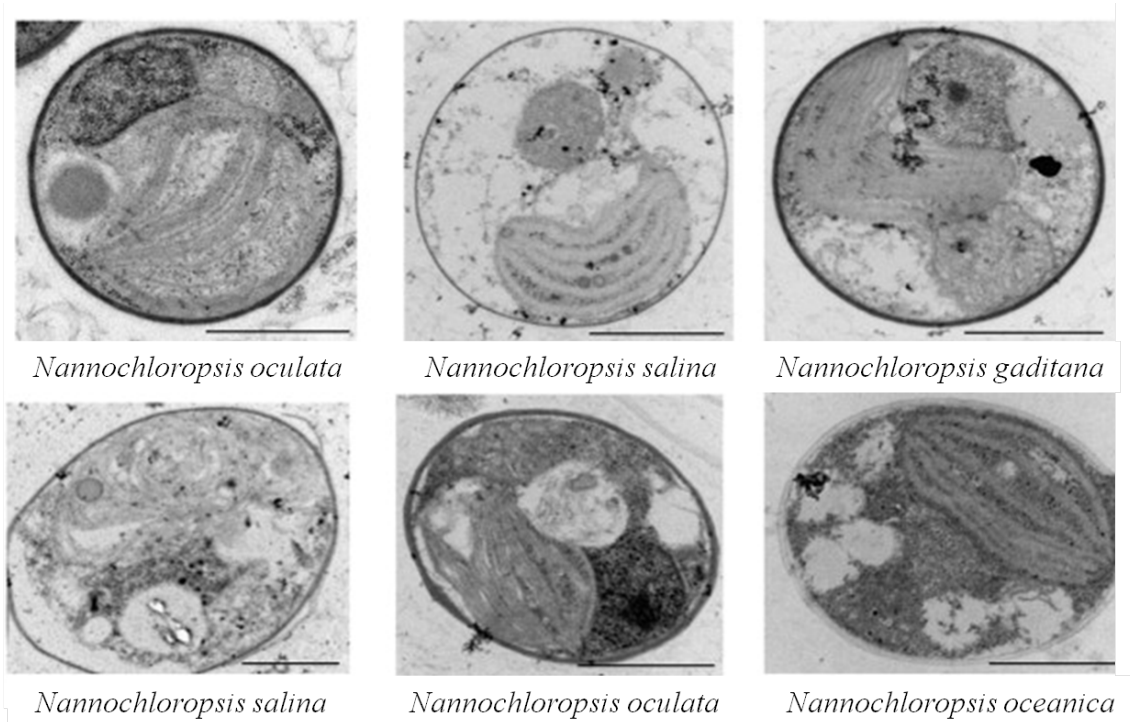
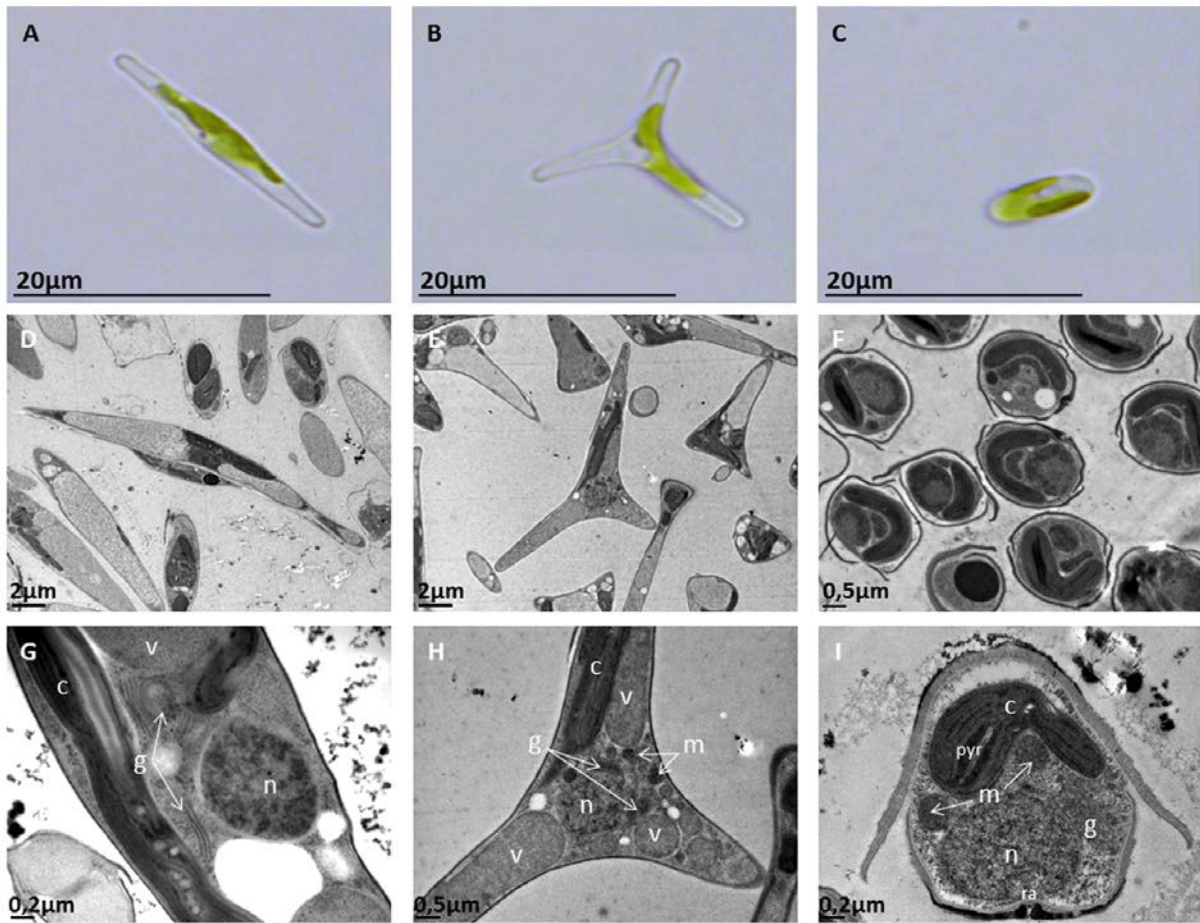


Figure I.5: Transmission electron micrographs of representative images of *Nannochloropsis* strains (*Nannochloropsis oculata*, *Nannochloropsis salina*, *Nannochloropsis gaditana*, and *Nannochloropsis oceanica*) (Beacham et al., 2014).

➤ *Phaeodactylum tricornutum*

The microalga *P. tricornutum*, a typical unicellular diatom, was found throughout marine and freshwater environments (Xu et al., 2010). The cell wall of *P. tricornutum* is unique, not only because of it is poor in silica and mainly composed of inorganic components (sulphated glucuronomannan), but also it is the only microlagal specie existed in three morphotypes (fusiform, triradiate, and oval) (Le Costaouec et al., 2017). Figure I.6 shows the light microscopy and TEM micrographs of the three morphotypes of *P. tricornutum* cells. The cell wall of fusiform phenotype *P. tricornutum* exhibits a three-layer construction: a thin (3 nm) electron opaque layer facing the cell interior is followed by a thicker (4–6 nm), less opaque middle layer, and an outer, more opaque layer the basal part of which is approximately of the same width as the interior layer (Reimann and Volcani, 1967)





*Figure I.6: The light microscopy micrographs of *P. tricornutum* cells alive: fusiform morphotype (A); triradiate morphotype (B) and oval morphotype (C); Transmission electron microscopy (TEM) micrographs of the three morphotypes (D–F); enlarge views of the TEM micrographs showing general cellular distribution of organelles in the fusiform cells (G), in the triradiate one (H), and in the oval cell type (I). n: nucleus; g: Golgi apparatus; v: vacuole; m: mitochondria; pyr: pyrenoid; c: chloroplast; ra: raphe (Ovide et al., 2018).*

#### I.1.4 Chemical composition

Microalgae have a large diversity in the chemical composition, not only because of the enormous evolutionary diversity, but also the effect of species and adopted growth conditions (light intensity, temperature, and nutrient availability, etc) (Hu, 2004). They can be manipulated to high proteins, carbohydrate or lipids content as required, as the energy feedstock for different bio-products (Figure I.7).

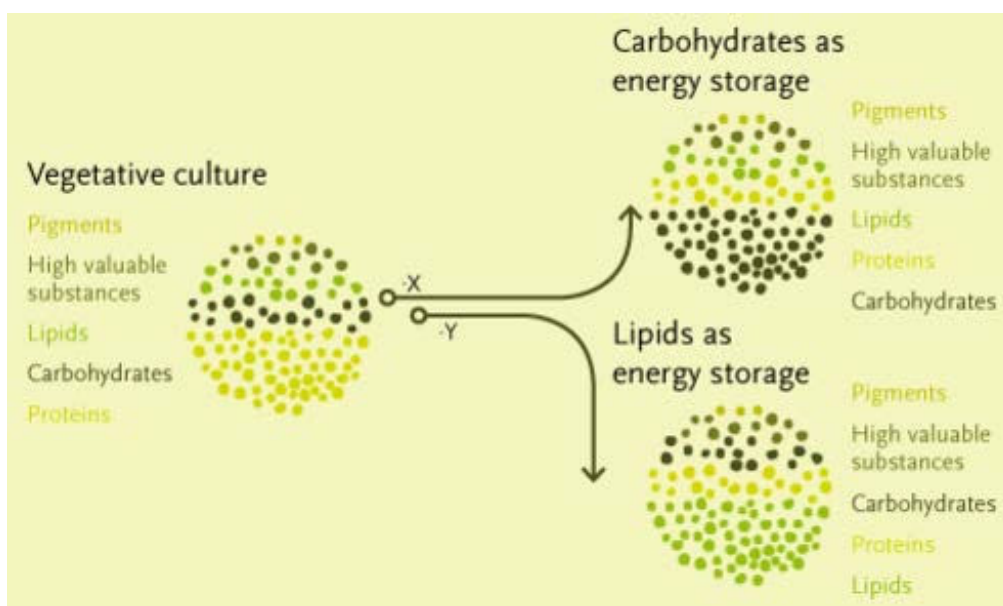


Figure I.7: Microalgae can be manipulated to high proteins, high carbohydrates or high lipids content as required (<https://subitec.com/en>).

Table I.1 compiled biomass profiles of several common microalgal species. The ranges of proteins from 9 to 77%, and 6-54% of carbohydrates, and 4-74% of lipids, were observed. Depending on the species and cultivation conditions, microalgae can be selected to produce a wide variety specific product for biofuel and production of nutraceuticals. Examples of lipid-rich microalgae (> 40%) are *Schizochytrium* sp. and some strains of *Nannochloropsis* sp.. Some microalgae possess a high proteins content (> 50%), such as, *Arthrospira platensis* (*Spirulina*), *Chlorella vulgaris*, *Dunaliella* sp., *Haematococcus pluvialis* and *Porphyridium cruentum*. In general, however, the higher lipids content of microalgal biomass, the lower the amount of proteins and carbohydrates.

Table 1.1: Proximate biomass composition of different microalgal species, expressed as percentage of dry biomass (%)(Bernaerts et al., 2019a).

Microalgal species	Proteins (%)	Carbohydrates (%)	Lipids (%)
<i>Arthrospira platensis</i> ( <i>Spirulina</i> )	43-77	8-22	4-14
<i>Chlorella vulgaris</i>	38-53	8-27	5-28
<i>Diacronema vlkianum</i>	24-39	15-31	18-39
<i>Dunaliella</i> sp.	27-57	14-41	6-22
<i>Haematococcus pluvialis</i>	10-52	34	15-40

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<i>Isochrysis galbana</i>	12-40	13-48	17-36
<i>Nannochloropsis</i> sp.	18-47	7-40	7-48
<i>Odontella aurita</i>	9-28	30-54	13-20
<i>Pavlova lutheria</i>	16-43	15-53	6-36
<i>Phaeodactylum tricornutum</i>	13-40	6-35	14-39
<i>Porphyridium cruentum</i>	27-57	12-39	5-13
<i>Scenedesmus</i> sp.	31-56	6-28	8-21
<i>Schizochytrium</i> sp.	10-14	12-24	46-74
<i>Tetraselmis</i> sp.	14-58	12-43	8-33

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#### I.1.4.1 Proteins

Microalgal biomass are rich in proteins that compete favorably, in terms of quantity and quality, with conventional food proteins (Ejike et al., 2017). Several factors can affect the amount of accumulated proteins in microalgae, including species type, light quality, nutrient adjustments, and environmental stress. An example of *Spirulina* contains about 43-77% proteins depending on the strain (Table I.1). Importantly, microalgael proteins contain well-balanced amino acid profiles, their amino acid pattern compares favorably with that of other food proteins (Ejike et al., 2017). Microalgae synthesize all 20 proteinogenic amino acids and can be unconventional sources of essential amino acids for human nutrition (Spolaore et al., 2006). Microalgae *Spirulina* and *Chlorella vulgaris* are most commonly produced as protein sources and have been selected for large scale production (Khanra et al., 2018; Pulz and Gross, 2004). In particular, *Spirulina* showing favorable amino acid profiles and good digestibility (Becker, 2004).

In terms of cell structure, the first group proteins existed in the cytoplasm (“storage” role) is water-soluble and readily available. The second group proteins; associated with cell membrane and organelle, have a more metabolic “function” and are often bound to pigments and lipids. The third group of proteins conducted a more “structural” role, for example; as part of the outer cell wall and membrane. Proteins associated with membranes and pigments display a more hydrophilic nature or are embedded in the cell-wall polysaccharides, and therefore they cannot be extracted in an aqueous medium by simple mechanical shear (Garcia, 2019).

#### I.1.4.2 Carbohydrates

Carbohydrates make up another important fraction of microalgal biomass, which mainly compose of two types: storage polysaccharides and structural polysaccharides (Garcia, 2019). The storage polysaccharides located in the microalgal cell differs (Figure I.2), including starch, floridean starch, glycogen, chrysolaminarin and paramylon (Figure I.8) (Bernaerts et al., 2019a). For instance, starch is stored in the chloroplasts, while chrysolaminarin is accumulated in the vacuoles. The other three types (floridean starch, paramylon, and glycogen) are located as granules in the cytosol. Thereinto, glucose is the dominant sugar in storage polysaccharides.

The structural polysaccharides (i.e. cell wall related polysaccharides) are chief ingredient of microalgal cell wall, which are generally composed of multiple monosaccharide residues. In the study of Bernaerts et al. (Bernaerts et al., 2018), the amount of cell wall related polysaccharides were determined from 10 microalgal species. They concluded that these polysaccharides generally account for approximately 10% of the dry biomass. Moreover, some microalgal species displayed lower amounts of cell wall polysaccharides (3.8–7.4%), but the authors attributed this to the presence of non-polysaccharide substances in their cell walls, such as algaenan polymers in *Nannochloropsis* sp. and a silica frustule in *Odontella aurita*.

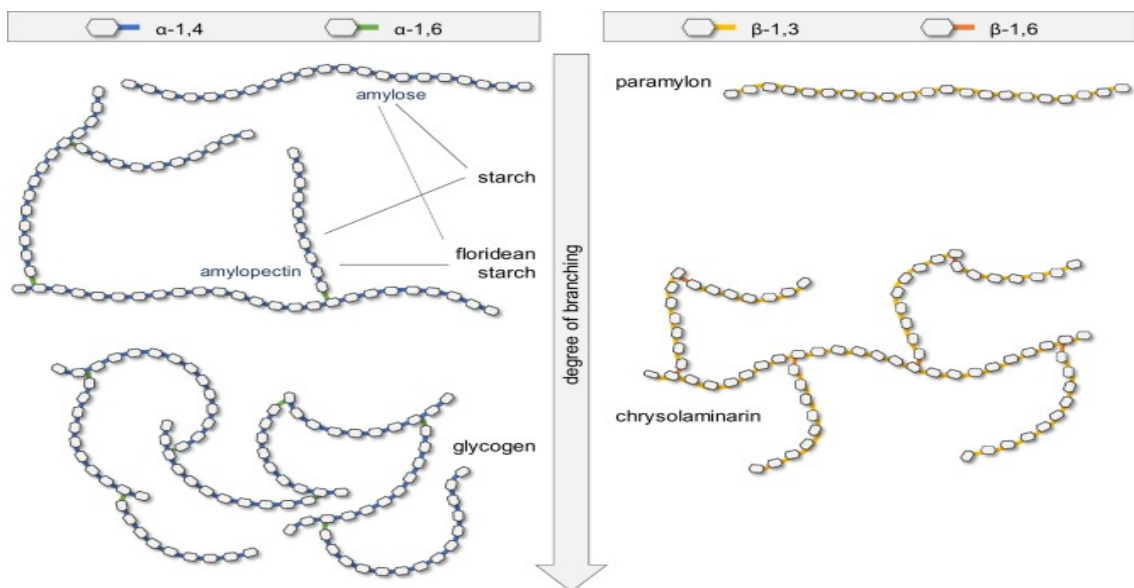


Figure I.8: Schematic representation of the five types of storage polysaccharides in microalgae (Bernaerts et al., 2019a).



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### I.1.4.3 Lipids

Lipids are mainly found in lipid bodies (storage) or membrane lipids (structure), depending on the microalgal strain and cultivation conditions (Garcia, 2019). It was reported that microalgae can accumulate a high percentage of lipids in the cultivation conditions of higher carbon to nitrogen (C/N) ratio, nitrogen starvation, high temperature, pH shift and high salt concentration (Kwak et al., 2016). Microalgal lipids can be classified into two groups: polar (phosphor- and glycolipids) and neutral (free fatty acids, mono-, di- and triacylglycerols) (Rivera et al., 2018). Microalgae use neutral lipids as energy reserved source and polar lipids to form cell membranes (D'Alessandro and Antoniosi Filho, 2016).

Microalgae are considered as the third generation of biodiesel feedstock, because of their high capacity to produce high oil contents' biomass, with higher growth rate and productivity than edible and non-edible feedstock (Table I.2) (Bodenes, 2017b). From the Table I.2, the oil yields obtained from microalgae can be up to 25 and 250 times higher than those obtained to palm and soybean respectively. Among all the sources of renewable biodiesel feedstock, microalgae seem the only one capable of meeting the global demand for transport (Atabani et al., 2012; Yusuf Chisti, 2007) regarding the arable area available (5,000 Mha arable land with 1,400 Mha are used for agriculture (Bodenes, 2017b) in 2016).

*Table I.2: Estimated oil productivity of different biodiesel feedstocks (Bodenes, 2017b).*

Plant source	Biodiesel (L/ha/year)	Area to satisfy global oil demand (10 <sup>6</sup> ha)
Cotton	325	15002
Soybean	446	10932
Mustard seed	572	8524
Sunflower	952	5121
Rapeseed/canola	1190	4097
Jatropha	1892	2577
Oil palm	5950	819
Algae	12000-136900	35-406

However, steroids and pigments as microalgal fatty acid free components are not converted into biodiesel. Consequently, the higher production of pigments implies lower production of fatty acids (Halim et al., 2012a).

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In addition, it is important to assess the types of fatty acids in microalgae, since they influence biodiesel quality, especially oxidative stability, cold filter plugging point, and contents of mono-, di- and triglycerides (D'Alessandro and Antoniosi Filho, 2016). For biodiesel applications, the lipids fraction of major interest is triacylglycerides of saturated fatty acids (Angles et al., 2017). Large amounts of saturated fatty acids have excellent combustion properties, while polyunsaturated fatty acids negatively affect oxidative stability (Knothe, 2005). Thus, the European standard EN14214 states that the content of linolenic acid, and consequently tri-unsaturated fatty acids, must be at most 12%, and at most 1% of polyunsaturated acids (D'Alessandro and Antoniosi Filho, 2016).

#### I.1.4.4 Pigments

Natural pigments have an important role in the photosynthetic metabolism and pigmentation in algae. Three major classes of photosynthetic pigments occur among the algae: phycobilins, chlorophylls, and carotenoids. They are present in sac-like structures called thylakoids. The thylakoids are arranged in stacks in the granum of the chloroplasts (Figure I.2). Different groups of microalgae have different types of pigments and organization of thylakoids in chloroplast.

Phycobilins (phycobiliproteins) are brilliantly colored water-soluble protein components, found in blue-green algae (Cyanophyta), red algae, and cryptomonads (Kuddus et al., 2013). These proteins are classified into two large groups based on their colors, the phycoerythrin (red), and the phycocyanin (blue) (Figure I.9). The phycocyanins are the major photosynthetic accessory pigments in microalgae, including C-phycocyanin (C-PC), R-phycocyanin (R-PC), and allophycocyanin (A-PC) (Chen et al., 1996). They are easy to be isolated and purified, because they comprise a large portion of the total cell protein (D'Alessandro and Antoniosi Filho, 2016). C-PC is the chief pigment in Cyanophyta. Example of *Arthrospira platensis* (Cyanophyta) with up to 40% of its total proteins as C-PC (Zhou et al., 2005).

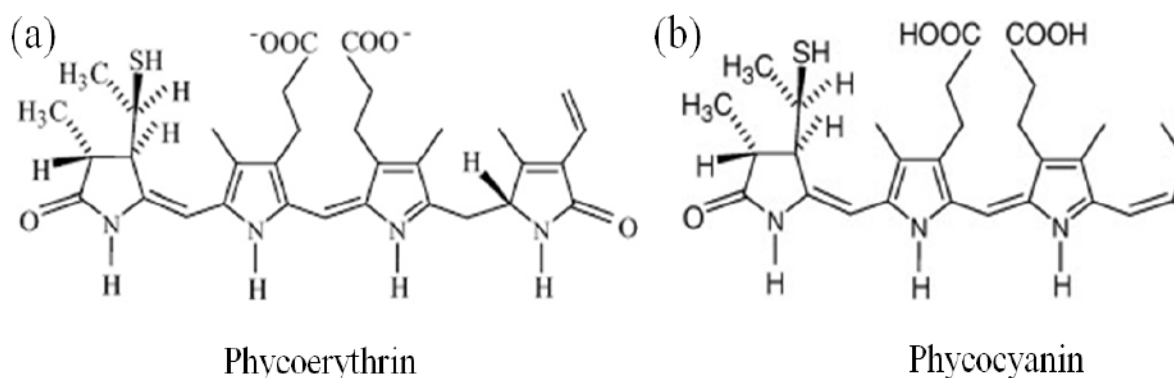


Figure I.9: Phycobilin structures: phycoerythrin (a) and phycocyanin (b).

Chlorophylls are the green coloured and lipid soluble pigments present in microalgae. They are responsible for converting solar energy into chemical energy in photosynthesis (D'Alessandro and Antoniosi Filho, 2016). The chlorophylls in microalgae are chlorophyll *a*, *b* types (Figure I.10). Chlorophyll *a* is almost present in all classes of microalgae. Chlorophyll *b* is primary pigment of Chlorophyta.

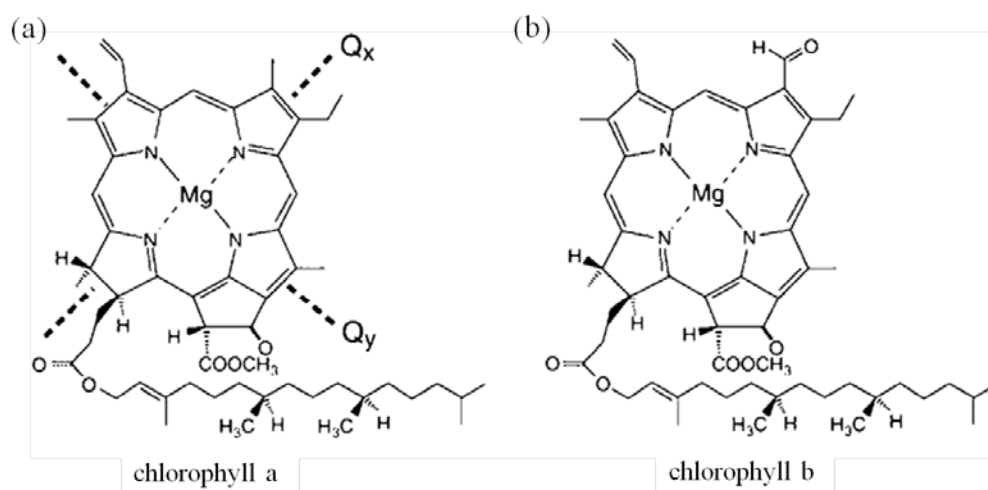


Figure I.10: The molecular structures of chlorophyll *a* (a) and chlorophyll *b* (b).

Carotenoids are lipid soluble pigments, which typically appear to be orange, red or yellow. They perform two key roles in photosynthesis: i) absorb light in regions of the visible spectrum, in which chlorophylls does not absorb efficiently; ii) photoprotect the photosynthetic systems. Photoprotection mechanisms remove the most energetic states of chlorophylls, resulting from the excessive absorption of light radiation. This hinders the formation of reactive oxygen species, makes carotenoids good antioxidants (Varela et al., 2015). The main carotenoids of microalgae are  $\beta$ -carotene, astaxanthin and lutein (Figure I.11). Of these,  $\beta$ -carotene was found in all classes of microalgae. *Dunaliella salina* was

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considered as a rich source of  $\beta$ -carotene due to the highest carotenoids content ( $\approx 10\%$  dry matter) among all the microalgae species (Prieto et al., 2011).

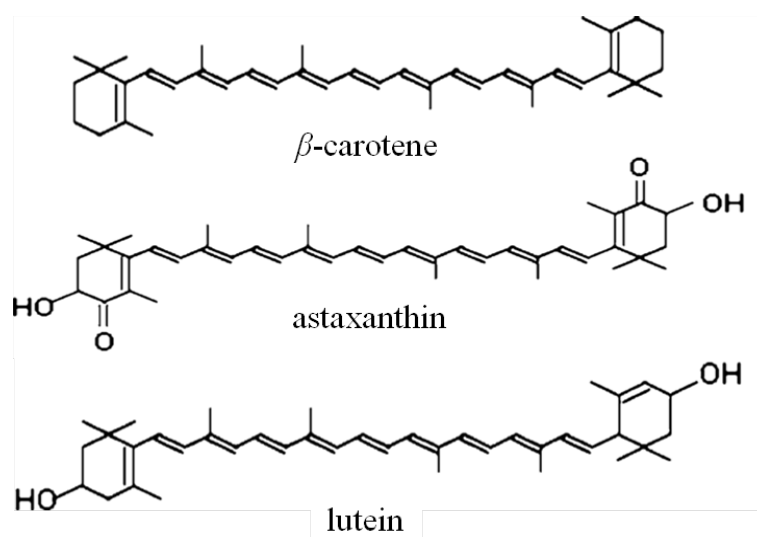


Figure I.11: Chemical structure of  $\beta$ -carotene, astaxanthin and lutein, main carotenoids from microalgae.

## I.2 Microalgae processing

### I.2.1 Overview of microalgae biorefineries

Biorefineries are found in widespread sectors at industrial scale, and this allows the biorefineries to concentrate on multiple products processing. This process is a promising way to mitigate greenhouse gas emission, and allows producing value-added bio-products through biomass transformation and process equipment. In the biorefineries, the valorisation of microalgae could be achieved by process integration. Upstream processing and downstream processing are the main stages of the microalgae biorefineries (Chew et al., 2017). Figure I.12 shows outline of the formation process of microalgal biomass and bio-products.

Upstream processing of microalgae biorefineries refers to four important factors: i) microalgae strain, ii) supply of  $\text{CO}_2$ , iii) nutrient source (e.g. nitrogen and phosphorus) and iv) source of illumination (Vanthoor-Koopmans et al., 2013). Conventional downstream processing involves all unit processes that occur within the photobioreactor (Chew et al., 2017). This processing facilitates the integration of the biomass conversion processes and equipment for the production of several fractions of interest through the use of mild separation technology (Jacob-Lopes et al. 2015).

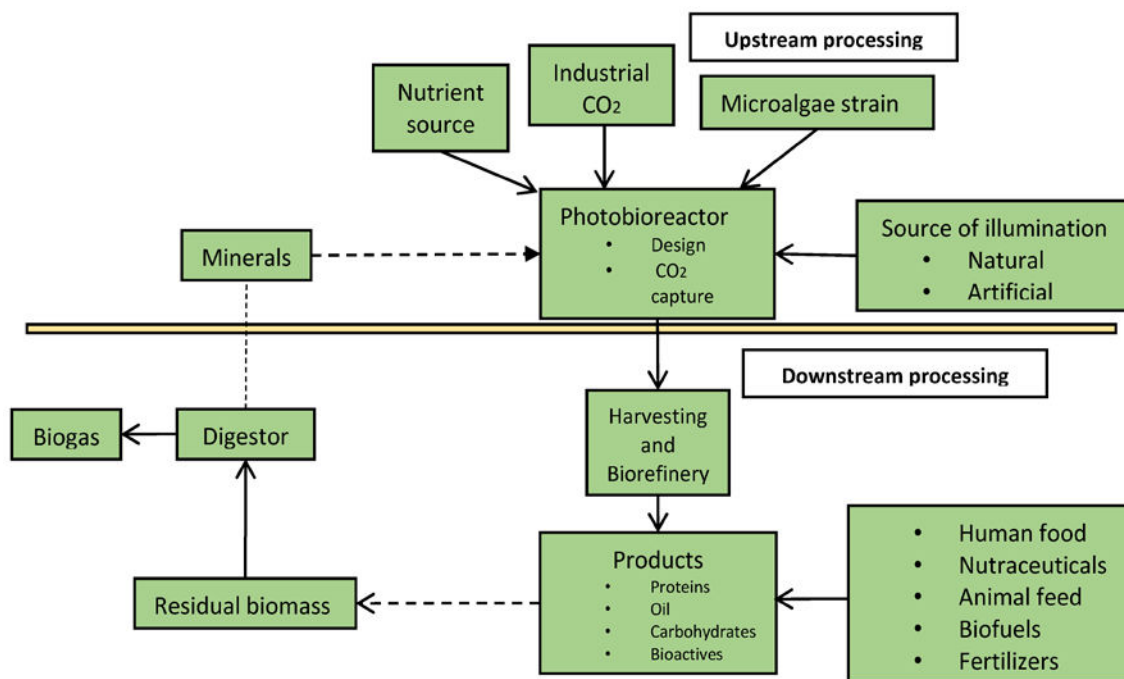


Figure I. 12: Outline of the formation process of microalgal biomass and bioproducts (Jacob-Lopes et al. 2015).

### I.2.2 Cultivation

The growth characteristics and chemical composition of microalgae are known to significantly depend on the cultivation conditions (Chojnacka and Marquez-Rocha, 2004). The main growth limiting factor of microalgae are: concentration and quality of nutrients, CO<sub>2</sub> concentration, water supply, temperature (16–27 °C), exposure to light (1 000–10 000 lx), pH values (4–11), culture density, salinity (12–40 g/L), turbulence, biological factors, presence of toxic compounds, heavy metals and synthetic organisms, as well as bioreactor operating conditions (Jankowska et al., 2017). This section describes three distinct mechanisms of microalgae cultivation, including photoautotrophic, heterotrophic and mixotrophic cultivation, all of which follow the natural growth processes. Table I.3 compares the characteristics of different cultivation conditions.

Table I.3: Comparison of the characteristics of different cultivation conditions (Chen et al., 2011).

Cultivation condition	Energy source	Carbon source	Cell density	Reactor scale-up	Cost	Issues associated with scale-up
Photoautotrophic	Light	Inorganic	Low	Open pond or photobioreactor	Low	Low cell density High condensation cost
Heterotrophic	Organic	Organic	High	Conventional fermentor	Medium	Contamination High substrate cost
Mixotrophic	Light and organic	Inorganic and organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost
Photoheterotrophic	Light	Organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost

#### I.2.2.1 Photoautotrophic cultivation

Currently, photoautotrophic production is the only method which is technically and economically feasible for large-scale production of microalgal biomass for non-energy production (Borowitzka, 1997). Three systems that have been deployed are based on open pond, closed photobioreactor and hybrid cultivation technologies (Borowitzka, 1999).

##### ➤ *Open pond systems*

Microalgae cultivation in open pond systems (OPR) has been utilized since the 1950s (Brennan and Owende, 2010a). OPR are reactors open to the environment, that can be classified into natural and artificial pond systems (Kroger and Muller-Langer, 2012). Raceway ponds are the commonly used types in concrete (Figure I.13) (Passos and Ferrer, 2014). OPR is a relative cheap, easy to operate and can be large-scale cultivation method. However, OPR requires long cultivation periods in which that do not exclude the contamination with other algae species and predators, or vaporization and the lack of control of the growth parameters (Koller et al., 2012). Moreover, OPR has a relatively low biomass productivity (Borowitzka, 1992), it is approximately 10–25 g dry matter of microalgal biomass per day per m<sup>3</sup>.



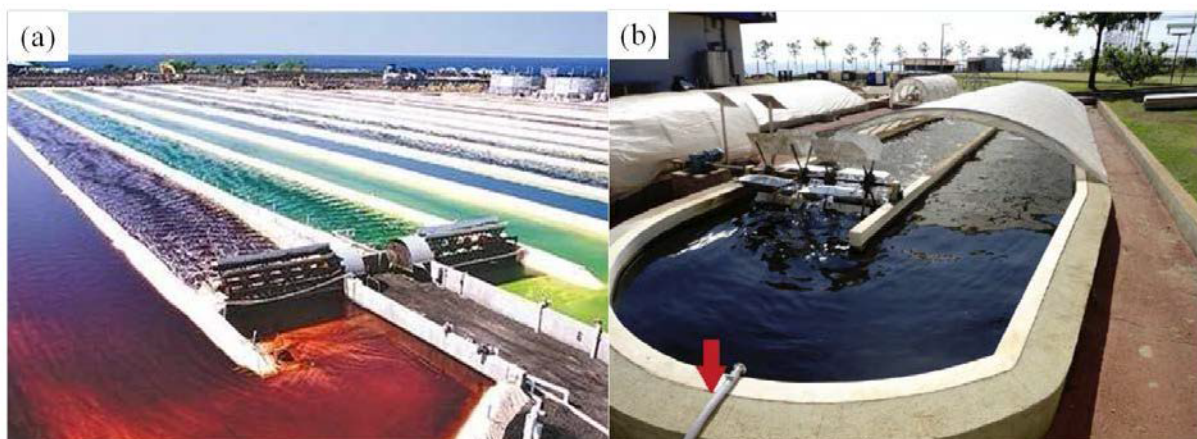


Figure I. 13: Microalgae cultivated in raceways, Cyanotech Hawaii (a), and in partially covered raceways at Ourofino Agronegocio, Brazil (b) (Bodenes, 2017b).

➤ *Closed photobioreactor systems*

The second method for microalgae photoautotrophic cultivation is in closed photobioreactors (PBRs). Frequently used types of PBRs include tubular, flat-tank, bubble column and serpentine (Jankowska et al., 2017). This technology is designed to overcome some of the major problems (such as contamination risks) that occur in the OPR systems. Moreover, PBRs systems have higher biomass productivity (20-100 g dry matter of microalgae biomass per day per m<sup>3</sup>) as compared to OPR systems (Miron et al., 1999). Nevertheless, PBRs systems have some disadvantages: higher operating and maintenance costs than open systems (Brennan and Owende, 2010a).

➤ *Hybrid cultivation systems*

The hybrid cultivation is a two-step system that refers to combine photobioreactors and OPR growth stages. The first cultivation step occurs in a photobioreactor that allows minimizing contamination from other organisms and favouring continuous cell division. The second cultivation step is aimed at accumulating desired products like lipids by exposing the cells to nutrient stresses (Brennan and Owende, 2010a). For example, this two-step system has been used for production of both lipids and astaxanthin from *Haematococcus pluvialis* (Huntley and Redalje, 2007).

#### I.2.2.2 Heterotrophic cultivation

Heterotrophic cultivation uses organic carbon (e.g. glucose, acetate, crop flours, wastewater and others) as substrates to reproduce microalgae in stirred tank bioreactors or

fermenters (Tan et al., 2018). In this process, the growth of microalgae is independent of solar or light energy, using their respiration metabolism (Figure I.14) (Brennan and Owende, 2010a; Lutz, 2012; Perez-Garcia and Bashan, 2015; Zhang et al., 2014). This system has a high degree of cell production and densities achieved thus promoting easy harvest (Chen and Chen, 2006). However, heterotrophic cultivation might cost more energy than photoautotrophic cultivation because this system cycle requires organic carbon source (Brennan and Owende, 2010a).

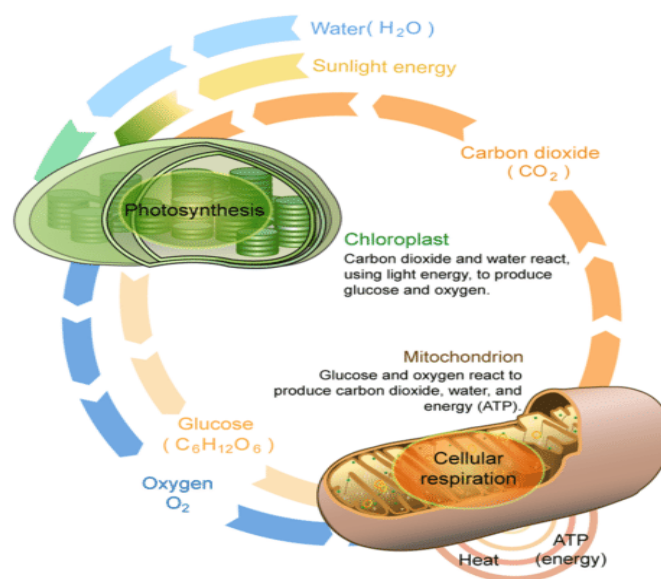


Figure I. 14: Photosynthesis and cellular respiration (Bodenes, 2017b).

Heterotrophic cultivation has also been successfully applied for microalgal biomass and metabolites. It was demonstrated that heterotrophic cultivation of *Chlorella protothecoides* resulted in the accumulation of 55% lipid content in cells, that was 4 times higher than cultivated under photoautotrophic environment (Miao and Wu, 2006).

### I.2.2.3 Mixotrophic cultivation

Mixotrophic cultivation is a process wherein microalgae can be reproduced under phototrophic and heterotrophic conditions. This means that microalgae can utilize both light energy and organic carbon as substrates to sustain their growth (Brennan and Owende, 2010a; Tan et al., 2018). Compared with phototrophic and heterotrophic cultivation systems, mixotrophic cultivation is rarely used in microalgal lipids production (Chen et al., 2011). Example of the cultivation of *Spirulina* sp. in photoautotrophic, heterotrophic and mixotrophic systems were compared by Chojnacka and Noworyta (Chojnacka and Noworyta,



2004). They reported that mixotrophic cultivation has lower photoinhibition and higher growth rates as compared with both photosynthetic and heterotrophic cultivations. Successful production of mixotrophic *Spirulina* sp. allowed integrating both photosynthetic and heterotrophic components during day and night cycle. This process can attenuate the impact of microalgal biomass loss during dark respiration and reduces the amount of organic substances used during growth. These findings indicated that mixotrophic cultivation may be an important part of the microalgae-to-biofuels process (Brennan and Owende, 2010b).

### I.2.3 Harvesting

When the biochemical process in the photobioreactor have finished, the upstream processing ends and gives way to downstream processing and harvesting of the biomass and refining of the bio-products in the biorefinery. Microalgal biomass usually contains high water content and hence, downstream processing is required to eliminate the water content. Harvesting refers to biomass recovery by one or more solid-liquid separation steps or detachment of microalgae from their growth medium, and accounts for 20-30% of the total costs of microalgae production (Mata et al., 2010; Singh and Patidar, 2018). Regardless of the objective of harvesting process, low cell densities (0.02-0.05% dry microalgae) and the small cell size ( $< 30 \mu\text{m}$ ), make harvesting process a challenging task (Brennan and Owende, 2010a). The selection of harvesting method depends on the physiognomies of the microalgae, cell density and size, as well as specifications of the desired products and on allowability for reuse of the culture medium (Mata et al., 2010). Experience has demonstrated that an universal harvesting method does not exist, the major techniques presently applied in the harvesting of microalgae include centrifugation, flocculation, flotation, and filtration or a combination of various techniques. The advantages and disadvantages of various harvesting techniques are presented in Table I.4.

*Table I.4: Advantages and disadvantages of various harvesting techniques (Abdelaziz et al., 2013; Barros et al., 2015; Mata et al., 2010).*

<b>Harvesting Technique</b>	<b>Advantages</b>	<b>Disadvantages</b>
Centrifugation	Fast and effective technique; High recovery efficiency (> 90%);	Expensive technique with high energy requirement; High operation and maintenance costs; Appropriate for recovery of high-valued

	Preferred for small scale and laboratory; Applicable to all microalgae	products; Time consuming and too expensive for large scale; Risk of cell destruction
Flocculation	Fast and easy technique; Used for large scale; Less cell damage; Applied to vast range of species; Less energy requirements; Auto and bioflocculation may be inexpensive methods	Chemicals may be expensive; Highly pH dependent; Difficult to separate the coagulant from harvesting biomass; Efficiency depends upon the coagulant used; Culture medium recycling is limited; Possibility of mineral or microbial contamination
Flotation	Suitable for large scale; Low cost and low space requirement; Short operation time	Needs surfactants; Ozoflotation is expensive
Filtration	High recovery efficiency; Cost effective; No chemical required; Low energy consumption (natural and pressure filter); Low shear stress;	Slow, requires pressure or vacuum; Not suitable for small algae; Membrane fouling/clogging and replacement increases operational and maintenance costs; High energy consumption (vacuum filter)
Electricity assisted techniques	Applicable to all microalgal species; No chemicals required	Metal electrodes required; High energy and equipment costs; Metal contamination

### I.2.3.1 Centrifugation

Most microalgae can be harvested from the culture medium using centrifugation. Centrifugation process depends on the size and structure density difference of microalgal cells, as well as slurry residence time in the centrifuge (Singh and Patidar, 2018). Centrifugation is preferred for harvesting of microalgal biomass and extended shelf-life concentrates for aquaculture (Grima et al., 2003). Laboratory centrifugation tests were usually conducted at 500–1000×g and showed that about 80–90% of recovery efficiency within 2–5 min (Chen et al., 2011). This process is rapid, and can reduce the use of chemicals solvents. However,

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centrifugation can lead to cell damage due to exposure of microalgal cells to the generated heat, high shear and gravitational forces applied (Goh et al., 2019).

### I.2.3.2 Flocculation

Flocculation involves a process that dispersed particles are aggregated together to form large particles for settling (Chen et al., 2011). Flocculation has been proposed to be the most cost effective methods for harvesting microalgal biomass as it can be used for large volumes of cultures (Vandamme et al., 2013). Since microalgal cells have a negative surface charge and found in dispersed state that results in slow natural sedimentation (Singh and Patidar, 2018). These microalgal cells can be successfully harvested by adding flocculants to cause cells aggregation or reduce the negative charge. The most used flocculants can be divided into two main types, inorganic and organic flocculants. Inorganic chemical flocculants are multivalent cations such as ferric chloride, aluminium sulfate, ferric sulphate and polyferric sulphate. Organic flocculants can be cationic, anionic, or non-ionic. It may also physically link one or more particles through a process called bridging, to facilitate the aggregation (Grima et al., 2003). The most suitable physically flocculants are multivalent metal salts, such as ferric chloride ( $\text{FeCl}_3$ ), aluminium sulphate ( $\text{Al}_2(\text{SO}_4)_3$ ) and ferric sulphate ( $\text{Fe}_2(\text{SO}_4)_3$ ). Furthermore, flocculation can occur spontaneously flocculates microalgae in suspension by other microorganisms produced some flocculants, named as bio-flocculation (Goh et al., 2019). The bio-flocculation technique has been implemented successfully in wastewater treatment plants, however, the underlying mechanism is still not very clear.

### I.2.3.3 Flotation

Flotation methods are based on the binding of microalgal cells using micro-air bubbles without adding any chemicals (Brennan and Owende, 2010a). Some microalgal species can naturally float on the water surface due to low density and self-float characteristics (Burton et al., 2009). Flotation is able to recovery particles in less than 500  $\mu\text{m}$  by collision and adhesion between the bubble and microalgal cells (Tan et al., 2018). Several important parameters, such as bubble size, surfactant concentration and pH, can affect the efficiency of flotation (Barros et al., 2015). Flotation processes can be classified into dissolved air flotation (DAF) and dispersed air flotation (DiAF), depending on the methods of bubble size production (Singh and Patidar, 2018; Tan et al., 2018). DAF is the most used method in the industrial wastewater treatment. During the process of DAF, small bubbles with the size range from 10

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to 100 µm are produced. Microalgal biomass harvesting can be achieved by DAF in three paths: i) saturation at atmospheric pressure and flotation under vacuum condition, ii) saturation in static head with flow upward causing bubble formation (micro-flotation) and iii) saturation with pressure which is higher than atmospheric (Tan et al., 2018). By contrast, DiAF is the process where continuous air bubbles are generated through porous material. This process requires less energy input as compared with microbubble production method. However, small bubbles are difficult to be generated.

#### I.2.3.4 Filtration

Conventional filtration operates under pressure or in a vacuum (suction), which is used to harvest large quantities of microalgae (> 70 µm), such as *Coelastrum* and *Spirulina* (Brennan and Owende, 2010a; Mata et al., 2010). Tangential flow filtration is a high rate method with the advantage of maintaining the integrity of microalgae biomass. Petrusevski et al (Petrusevski et al., 1995) have successfully recovered 70-89% of fresh microalgae like *Stephanodiscus hantzschii*, *S. Astraea*, *Cyclotella* sp. and *Rhodomonas minuta* by using tangential flow filtration.

Alternative, membrane microfiltration and ultrafiltration process are the appropriate methods for harvesting smaller size of microalgae (< 30 µm) like *Scenedesmus*, *Dunaliella* and *Chlorella* (Brennan and Owende, 2010a; Tan et al., 2018) or fragile microalgal cells (Borowitzka, 1997; Mata et al., 2010). At larger scales of production (> 20 m<sup>3</sup> per day) membrane filtration may be a less economic method than centrifugation because of the need for membrane exchange and pumping. However, for processing of small volumes (< 2 m<sup>3</sup> per day), it can be more cost effective compared to centrifugation (MacKay and Salusbury, 1988). Mohn et al. (Mohn, 1980) have utilised chamber membrane filter press to harvest *Coelastrum proboscideum*. They obtained 27% solids of sludge that was 245-fold higher concentration than original concentration.

#### I.2.3.5 Electricity assisted techniques

Electricity is able to improve the efficiency of microalgae harvesting. These techniques can be deemed as environmentally friendly due to they require low chemical usage and low power consumption (Goh et al., 2019). Among them, the mechanism of electrocoagulation refers to three consecutive stages: i) generating coagulants by electrolytic oxidation of sacrifice electrode, ii) destabilization of particulate suspension and breaking of

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emulsion, and iii) forming flocs by reaggregating the destabilized phases. The continuous flow electrocoagulation has been successfully applied to harvesting microalgae from industrial waste-water (Azarian et al., 2007).

Additionally, electricity is also applied to improve the efficiencies of flocculation and floatation, these techniques are named as electrolytic floatation and electrolytic flocculation (Goh et al., 2019). Electrolytic floatation is achieved by formation of fine hydrogen bubbles at the cathode that will capture floating particles and allows for better microalgae separation (Baierle et al., 2015). Electrolytic flocculation utilizes charge neutralization which creates sorption affinity for negatively charged particles (Shi et al., 2017). Poelman et al. (Poelman et al., 1997) have successfully recovered 80-95% of microalgae by using electrolytic flocculation. The efficiency of the process depends on electrode material, electrolysis time, current density, pH and composition of the microalgae suspension (Singh and Patidar, 2018).

#### I.2.4 Drying

The recovered microalgal slurry (typical 5-15% dry solid content) is perishable and must be processed rapidly after harvesting; drying is commonly used to extend the viability depending on the final product required (Brennan and Owende, 2010a). Example of drying of wet microalgal biomass is one of the important steps prior to biodiesel production. High moisture content presented in the microalgal biomass can affect the yield and efficiency of the biodiesel processing (Tan et al., 2018). Methods that have been applied to drying microalgae include sun drying, convective drying, spray drying and freeze drying.

##### I.2.4.1 Sun drying

Sun drying is the cheapest drying method by utilizing natural sunlight. However, this method requires long drying times and large drying surfaces. Moreover, it is difficult to maintain the quality of the end biomass because of the slow drying rate can cause biomass degradation and thus a rise in the bacterial count (Chen et al., 2015).

##### I.2.4.2 Convective drying

Convective drying is also a popular drying method for microalgae dehydration, which is commonly done by a type of convective hot air drying, such as oven drying (Chen et al., 2015). A wide range of temperature (20-60 °C) and time (18-48 h) have been utilized for convective drying. But generally, 60 °C temperature and overnight drying duration is used

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(Rubio et al., 2010). Oliveira et al. (Oliveira et al., 2010) have demonstrated the optimal temperature range for *Spirulina* sp. using this method is 40-55 °C. They found that the phycocyanin loss percentage is approximately 37%, while the fatty acid composition is not significantly different between dried biomass and fresh biomass.

#### I.2.4.3 Spray drying

Besides drying using sunlight and convective hot air, spray drying is commonly used to dry high value microalgal products. However, this method is relatively expensive and may cause deterioration of microalgal pigments (Brennan and Owende, 2010a). Additionally, spray drying can retain higher yields of nutrients compared with convective drying.

#### I.2.4.4 Freeze drying

Like spray drying, freeze drying is also costly, especially for large scale process. For freeze drying, the drying temperatures are within -50 to -80 °C with time duration around 24-48 h (Khanra et al., 2018). Therefore, freeze drying is used instead of thermal drying when the final bio-products are living system or thermal sensitivity (Khanra et al., 2018). Compared with convective drying and spray drying, freeze drying keeps the most amount of proteins in dried microalgal biomass, with the protein loss being below 10% (Desmorieux and Hernandez, 2004).

Moreover, lipids are difficult to extract from wet biomass with solvents without cell disruption freeze drying, but are extracted more easily from freeze dried biomass. This is because microalgal biomass freezes slowly, larger intracellular ice crystals form, causing disruption of the cell wall (Chen et al., 2015).

#### I.2.5 Cell disruption techniques

Disruption of microalgae is very important step in biorefinery of valuable bio-molecules, which are present inside the cells. Figure I.15 implies cell disruption aims to permeabilize or completely break microalgal cell wall and membrane to allow direct access of water/solvent to the intracellular bio-molecules, thus realizing a simple extraction or release of intracellular bio-molecules (Goh et al., 2019; Postma et al., 2016). However, obtaining these bio-molecules is not an easy task and requires application of special techniques for cell disruption and extraction.

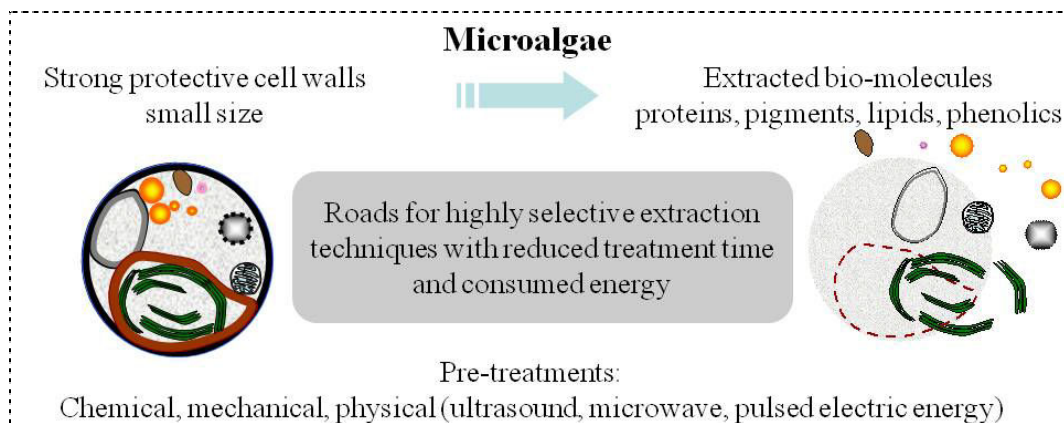


Figure I.15: The action of cell disruption methods for microalgae (Zhang et al., 2019b).

State of the art cell disruption techniques include: mechanical (e.g. shear forces, electrical pulses, waves or heat) and non- mechanical (e.g. chemical or biological) (Lee et al., 2017). Figure I.16 shows the classification of cell disruption methods used for microalgae biorefineries.

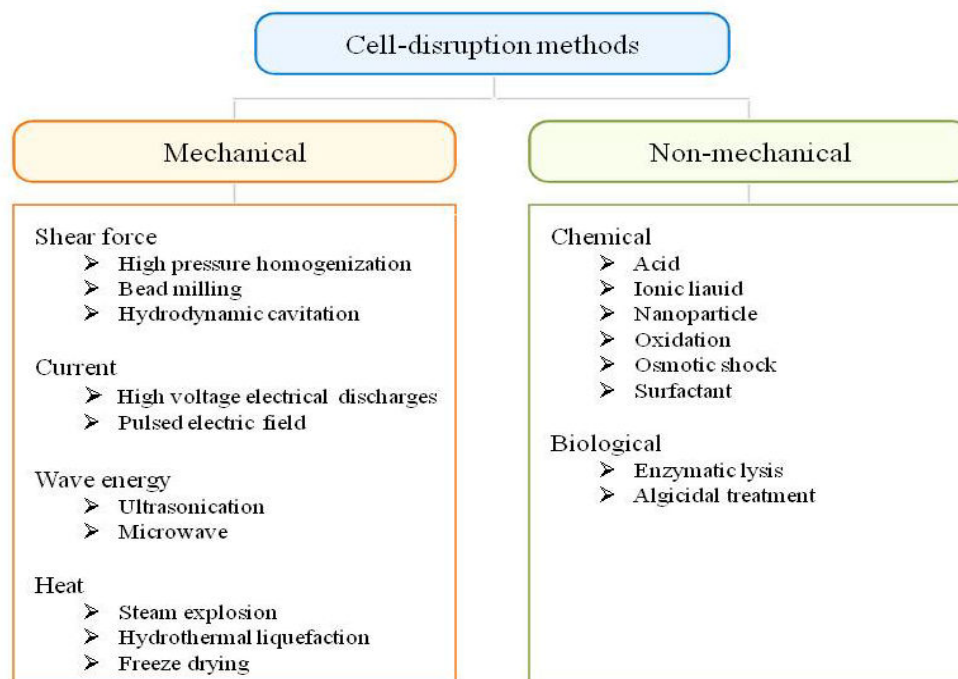


Figure I.16: Classification of cell disruption methods for microalgel biorefineries (Lee et al., 2017).

However, the appropriate cell disruption technology is selected based on the given microalgal species' cell-wall characteristics and status (wet/dried), as well as on the target bio-molecules nature (Show et al., 2015). This section represents the basic mechanisms of several alternative cell disruption techniques (High voltage electrical discharges (HVED),



pulsed electric fields (PEF), ultrasonication (US) and high pressure homogenization (HPH) and their relating case studies. An overview of recent studies on the cell disruption for microalgae is summarized in Table I.5.

Table I.5: An overview of recent studies investing cell disruption of microalgae.

Microalgae	Treatment conditions	Major findings	Refs.
<b>HVED</b>			
<i>Nannochloropsis</i> sp.	40 kV/cm, 4 ms	HVED allowed selective extraction of water-soluble ionics and small molecular weight organic compounds.	(Grimi et al., 2014)
<i>P. kessleri</i>	40 kV/cm, 8 ms	HVED was effective for the extraction of ionics and carbohydrates, while it was ineffective for pigments and protein extraction.	(Zhang et al., 2019a)
<b>PEF</b>			
<i>C. reinhardtii</i>	0.5–15 kV/cm; 0.05–0.2 ms	70% of the proteins could be released	(t Lam et al., 2017)
<i>A. protothecoides</i>	23–43 kV/cm; 36-167 g <sub>dw</sub> /kg <sub>sus</sub>	Cell disintegration efficiency increased with increasing specific energy input, whereas the field strength hardly had any influence.	(Goettel et al., 2013)
<i>C. vulgaris</i>	27–35 kV/cm, 10.8 and 14 kV, 1-6 Hz	PEF treatment induced irreversible permeabilization of microalgae cells, and improving extraction yield of ionics, carbohydrates and phenolic compounds.	(Pataro et al., 2017)
<b>US</b>			
<i>H. pluvialis</i>	18.4 W; 60 min 45 °C	55-60 % yield increase of astaxanthin after US treatment	(Ruen-ngam et al., 2010)
<i>C. vulgaris</i>	200 W; 78.7 min; 61.4 °C	Enhanced chlorophyll recovery (59 %)	(Kong et al., 2014)
<i>Cryptocodinium</i>	19–300 kHz	US increased oil yield (25.9%) compared	(Cravotto et al., 2008)



<i>cohnii</i>		conventional treatment	
<b>HPH</b>			
<i>N. oculata</i>	75–350 MPa, 1.6 % dw	HPH treatment obtained 22.7-50.4 mg/g proteins and 55-62.5 mg/g sugars.	(Shene et al., 2016)
<i>C. vulgaris</i>	150 MPa, 1.2 % dw	HPH resulted in 1.1 and 10.3 folds higher yields than PEF, respectively of carbohydrates and proteins.	(Carullo et al., 2018)
<i>T. suecica</i> , <i>Chloroocccum</i> sp.	517 or 862 bar	Mean disruption rate constant for HPH was about 7-fold for US.	(Halim et al., 2013)
<i>Chloroocccum</i> sp.	500 or 850 bar, 15 min	HPH resulted in 73.8% average disruption of initial intact cells.	(Halim et al., 2012b)

<sup>†</sup>dw: dry weight; *P. kessleri*: *Parachlorella kessleri*; *C. reinhardtii*: *Chlamydomonas reinhardtii*; *A. protothecoides*: *Auxenochlorella protothecoides*; *C. vulgaris*: *Chlorella vulgaris*; *H. pluvialis*: *Haematococcus pluvialis*; *C. cohnii*: *Cryptocodinium cohnii*; *N. oculata*: *Nannochloropsis oculata*; *T. suecica*: *Tetraselmis suecica*;

#### I.2.5.1 High voltage electrical discharges (HVED)

The HVED treatment is one of the applications of liquid phase discharge technology, and is lately developed as an innovative alternative cell disruption technique to conventional extraction methods. The HVED treatment are commonly applied to aqueous wet biomass using needle-plane electrode geometry, such treatment happened accompanies by electrical and mechanical process.

The electrical discharge process is comprised of the streamer discharge process (pre-breakdown phase) and the electric arc process (breakdown phase) (Boussetta and Vorobiev, 2014). The probable action mechanisms and phenomenon and of HVED were shown in Figure I.17. During the streamer discharge process, on one hand, the relatively weak shock wave and a small number of little bubbles cavitations appeared in water. On the other hand, it also generates high-intensity UV radiation and active radicals (Li et al., 2018). When the streamer reaches the grounded plane electrode, the pre-breakdown phase transits to the breakdown phase. During the electric arc process, more intensive electrohydraulic effects happened resulted in stronger shock waves, liquid turbulence and UV radiation, as well as produce highly concentrated free radicals (Barba et al., 2015a). Hence, in short, important effects of HVED on wet biomass include electrical breakdown and some secondary

phenomena in water. By means of these phenomena lead to microalgal cell structure damage and particle fragmentation, consequently facilitating the release of bio-molecules (Zhang et al., 2019b).

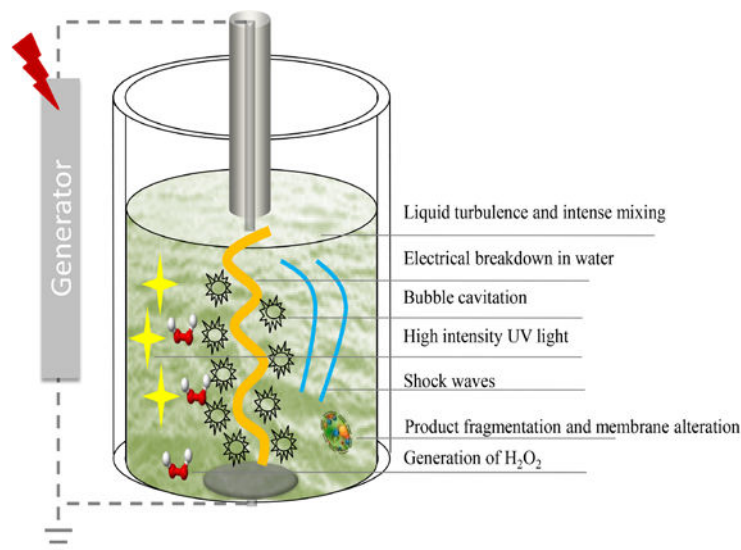


Figure I.17: The probable mechanisms of action of HVED treatment (Zhang et al., 2019a).

In recent years, our team have tried to recovery of ionic components, proteins, carbohydrates and pigments from *Nannochloropsis* sp. and *P. kessleri* by application of HVED treatment (Grimi et al., 2014; Zhang et al., 2019a). For example, Figure I.18 presents the effects of different cell disruption techniques (PEF, HVED, US and HPH) on extraction of ionic components and chlorophylls from *Nannochloropsis* sp. in aqueous phase. A sequentially extraction procedure (PEF → HVED → US → HPH) were applied, and after each extraction, the supernatants were replaced by the deionized water. The data demonstrated that HVED treatment allowed significantly increase recovery ratio of ionic components (Figure. 1.18a). Moreover, after application of the first PEF and HVED steps, the next sequential US and HPH steps gave rather small additional input to the extraction. However, pulsed electric energy (PEF and HVED) steps were ineffective for extraction of chlorophylls (Figure. 18b) and gave only  $\approx 1\%$  of extraction level. The noticeable recovery of chlorophylls was only obtained after application of sequential steps US → HPH. Interestingly, it was observed that microalgal cells after the HVED treatment were highly agglomerated by microscopic analyses (see inset to Figure. 18a). The author attributed this phenomenon to that HVED treatment changes the surface charge of the microalga results in the loss of the stability of the suspension.

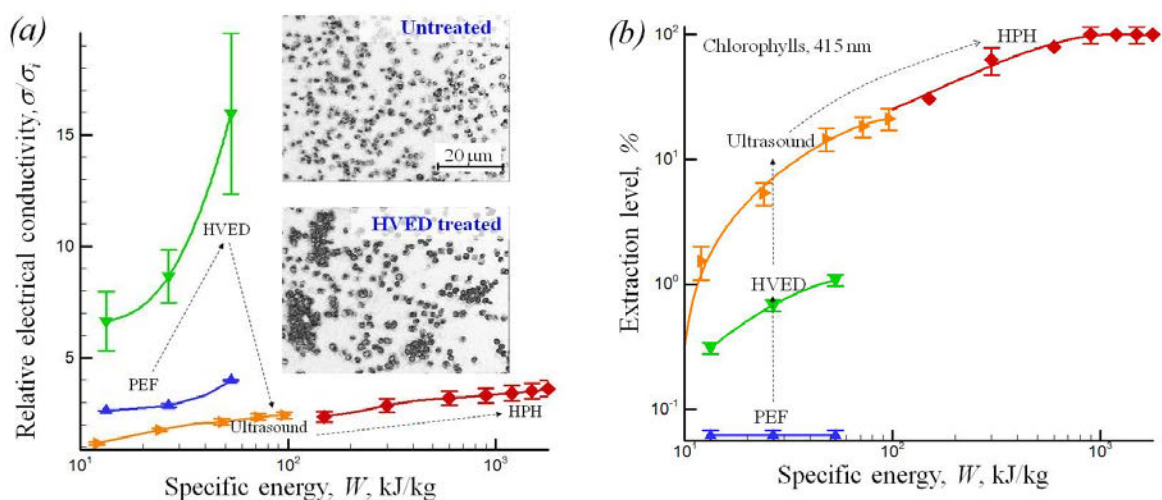


Figure I.18: Ratio of electrical conductivities,  $\sigma/\sigma_0$ , after and before treatment (a) and extraction level of chlorophylls ( $\lambda = 415$  nm) versus the specific energy,  $W$ , (b). Insert a shows the microscopy images of untreated and HVED treated *Nannochloropsis* sp. (Grimi et al., 2014).

In general, the HVED treatment is simple, fast, can be energetically efficient and combined with other extraction techniques. However, there is still lack of more informations about the effect of HVED treatment on bio-molecules recovery from microalgae.

### I. 2.5.2 Pulsed electric fields (PEF)

The PEF treatment is an innovative and promising method for non-thermal processing of cell disruption. This minimally invasive (mild) cell disruption allows avoidance of undesirable changes in a biological material, and acceleration of extraction by electrical breakage of cellular membranes (Vorobiev et al., 2012).

The action of PEF caused cell disruption is reflected by the loss of membrane barrier functions. A membrane envelope around the cell restricts the exchange of inter- and intracellular media. The application of PEF induces the formation of pores inside the membrane and increases its permeability. Traditionally this phenomenon is called “electroporation” or “electropermeabilization” (Weaver and Chizmadzhev, 1996). The degree of electroporation depends on the potential difference across a membrane, or the transmembrane potential. Depending on the duration of cell’s PEF exposure time, a reversible (temporary) or irreversible (permanently) loss of barrier function may occur (Figure I.19). If the field strength exceeds what is known as reversible threshold and exposure is of sufficient duration, so-called reversible electroporation occurs; the membrane is permeabilized and remains in a state of higher permeability for a period of time, but is eventually able to

spontaneously return to its original state by means of membrane resealing, a process in which the pores close and the cell restores its normal transmembrane potential. By contrast, if the field strength and amount of delivered energy are too high, however, irreversible electroporation occurs, resulting in loss of cell homeostasis (and possibly in a complete breakdown of the plasma membrane), effectively killing the cell (Mahnic-Kalamiza et al., 2014).

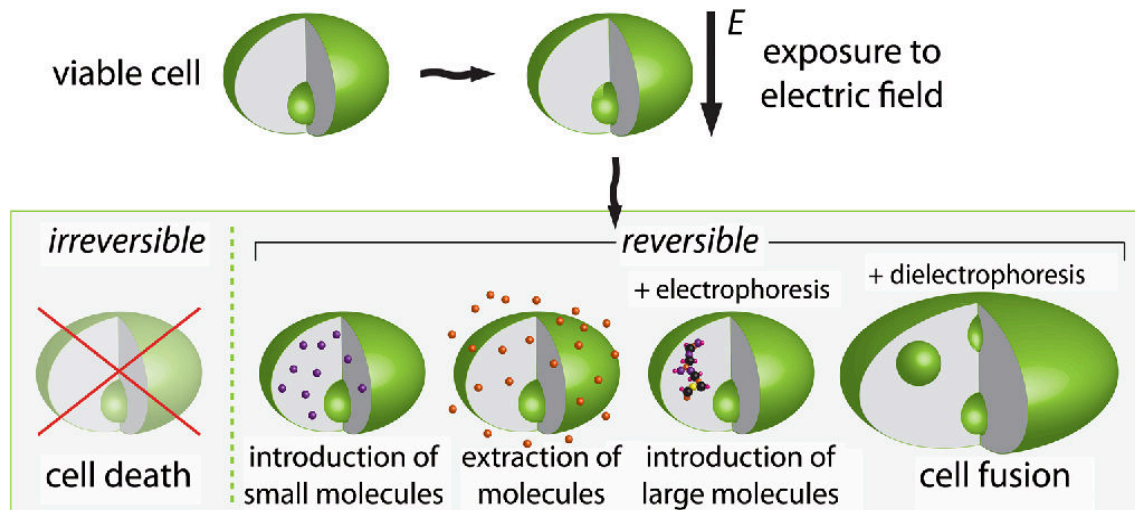


Figure I.19: A schematic representation of cell electroporation with possible outcomes depending on the pulsing protocol (amplitude, shape, duration of pulses) and additional cell manipulation techniques, e.g. (di)electrophoresis (Mahnic-Kalamiza et al., 2014).

Nowadays, the PEF treatment is widely used for food and biomaterials. Figure I.20 gives a schematic representation of exposure of a biological cell to an external electric field, and corresponding processing intensity and energy input for PEF. For microalgal cells it was shown that an application of 15-40 kV/cm is sufficient to induce pore formation, and results in specific energy input of 400-1000 kJ/kg (Mahnic-Kalamiza et al., 2014; Topfl, 2006). PEF assisted extraction from biological cell is expected to be highly selective with respect to low and high molecular weight bio-molecules, and have a small influence on the cell wall due to non-thermal processing (Vorobiev et al., 2012). However, the efficiency of PEF treatment for bio-suspensions may be dependent on multiple factors, including cell characteristic (cell shape, size and aggregation state) and suspension properties (electrical conductivity, salinity, pH and cell density) and others (Vorobiev et al., 2012).

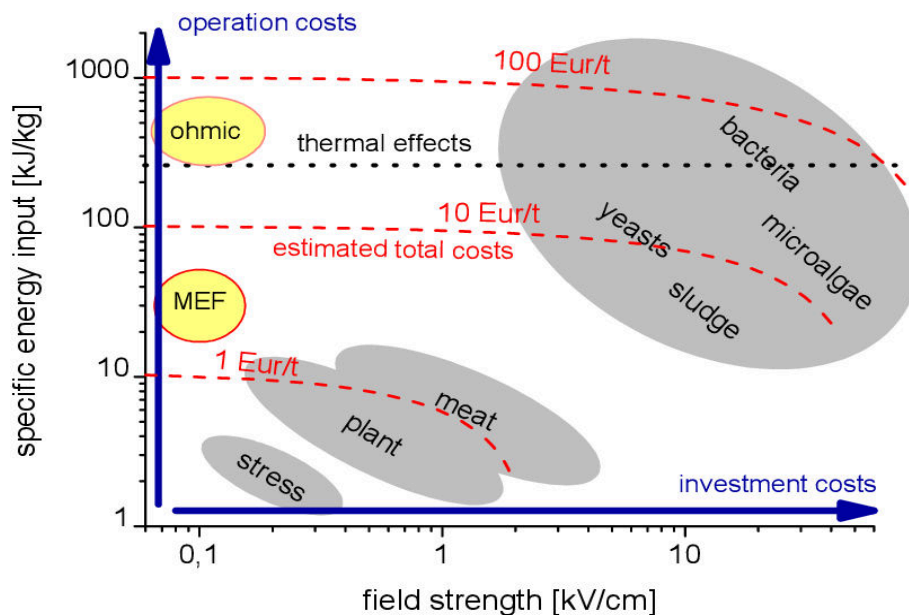


Figure I.20: Overview of required processing intensity for PEF application to induce stress reactions, disintegration of plant or animal cells and microbial inactivation (Topfl, 2006).

In the last decades, there exist many successful examples of PEF application for the enhancement of extraction of different valuable components from microalgae. For example, PEF-assisted extraction of pigments from *Chlorella vulgaris*, proteins, carbohydrates and phenolics from *Nannochloropsis* sp., cytoplasmic proteins from *Nannochloropsis salina* and *Chlorella vulgaris*, and lipids from *Auxenochlorella protothecoides* have been recently tested (see, (Barba et al., 2015) for a recent review). In many cases, it was supposed that observed effects reflect the cell membrane permeabilisation.

For the extraction of water-soluble hydrophilic components (ionic components, carbohydrates, proteins), the aqueous media, or mixed solvents and pH regulation have been used. It was demonstrated that PEF treatment allowed extraction of ionic components, amino-acids and small water-soluble proteins from *Nannochloropsis* sp. (Grimi et al., 2014). However, the PEF treatment was neither successful for protein release (10% proteins, w/w) nor energy-efficient. For better efficiency of PEF treatment, the use of the binary mixture of organic solvent and water were tested. PEF-assisted extraction of different bio-molecules (total chlorophylls, carotenoids, proteins and phenolics) from *Nannochloropsis* spp. using the mixture of organic solvents (dimethyl sulfoxide, DMSO and ethanol, EtOH) and water was investigated (Parniakov et al., 2015c). Two-step procedure was applied, including a PEF (20 kV/cm, 4 ms) treatment in water at the first step and extraction in a binary mixture at the second step. This applied procedure allowed efficient extraction of proteins at the first step

with a better extraction of pigments and other high-added value bio-molecules at the second step.

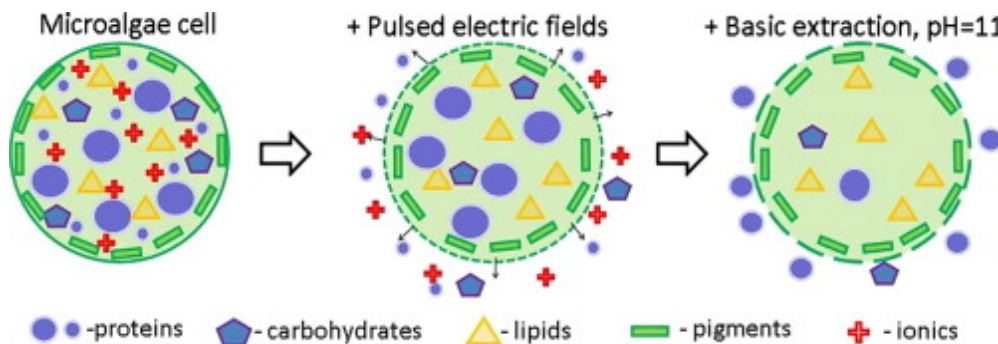


Figure I.21: Schematic representation of PEF treatment and pH assisted selective extraction of bio-molecules from microalgae (Parniakov et al., 2015b).

Moreover, the PEF treatment combined with pH-assisted aqueous extraction was also used selective extraction bio-molecules from *Nannochloropsis* sp. (Parniakov et al., 2015b). Figure I.21 presents a schematic representation of PEF treatment and pH assisted selective extraction of bio-molecules from microalgae.

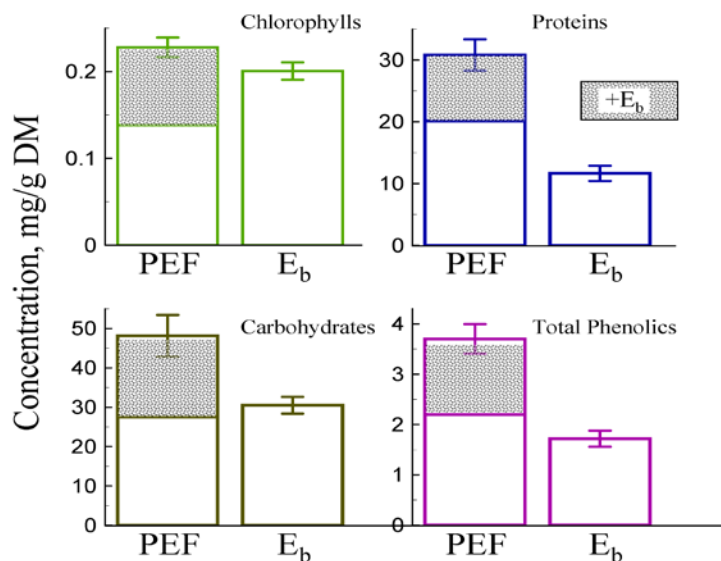


Figure I.22: Concentration of chlorophylls, proteins, carbohydrates and total phenolics extracted from *Nannochloropsis* sp. The data are presented for extracts, obtained after PEF treatment, and aqueous extraction in the basic medium, E<sub>b</sub>. The effects of supplementary aqueous extraction + E<sub>b</sub> are also shown (Parniakov et al., 2015b).

In this study, the extraction efficiencies of various components (chlorophylls, proteins, carbohydrates and phenolic compounds) stimulated by PEF treatment was comparable with that obtained for aqueous extraction in a basic medium. However, supplementary basic



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extraction at pH 11 (+  $E_b$  is shown as dashed section of bars)) after the PEF treatment allowed a noticeable increase in the concentrations of all components in the extracts (Figure I.22). Thus, it was demonstrated that PEF pre-treatment has an excellent potential as a preliminary step of aqueous extraction of *Nannochloropsis* sp. components.

Additionally, the impact of PEF treatment for the extraction of cytoplasmic proteins from *Nannochloropsis salina*, *Chlorella vulgaris* and *Haematococcus pluvialis* was also demonstrated (Coustets et al., 2015). The results evidenced the PEF's potential for selective extraction of these compounds and higher purity of obtained extracts. The PEF treatment was also applied for the extraction of chlorophylls and carotenoids from microalgae. Due to the hydrocarbon structure, these pigments are hydrophobic substances, soluble only in organic solvents, oils and fats, and practically insoluble in water. For example, chlorophylls can be dissolved easily in acetone, and alcohol, but they have low solubility in alkanes (such as hexane and butane) and are practically insoluble in water. However, the complexes of chlorophylls binding molecules can be dissolved in water. The influence of treatment medium temperature (10-40 °C) on the extraction efficiencies of pigments (carotenoids, chlorophylls) and Lutein (carotenoid) from *Chlorella vulgaris* assisted by PEF treatment were investigated (Luengo et al., 2015). Higher temperature increased the sensitivity of microalgal cells to irreversible electroporation. It was demonstrated that irreversible "electroporation" required electric field strengths of order  $\geq 4$  kV/cm and  $\geq 10$  kV/cm for pulse durations in the millisecond and microsecond ranges, respectively. Moreover, the induction period was observed and the extraction yield of carotenoids was significantly increased for the extraction applied after 1 h of the PEF treatment.

Furthermore, PEF-assisted extraction of hydrophobic intracellular lipids from microalgae requires application of organic solvents or strong mixtures to penetrate the cell wall and outer membranes. The green solvent (ethyl acetate) used as supporting solvent allowed significant improvement the lipid recovery for PEF-assisted extraction from *Ankistrodesmus falcatus* (Zbinden et al., 2013). In absence of PEF, the extraction efficiency for ethyl acetate was lower (83–88%) than that of chloroform. Focused-pulsed (FP) assisted extraction applied for *Scenedesmus* yielded 3.1-fold more crude lipids and fatty acid methyl ester (FAME) (using hexane over control) after recovery in different solvent mixtures (Lai et al., 2014). FP assisted extraction also increased the FAME-to-crude-lipid ratio for all tested solvents.

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The effects of PEF treatment on lipids recovery from *Auxenochlorella protothecoides* were tested in several works (Eing et al., 2013; Silve et al., 2018). The evaluated lipids content for this microalga is rather high (30–35% of cell dry weight). PEF treatment (23–43 kV/cm, 52–211 kJ/kg) was applied to  $\approx 10\%$  aqueous suspension and after extraction of water-soluble cell components during the first step, the lipid extraction from residual biomass was applied using 70% ethanol (EtOH) as solvent at the second step (Eing et al., 2013). The proposed extraction procedure from the wet biomass had the comparable efficiency with extraction from dry biomass. The proposed PEF assisted extraction of lipids from wet biomass is economically expedient, because the energy requirements (1.5 MJ/kg DW) is lower compared to the required energy for dried biomass (7 MJ/kg DW). In another work (Silve et al., 2018), the PEF treatment (10 kV/cm, 150 kJ/kg) was applied to concentrated biomass (10% w/w solids) as pre-treatment prior to organic solvent extraction of lipids in the triple mixture of water/ethanol/hexane (1: 18: 7.3, v/v/v). Experiments were performed with mixotrophic and autotrophic cultures. For PEF untreated the extraction yield was up to 10% of total lipids content. PEF treatment enabled to recover 92% (mixotrophic), and 72% (autotrophic) of the evaluated lipid content after 2 h of extraction, and 97% (mixotrophic), and 90% (autotrophic), after 20 h of extraction.

In general, the direct effects of PEF on the cell walls and disruption of them are marginal. The PEF treatment did not alter proteins, pigments, lipids and fatty acids compositions. The PEF-assisted extraction technique can be applied in highly selective modes for the extraction of non-degraded ionic components, phenolic compounds, proteins, pigments and lipids from microalgae. This technique show promising perspectives for industrial upscaling. However, the extraction efficiency of this technique for high molecular weight and hydrophobic components may rather low. Moreover, in practical application the thorough optimization of PEF treatment protocols, temperature, pH and supporting solvents are required for different species of microalgae.

#### I. 2.5.3 Ultrasonication (US)

US is also a sustainable and innovative cell disruption technology. Several classes of valuable bio-molecules such as aromas, pigments, antioxidants, and other organic and mineral compounds have been extracted efficiently from a variety of matrices (mainly animal tissues, microalgae, yeasts, food and plant materials) (Chemat et al., 2017). In order to meet the



requirements of “green extraction”, using US-assisted extraction can be now be completed in short time with high recovery efficiency, reducing the used solvent and energy input (Chemat et al., 2017). This evolution or revolution of extraction of natural products is resumed in Figure I.23.

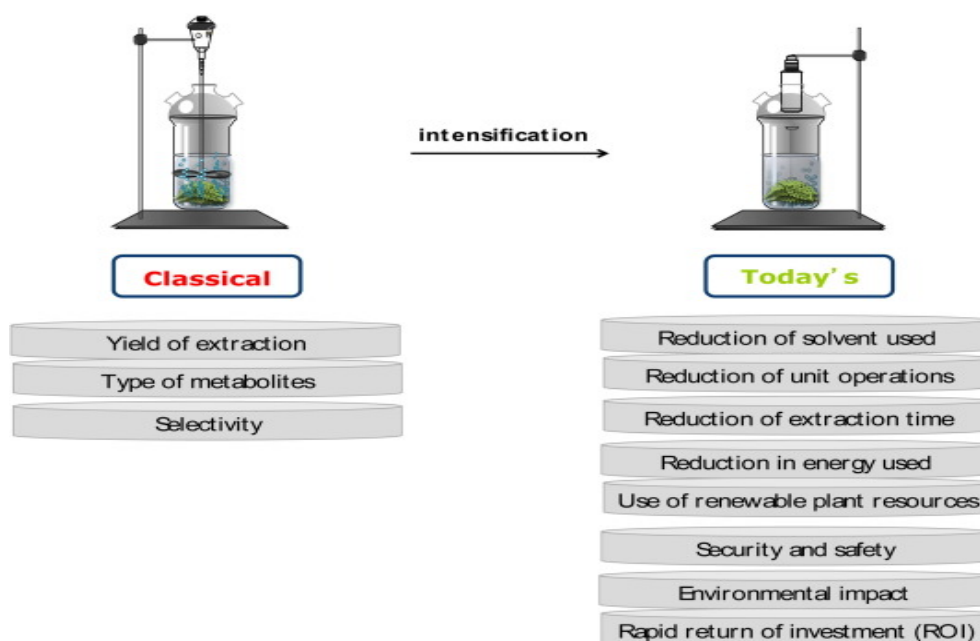


Figure I.23: Ultrasound-assisted extraction: evolution or revolution (Chemat et al., 2017).

Ultrasound waves are high frequency (20 kHz to 1 MHz) sound waves beyond our human hearing limit (Zhang et al., 2019b). The basic principle of US is acoustic cavitation and micro-streaming. When high power ultrasound waves propagate through any medium, a sequence of compressions (positive pressure) and rarefactions (negative pressure) is induced in the molecules of the medium causing pressure alteration. The developed negative pressure during the rarefaction phase advances above tensile strength of the fluid causing the formation of cavitation bubbles from the gas nuclei of the medium. These bubbles grow over a number of cycles until they become unstable and finally violently collapse/implodes (Kumari et al., 2018). This phenomenon of creation, expansion, and implosive collapse of bubbles in ultrasonicated medium is called acoustic cavitation phenomenon (Tiwari, 2015). Figure I.24 depicts the schematic representation of the acoustic cavitation mechanism. Since frequency is inversely proportional to the bubble size so in case of power US treatment, larger cavitation bubbles are formed. This implosion generates high temperature and pressure which in turn results into high shear energy waves and turbulence causing combination of mechanical effect on the material. It also develops strong micro-streaming currents (Kumari et al., 2018). These

perturbations can shatter the cell walls, improve penetration of solvent inside biomass and accelerate diffusion.

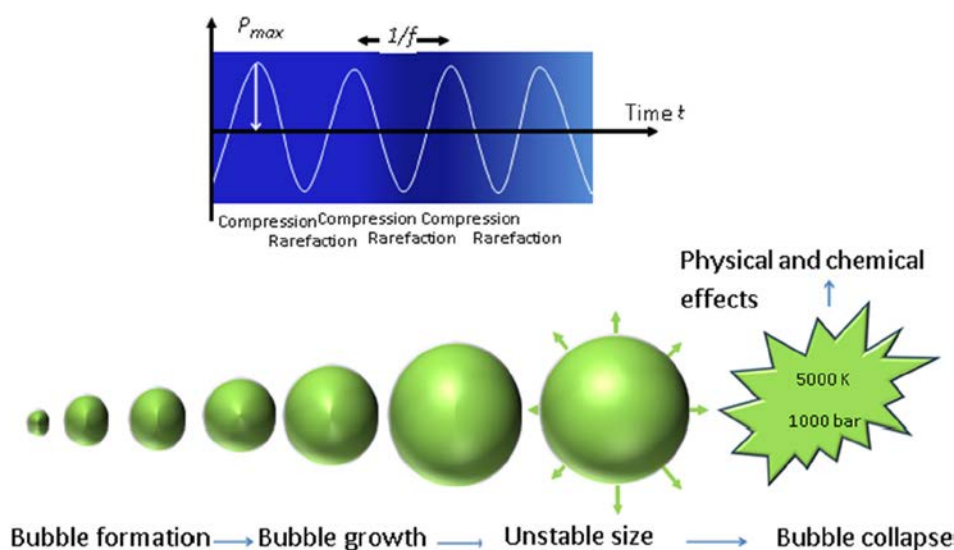


Figure I.24: Schematic representation of the acoustic cavitation mechanism (Lorimer and Mason, 1987).

The mechanism of US disruption in suspensions of five strains of microalgae (including *Chlamydomonas reinhardtii* (wild type and mutant strain), *Thalassiosira pseudonana*, *Isochrysis galbana*, *Nannochloropsis oculata*) with different sizes and cell wall compositions was studied (Greenly and Tester, 2015). The most significant cell disruption and a small difference between species were observed during the initial seconds of US. At longer exposure times, differences between species became more pronounced. The US-assisted extraction of lipids from several microalgal species (*Chlorella* sp., *Tetraselmis suecica* and *Nannochloropsis* sp.) has also been examined (Natarajan et al., 2014). The cell disruption efficiency correlated well with US energy consumption. For freshwater *Chlorella* sp. with rigid cell walls, the lipids were easily released to the aqueous phase whereas for other species *Tetraselmis suecica* and *Nannochloropsis* sp. the cells retained the membrane lipids after the disruption. The US-assisted extraction method (booster horn, 20 kHz, 1000 W), with water as a solvent, was tested to extract lipids from fresh *Nannochloropsis oculata* biomass (Adam et al., 2012). After extraction, the oil/water emulsion was demulsified by using the saline solution and centrifugation step. Finally, water and oil were separated into two distinct phases that simplified the oil recovery. Scanning electron microscope (SEM) analysis had shown that external structure of the surface of the cells was modified after US treatment. Cells were smaller and their parietal system and cell walls were damaged. During 30 min of extraction,

the oil recovery continuously increased with temperature increases from 1 to 35 °C. The maximum lipids recovery at optimum conditions (1000 W ultrasonic power, 30 min extraction time and dry weight content at 5.0%) was around 0.21%. The US process implied less solvent consumption, and a marked reduction in treatment time and temperature compared to conventional extraction.

The US-assisted extraction of phenolics and pigments from *Nannochloropsis* sp. has been tested (Parniakov et al., 2015a). The authors found that the extraction yields for US-assisted method was  $\approx 2$  times higher than that of conventional water extraction. Moreover, they reported that US-assisted extraction from concentrated suspension is less power consuming. For example, the increase of concentration from 1% wt to 10% wt resulted in  $\approx 10$ -folds decrease of US power consumption at approximately the same efficiency of extraction of total phenolic compounds and total chlorophylls (Figure I.25).

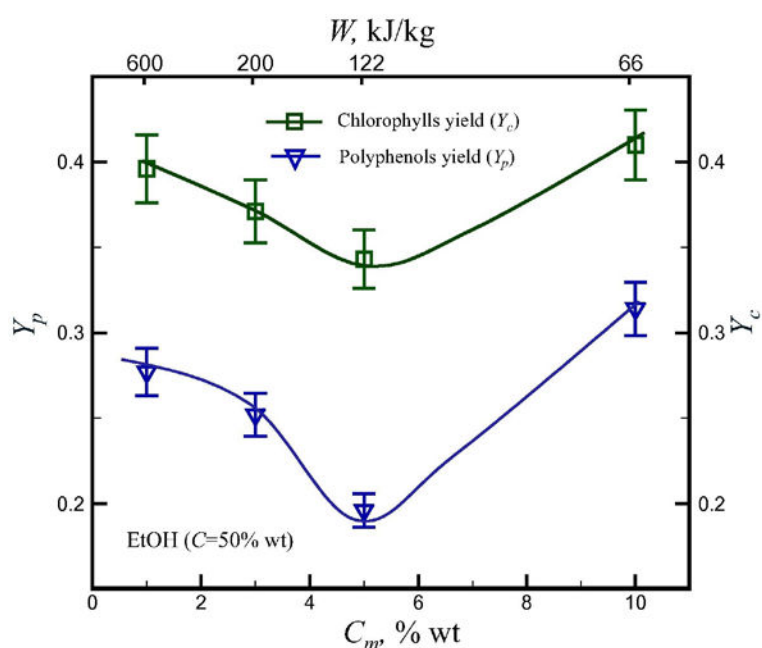


Figure I.25: Yields of total phenolic compounds,  $Y_p$ , and total chlorophylls,  $Y_c$ , versus the concentration of microalgae in suspension,  $C_m$ , for US-assisted extraction in binary mixture of solvents  $H_2O + EtOH$  ( $C = 50\%$  wt). The ultrasound power was 400 W and the extraction time was 5 min. The upper horizontal axis presents the energy input per kg of microalgae (Parniakov et al., 2015a).

The effectiveness high-frequency focused ultrasound (HFFU, 3.2 MHz, 40 W) and low-frequency non-focused ultrasound (LFNFU, 20 kHz, 100 W) techniques for disruption of *Nannochloropsis oculata* has been compared (Wang et al., 2014). HFFU treatment was more

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energy efficient as compared with LFNFU. Moreover, the combination of high and low-frequency treatments was even more effective than single frequency treatment. The effectiveness of a continuous ultrasonic flow system (2 kW) for disruption of *Nannochloropsis oculata* has been studied (Wang and Yuan, 2015). Cell recirculation was found beneficial to cell disruption. Nile red stained lipid fluorescence density and cell debris concentration in treated systems treatments increased up to 56.3% and 112%, correspondingly, compared to the control.

The novel technique combining simultaneous US and enzymatic hydrolysis treatment was used for extraction of reducing sugars from *Chlamydomonas mexicana* with improved yield by 4-fold as compared with the US pretreatment under optimum conditions (Eldalatomy et al., 2016).

#### I. 2.5.4 High pressure homogenization (HPH)

The HPH treatment is a desirable cell disruption method for microalgae with a recalcitrant cell wall structure (Yap et al., 2015). The underlying mechanism of cell disruption of HPH treatment have been investigated by Shirgaonkar et al. (Shirgaonkar et al., 1998), Kleinig and Middelberg (Kleinig and Middelberg, 1998), and Brookman (Brookman, 1974). In an HPH unit, the cell suspension is forced to flow through a narrow nozzle under high pressure where mechanical effects, including torsion and shear stresses, turbulence, impingement, shock waves, cavitation, and heating, promote cell disruption. The probable mechanisms of action of HPH treatment are shown in Figure I.26.

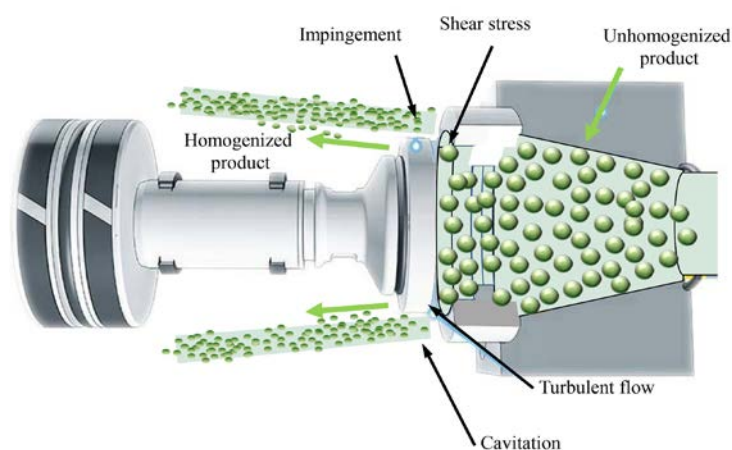


Figure I.26: The probable mechanisms of action of HPH treatment (Zhang et al., 2019a).

The degree of cell disintegration in HPH is mainly determined by the pressure at the valve (loading pressure) and the cell-suspension properties (viscosity, suspension concentration, cell size, etc.) (Lee et al., 2012). Among these technologies, HPH is widely used for the large-scale disruption of cells to recover bio-molecules from bio-suspension (Zhang et al., 2019b). It allowed for the release of numerous intracellular compounds including high- and low-weight molecules due to intensive cell disintegration (Lee et al., 2017).

Halim et al. (Halim et al., 2012b) reported that a higher applied homogenizer pressure of HPH treatment was beneficial for enhancement of *Chlorococcum* sp. cell disintegration efficiency. Later, they investigated the impact of applied homogenizer pressure and cell concentration on the cell disruption efficiency, using *Tetraselmis suecica* and *Chlorococcum* sp. (Halim et al., 2013). They found that the disruption rate was inversely proportional to cell suspension but positively correlated to homogenizing pressure.

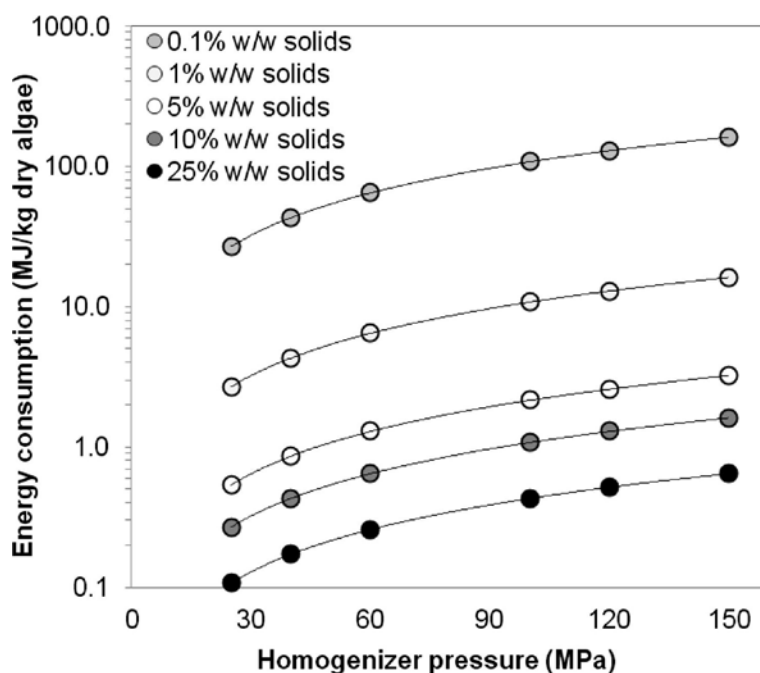


Figure I.27: The effect of cell concentration on the energy consumption per unit mass of dry algae through a process-scale high pressure homogeniser (Yap et al., 2015).

In a recent study, Yap et al. (Yap et al., 2015) analyzed the specific energy consumption of HPH treatment for rupturing *Nannochloropsis* sp. cells. The relationship among cell concentration (solid content, %), applied homogenizer pressure was evaluated. They reported that that energy efficiency is critically dependent on operating conditions and

cell concentration. This study also indicates that HPH treatment can be feasibly scaled to levels required for industrial algae processing.

Moreover, the impact of HPH (100 MPa) and ultra high pressure homogenization (UHPH, 250 MPa) on the degree of cell disruption was investigated for *Nannochloropsis* sp. suspensions (Bernaerts et al., 2019b). Applying an UHPH treatment obviously reduced the number of passes required to obtain a specific degree of cell disruption compared to HPH treatment. Figure I.28 presents that representative SEM images of cell suspensions before (untreated) and after different passes of HPH and UHPH treatment. The larger degree of cell disruption by UHPH is obvious from comparing the abundance of intact cells in contrast to HPH at the same number of passes. However, heating of the sample occurred in UHPH treatment resulting in extensive cell debris aggregation after multiple homogenization passes.

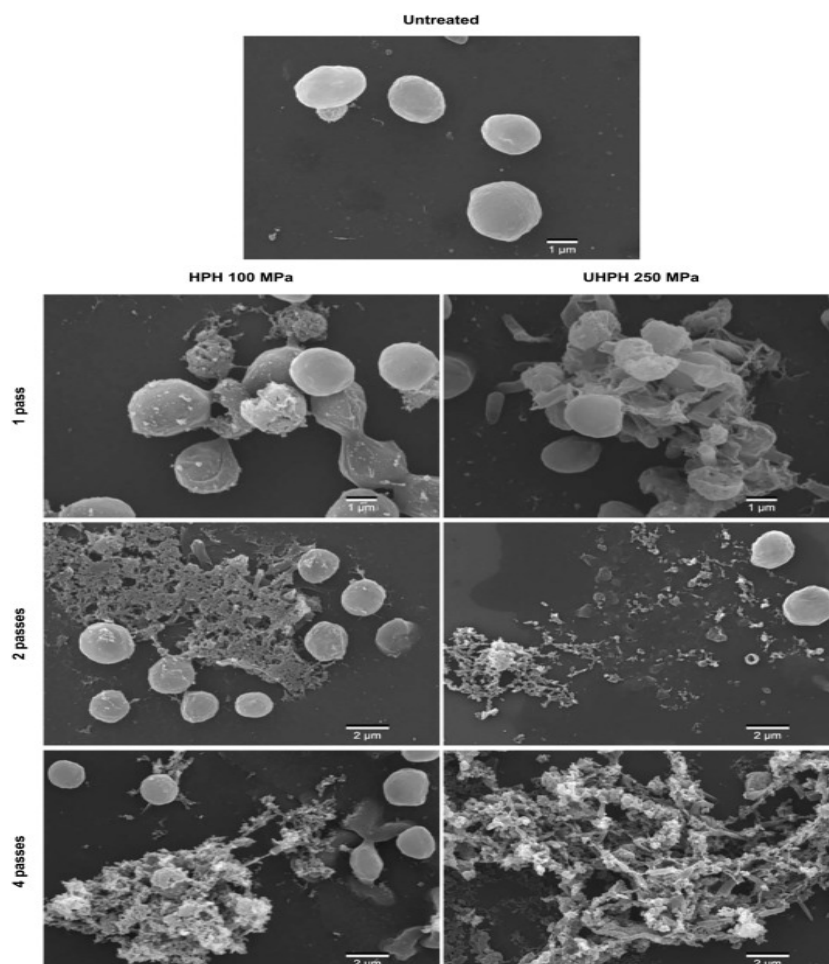


Figure I.28: Representative scanning electron microscopy (SEM) images of *Nannochloropsis* sp. suspensions before (untreated) and after different passes of HPH (100 MPa) and ultra UHPH (250 MPa) (Bernaerts et al., 2019b).



### I.2.5.5 Comparison of different cell disruption methods

#### ➤ *Cell disruption effectiveness and selectivity bio-molecules extraction*

The sustainability of valuable microalgae bio-products production largely depends upon efficient extraction of the bio-molecules. Cell disruption effectiveness was found to differ according to the microalgal species, cell wall strength, and disruption methods. An ideal extraction method should be more selective towards extraction of specific microalgal bio-molecules and simultaneously minimize the co-extraction of contaminants. Therefore, it is important to find appropriate cell disruption methods in order to improve the extraction effectiveness. Example of the disruption effectiveness of different techniques on *Nannochloropsis* sp. in terms of disrupted cells, recovery of pigments, proteins and lipids, as well as other compounds were summarized in Table I.6.

*Table I.6: Comparison cell disruption effectiveness of varied techniques for Nannochloropsis sp..*

<b>Methods</b>	<b>Major cell disruption effectiveness</b>	<b>Ref.</b>
HPH	≈ 91 % protein extraction	(Grimi et al., 2014)
US	0.21 % oil yields increase after US; pigments yield (≈ 1.5-fold) was higher after US	(Adam et al., 2012) (Grimi et al., 2014)
PEF	proteins yield (≈ 5-fold) was higher after PEF	(Coustets et al., 2013; Parniakov et al., 2015)
HVED	Increase of pigments and proteins extraction after HEVD compared to control sample	(Grimi et al., 2014)

#### ➤ *Cost-effectiveness*

Cell disruption techniques often acquire high energy input. Cost-effectiveness in cell disruption is related to several factors such as energy consumption per kilogram of dry weight, concentration of treated cell suspensions, time to obtain reasonable disruption yields and so on (D'Hondt et al., 2018). Most of the researchers reported current cell disruption techniques such as US as cost-effective technologies for bio-molecules extraction from microalgae. However, generalization is very complicated due to different operating conditions in varied methods and many unknown factors in different microalgal species. Comparison between treatments is also complicated because of the lack of knowledge concerning the relation between the extraction yield and the energy input. Table I.7 compares cell disruption

techniques for *Nannochloropsis* sp. in terms of energy consumption. A varied energy consumption of 0.1-1500 kJ/kg was obtained for these cell disruption techniques.

*Table I.7: Comparison of cell disruption techniques in terms of energy consumption for Nannochloropsis sp..*

<b>Cell disruption techniques</b>	<b>Biomass concentration</b>	<b>Experimental conditions</b>	<b>Specific energy consumption, kJ/kg dry biomass</b>	<b>Ref.</b>
HPH	1% dw	150 MPa, 1-10 passes	150-1500	(Grimi et al., 2014)
US	0.14% dw	40 W, 20 min	0.132	(McMillan et al., 2013)
	1% dw	200 W, 1-8 min	12-96	(Grimi et al., 2014)
PEF	1% dw	20 kV/cm, 1-4 ms	13.3-53.1	(Grimi et al., 2014)
HVED	1% dw	40 kV/cm, 1-4 ms	13.3-53.1	(Grimi et al., 2014)

➤ *Benefits and limitations*

Conventional cell disruption methods are hindered by longer treatment time, large toxic solvent requirements and production process with difficulties in scaling-up. Compared with conventional methods, the use of these alternative techniques allowed the recovery biomolecules avoiding toxic solvent, high temperature and treatment time. Most of them are potential for scale-up and have been used for commercial application. The main benefits and drawbacks of varied cell disruption techniques are summarized in Table I.8.

*Table I.8: Benefits and limitations of different cell disruption techniques.*

<b>Methods</b>	<b>Operates at industrial scale</b>	<b>Suitability for commercial application</b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>Ref.</b>
HPH	√	-	Destruction of	High energy	(Al Hattab



			cell walls, high efficiency; easy scale-up	input, temperature rise, very fine cell debris	et al., 2015; Spiden et al., 2013)
US	×	+++	Effective cell wall disruption, relatively rapid process, hazardous chemicals are not required	High operational costs and energy input	(Al Hattab et al., 2015)
PEF	√	+	High selectivity and extraction yield, non-thermal and mild process, relatively low energy usage	Still in its infancy	(Barba et al., 2015b; Goettel et al., 2013)
HVED	×	+	High extraction yield, avoid solvent usage, relatively low energy input, reduced heating effect	Still in its infancy	(Barba et al., 2015b)

### I.2.6 Extraction and fractionation

After harvesting and disintegrating the microalgal biomass, depending on the foreseen bio-products, bio-molecules separation using extraction and a possible further fractionation are applied (Postma et al., 2016). Solvents are usually utilised to extract bio-molecules such as pigments (astaxanthin,  $\beta$ -carotene, etc) and fatty acids from microalgal biomass. The process entails cell uptake of solvent molecules on exposure to a solvent, which causes alterations to the cell membrane to enhance the movement of globules toward the outside of the cell

(Brennan and Owende, 2010b). Example of organic solvent extraction of microalgal lipids, the proposed mechanism is shown in Figure I.29 and can be divided into 5 steps. When a microalgal cell is exposed to a non-polar organic solvent, the organic solvent penetrates through the cell membrane into the cytoplasm (1<sup>st</sup> step) and interacts with the neutral lipids using similar van der Waals forces (2<sup>nd</sup> step) to form an organic solvent-lipids complex (3<sup>rd</sup> step). This organic solvent–lipids complex, driven by a concentration gradient, diffuses across the cell membrane (4<sup>th</sup> step) and the static organic solvent film surrounding the cell (5<sup>th</sup> step) into the bulk organic solvent. As a result, the neutral lipids are extracted out of the cells and remain dissolved in the non-polar organic solvent (Halim et al., 2012a).

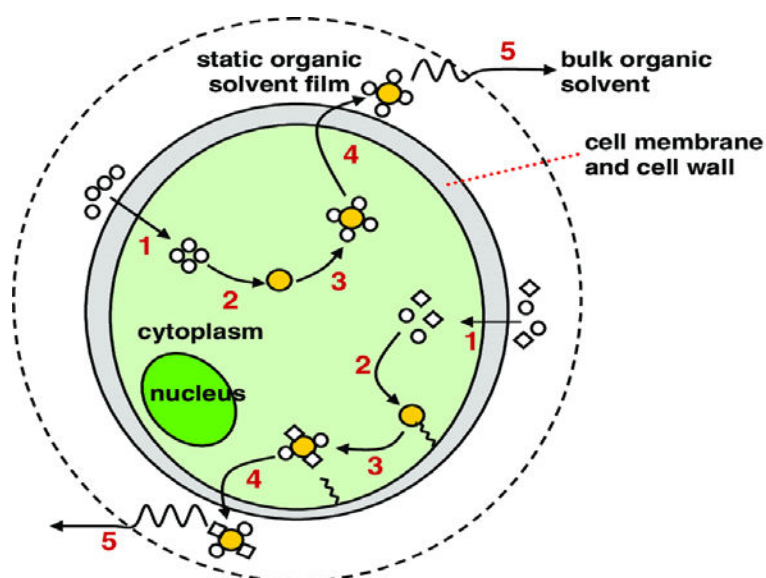


Figure I.29: Schematic diagram of the proposed organic solvent extraction mechanisms. Pathway shown at the top of the cell: mechanism for non-polar organic solvent. Pathway shown at the bottom of the cell: mechanism for non-polar/polar organic solvent mixture. lipids, ○ non-polar organic solvent, ◇ polar organic solvent. Both mechanisms can be described in 5 steps (Halim et al., 2012a).

Different process for the extraction of lipids from *Aphanothece microscopica Nageli*, *Phaeodactylum tricornutum*, *Isochrysis galbana* have been described. For extracting lipids, several organic solvent are suitable. Generally, methanol/chloroform shows the best yield due to the best polarity index of the mixture for extracting both the lipid classes (Halim et al., 2012b). On industrial scale, hexane is frequently preferred for oil extraction from oilseeds.

Moreover, application of subcritical ( $S_bFE$ ) and supercritical ( $S_pFE$ ) fluid extraction technology for recovery bio-molecules are also accord with green requirement. Several subcritical solvents used for microalgal species. For example, pressurized water was used for

extraction antioxidant and antimicrobial bio-molecules from *Spirulina platensis* and *Haematococcus pluvialis*; pressurized EtOH was used for extraction of carotenoids from *Haematococcus pluvialis* and *Dunaliella salina*; and pressurized propane was used for extraction fatty acid from *Nannochloropsis oculata* (Zhang et al., 2019b). Similarly, the S<sub>p</sub>FE using CO<sub>2</sub> as solvent has been applied to extraction different bio-molecules from several microalgal species. Example of the recovery of carotenoids from *Chlorella vulgaris*, β-carotene from *Dunaliella salina*, γ-linolenic acid (GLA) from *Arthrospira (Spirulina) maxima*, lipids, tocopherol, and polyunsaturated fatty acids from *Nannochloropsis oculata* and *Tetraselmis suecica* were tested (for a review see (Zhang et al., 2019b)).

### 1.2.7 Applications and potential interests

Microalgae are very attractive as a feedstock for bio-products due to an aerial productivity superior to traditional agricultural crops: realistic estimates for areal productivity are in the order of magnitude of 40–80 tonnes of dry matter per hectare per year depending on the technology used and location of production (Postma et al., 2016). It is estimated that the annual production of the microalgae industry in 2004 had reached 7000 tonnes of dry biomass (Pulz and Gross, 2004). Additionally, microalgae accumulated different bio-molecules can be used for different markets such as bulk and high added value bio-products (Figure I.30a).

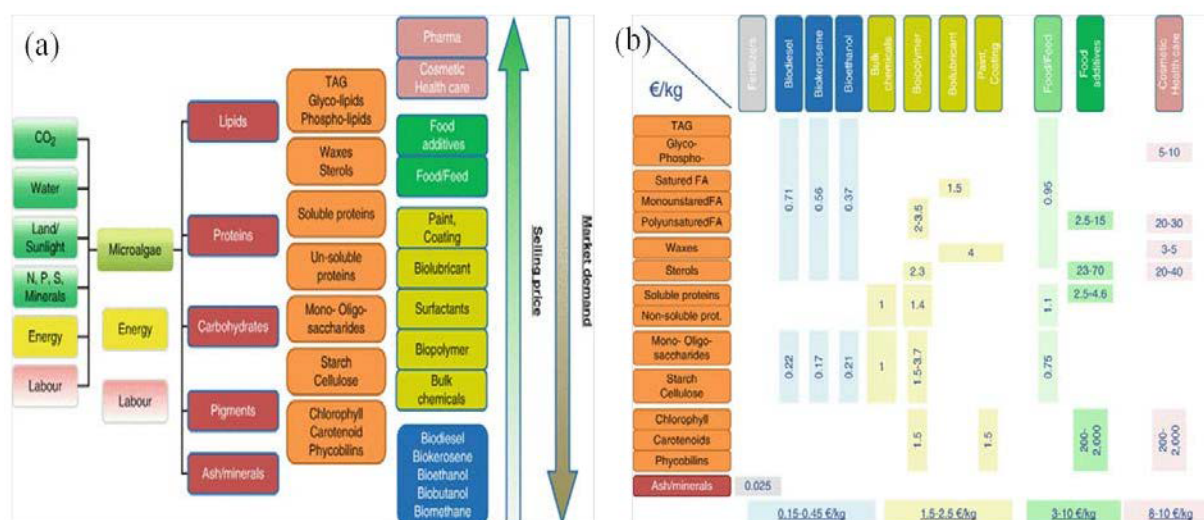


Figure I.30: Overall spectrum of microalgal component and their possible application (a), and selling prices of microalgal components in different market scenarios and derived overall biomass revenue (b) (Postma et al., 2016).

Thereinto, lipids and proteins are the most interesting fractions of the microalgae. Globally the need for lipids and proteins as food, feed, and fuel is especially rising in Europe,

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where currently 44% of the lipid and 68% of the protein requirement is imported (Postma et al., 2016). However, microalgae are nowadays only produced and commercialized for niche markets, either as whole biomass (food additives and feed for aquaculture) or as extracted valuable bio-molecules such as astaxanthin,  $\beta$ -carotene,  $\omega$ -3 fatty acids, and phycobiliproteins, with a very low market volume (10,000 MT/y) (Vigani et al., 2015). When exploiting the whole potential of microalgae in an overall biorefinery strategy, many different products have to be selective extracted and fractionated in order to turn the potential selling price of the microalgal biomass higher than the production and extraction costs (Figure I.30b) (Postma et al., 2016).

#### I. 2.7.1 Human nutrition

Because of the strict food safety regulations, commercial factors, market demand and specific preparation (Chisti, 2007), the source of microalgal biomass used in human consumption is restricted to very few species. *Chlorella*, *Spirulina*, and *Dunaliella* biomass have predominance in the market, generally in the form of capsules, tablets, extracts and powder as food additives (Brennan and Owende, 2010a). Especially, *Chlorella* have also a medicinal value, and can be used for protection against renal failure and growth promotion of intestinal lactobacillus (Yamaguchi, 1996). Nevertheless, despite these microalgae richness in nutrients that can provide human health benefits, they are rather considered as nutraceuticals instead of food products due to the lack of clear common official legislations in terms of quality and requirements regarding microalgae (Safi, 2013). Moreover, *Dunaliella* can be found in the market as a colorant resource. It has an annual production of 1200 tonnes per annum in where  $\beta$ -carotene is exploited for its content of up to 14% (Metting, 1996).

#### I. 2.7.2 Animal feed and aquaculture

Like human nutrition resource, specific microalgal species can be used for preparation of animal feed supplements. For example, *Chlorella*, *Scenedesmus* and *Spirulina* have showed beneficial aspects including improved immune response, improved fertility, better weight control, healthier skin and a lustrous coat (Pulz and Gross, 2004). To date, it is estimated that approximately 30% of microalgal production is provided for animal feed (Becker, 2007).

Moreover, microalgae are also the natural food source of many important aquaculture species like molluscs, shrimps and fish. For instance, *Chlorella vulgaris* can accumulate a high amount of carotenoids by stressing cultivation, and after feeding it to fish and poultry it

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showed interesting pigmentation potential for fish flesh and egg yolk in poultry, together with enhancing health and increasing life expectancy of animals (Safi, 2013). Furthermore, it was demonstrated that microalgae has a protective effect against heavy metals and other harmful compounds (Lead, Cadmium, Naphtalene) by reducing significantly the oxidative stress induced by these harmful compounds, and increasing the antioxidant activity in the organisms of tested animals (Shim et al., 2008; Vijayavel et al., 2007; Yun et al., 2011).

#### I. 2.7.3 Biofuel production

Biofuels are fuels that contain energy from geologically recent carbon fixation i.e. living organisms. Based on the feedstock types used and their current/future availability, biofuels are categorized from first to fourth generation biofuels (Shuba and Kifle, 2018). Recently, microalgae have attracted wide attention for the valuable natural bioproducts they generate, and their potential as energy crops. The third-generation biofuel is a biofuel that is derived from algae. This is the right move for the production of biofuels as algae possess enormous potential (like low-input, high-yield prospect) for renewable energy applications (Dismukes et al., 2008; Hu et al., 2008). Thus, this potential may enable to completely displace petroleum-derived transport fuels without the controversial argument “food for fuel” (Shuba and Kifle, 2018).

#### I. 2.7.4 Waste water treatment

Nitrates, nitrites and ammonium, as well as phosphates in wastewater, are the important nutrient sources for microalgae cultivation. Microalgae can assimilate these nutrients and other organic compounds in wastewater into the cells for their growth (Pittman et al., 2011). Hence, the ideology of producing microalgae in wastewater is not only to reduce the growth media components for cultivation, but also in favor of cleaning up the wastewater (Sydney et al., 2011). Recently, photobioreactor and high rate algal ponds (HRAP) microalgae cultivation form can be used to treat a large quantity of wastewater (Abinandan and Shanthakumar, 2015).

Furthermore, the obtained biomass from microalgae cultivation in wastewater can be used as a commercial value-added product. Globally, the consumption of water for domestic is 315 billion  $\text{m}^{-3}$   $\text{year}^{-1}$ , and then is released as wastewater (Flörke et al., 2013). If, 70% of the total is used for microalgae cultivation, it could generate ~23.5 billion tons of oil. Additionally, the obtained biomass can also be used in food, pharmaceutical or cosmetic

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industries since they possess high content of proteins (nearly 50%), lipids (nearly 23%) and carbohydrates (nearly 23%) (Abinandan and Shanthakumar, 2015).

### I.3 Conclusion and research objectives

Microalgae has attracted widely attention due to certain strains of microalgae can produce larger amounts of high value bio-molecules such as pigments, fatty acids, proteins and anti-oxidant, etc. Moreover, microalgae are considered as the third generation of biodiesel feedstock, because of their high capacity to produce high oil contents' biomass, with higher growth rate and productivity than edible and non-edible feedstock microalgae. However, several challenges arise in the aspects of intracellular molecules recovery, such as the scalability of the methods of extraction, energy consumption and viability of certain methods for scale up processing. The microalgae biorefineries system utilizes microalgal biomass for the production of valuable bio-products; the final yields and purity are usually low as the number of steps required to obtained specific purity level differs for each industry. Therefore, more efforts should be performed to reduce product loss and minimize energy costs while heading towards an environmental friendly large scale downstream processing for the extraction of high value molecules from microalgae.

The aims of this thesis are:

(1) Understand the impact of physical pre-treatments on cell disruption and release of intracellular bio-molecules from different microalgal species:

- Investigate the feasibility of physical treatments to assist extraction of bio-molecules from different microalgal species;

- Study and compare the impact of different physical treatments on extraction efficiencies and release behaviors of intracellular bio-molecules;

- Discuss the impact of microalgal cell structure (such as cell shape, size and location of molecules) on the effectiveness of different physical treatments;

- Analysis of correlations between selected extraction method and efficiency of extracted target bio-molecules.

(2) Propose a new strategy by combined treatment for selective extraction of intracellular bio-molecules from microalgae:

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- Investigate the feasibility of combined method (physical treatments + HPH) for improving the extraction efficiencies of bio-molecules;

- Verify whether the applied combined method can obtain the same or/even higher extraction efficiency with lower processing energy consumption.

(3) In order to realize the maximum valorisation of microalgal biomass, propose a new strategy for the selective extraction and fractionation of various bio-molecules from microalgae:

- Compare the performance of the two valorized procedure (continuous and discontinuous) of microalgal biomass;

- Optimizing the extraction and fractionation of microalgal bio-molecules assisted by multi-step extraction process.



## Chapter II Methodology and Protocols

The downstream process is less explored field of the microalgae biorefineries as compared to microalgae cultivation. The activity performed in this thesis is part of a program aiming at the exploitation of the different microalgal bio-molecules. The thesis covers three main aspects of microalgae biorefineries: cell disruption, extraction and fractionation. Figure II.1 presents the schematic representation of applied approaches of this thesis.

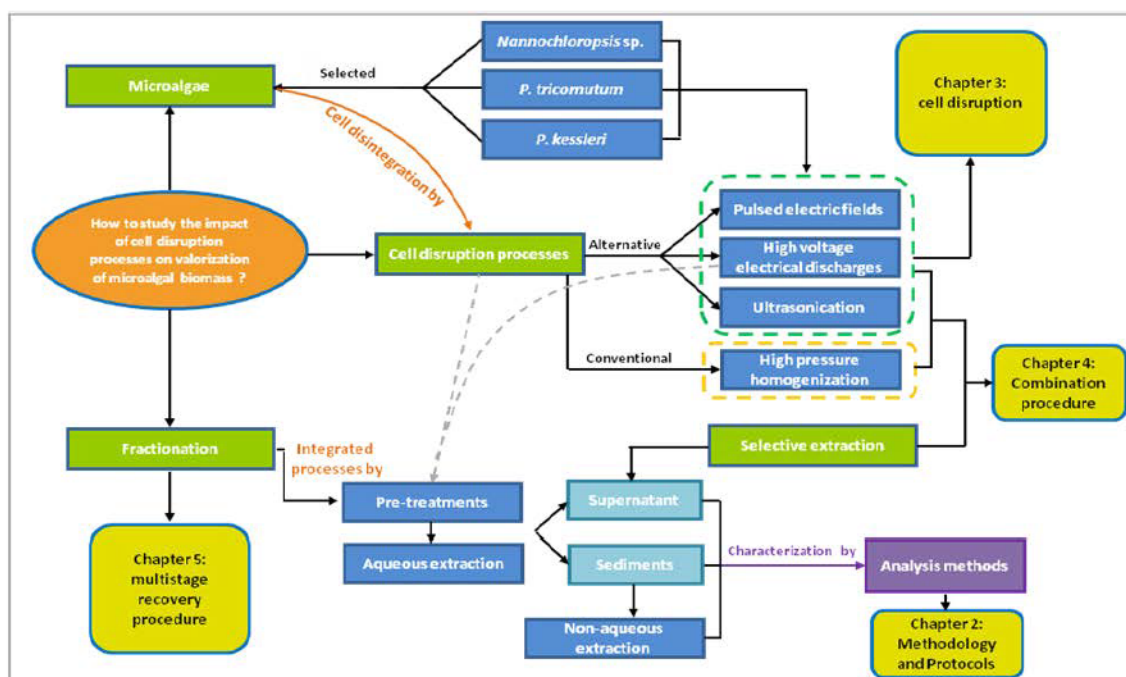


Figure 2.1: Schematic representation of applied approaches of this thesis.

### II.1 Effects of alternative physical treatments for cell disintegration of different microalgal species

This task was aimed to investigate the effects of physical treatments (PEF, HVED and US) on cell disintegration of different microalgal species. The cell disintegration was evaluated based on the extraction degree of water-soluble (relative small-size carbohydrates and larger size proteins) and water-insoluble (chlorophyll *a*) bio-molecules. Two marine microalgae, *Nannochloropsis* sp. and *P. tricomutum*, and one fresh microalga *P. kessleri*, were selected as representative strains. The biomass was produced by AlgoSolis (Saint-Nazaire, France), which means we do not control the production process. For the same reason, we did not have the possibility to control the biomass composition. The biomass was directly freeze-dried after harvesting, and then sent to our laboratory. In the experiments of this chapter, all



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the biomasses were preliminary washed with distilled water 3 times in order to remove salt or ice, then freeze-dried and, finally, 1% dry matter (DM, hereinafter %) suspension was prepared and treated using pulsed electric energy (PEF and HVED) or US techniques (physical treatments). For eliminating the effect of pre-freeze-drying on microalgal cells, 1% untreated suspensions was used as control.

On one hand, the effects of three technologies on extraction indexes of water-soluble compounds was investigated for the selected species. At the equivalent applied energy, the correlation between extraction of carbohydrates and proteins, and selectivity indexes were evaluated in order to better comparing the impact of physical treatments on cell damage, and to assess their potential for further biorefinery applications. On the other hand, the solvent extraction process after cell disruption regarded extraction behavior of bio-molecules by different technologies and the fragility of microalgal cell wall. Attention was also focused on the effects of physical treatments on extraction kinetics of chlorophyll *a*.

## II.2 Effect of combination process for selective and energy efficient extraction of bio-molecules from microalga *Parachlorella kessleri*

The higher extraction efficiency and the lower energy consumption is one of the critical issues for suitable biorefinery process. A combination procedure was designed, by alternative physical treatment (PEF, HVED or US) coupled with HPH treatment. Indeed, the HPH treatment is governed solely by two operating parameters (homogenizing pressure and number of passes), allows a maximum release of intracellular bio-molecules, but is not selective and have high energy consumption. While the physical technologies are relatively mild, but are by the same more flexible allowing, in theory, more selective extraction of target molecules than the HPH treatment. Therefore, performances were assessed by using combined pulsed electric energy treatments (E procedure) and HPH treatment (P procedure) (i.e. S + P procedure), and combined US treatment (S procedure) and HPH treatment (i.e. E + P procedure), respectively.

The microalga *P. kessleri* was selected as tested species in this part. For the comparison, the biomass was washed and then suspended in deionized water with respect to the combined E + P procedure, while the biomass was thawed and used directly afterwards in the experiment of combined S + P procedure. Moreover, the step of centrifugation was performed after E procedure only. Furthermore, a preliminary study was explored about the

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possibility of decrease energy consumption for using the combined method recovery of bio-molecules from microalgal biomass. Therefore, the effects of preliminary procedure (E or S) with different concentrations of suspension on total energy consumption was also investigated. Finally, the effects of applied procedures on the extraction efficiencies of bio-molecules (ionic components, carbohydrates, proteins and pigments) in the supernatant was evaluated.

### II.3 Effect of multistage extraction procedure on extraction and fractionation of bio-molecules from microalgae

The goal of this task was to investigate the integrated process for the continuous extraction and fractionation of different valuable bio-molecules from microalgal biomass. The integrated process included the application of cell disruption method, combined with aqueous and non-aqueous extraction procedures. Two microalgal species riched in lipids, *Nannochloropsis* sp. and *P. tricornutum*, were selected.

In this task, HVED treatment was performed as the preliminary cell disruption method due to its high extraction selectivity. Then, the feasibility of HVED as pre-treatment during the multi-step extraction process was investigated by two units. The extraction efficiencies of water-soluble molecules (ionic components, carbohydrates, and proteins) were evaluated during the cell disruption pre-treatment and aqueous extraction procedure. Then the extraction of liposoluble molecules (pigments and lipids) were evaluated during the following non-aqueous extraction procedure. Moreover, the effect of HVED pre-treatment on extraction of different bio-molecules during each step of multistage process was compared with control.

In all, we propose that new microalgae biorefineries must be based on the consideration of of maximal valorisation and application must prioritize mild, selective and integrated approaches.

### II.4 Organization of the manuscript

According to the objective of this thesis (investigate the impact of three physical treatments on cell disruption, selective and energy efficient extraction/fractionation of intracellular bio-molecules from microalgae), the experimental part of the manuscript was divided in three chapters (from chapter III to chapter V). Moreover, some specific points include research background, objective and experimental procedures (chapter introduction) were grouped with the results (chapter conclusion) in each of these three chapters, in order to

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improve the fluidity of the reading and the comprehension of the results. Finally, summarizing conclusion of the discussed papers and presents some suggestions for further work.

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## Chapter III Effects of alternative physical treatments for cell disintegration of different microalgal species

### III.1 Chapter introduction

Microalgae usually have rigid cell walls and complex cell structures (Eppink et al., 2013). However, most of high-added value bio-molecules from microalgae are commonly located intracellular, either in the cytoplasm, in complicated organelles or bound to cell walls (Postma et al., 2017). In order to take advantage of these valuable bio-molecules, the cells need to be disintegrated to permit complete access to these intracellular bio-molecules and facilitate the extraction process (Safi et al., 2014). Previously studies about the extraction of bio-molecules from microalgae have been reported by several research groups. The most commonly practiced cell disruption techniques including chemical hydrolysis (Duongbia et al., 2019; Lorenzo-Hernando et al., 2019; Sedighi et al., 2019), freeze/thaw cycles (Abdollahi et al., 2019), high pressure cell disruption (Bernaerts et al., 2019; Rivera et al., 2018), ultrasound (Skorupskaite et al., 2019; Zhang et al., 2019) and microwave (Chew et al., 2019; Zocher et al., 2019)-assisted extraction, or bead milling (Garcia et al., 2019; Rivera et al., 2018), etc. Recently, several alternative cell disruption techniques have been also reported. The application of mild pulsed electric energy technologies, such as PEF and HVED, were shown to be promising for intracellular extraction from bio-suspensions (Vorobiev et al., 2012). The application of US have been demonstrated rather effective and lead to destruction of the cell walls and membranes (Grimi et al., 2014).

However, the efficiencies of these techniques were usually evaluated by quantifying target bio-molecules before and after treatment from single microalgal specie. In fact, their efficiencies can depend on variant cell structure of different microalgal species. There are not a large number of studies conducted on the comparison of the disruption efficiencies for different microalgal species. The literature reviews only show one published work done to compare different techniques (grinding, ultrasonication, alkaline treatment, and HPH) to assist aqueous extraction of proteins from five microalgal species (*Haematococcus pluvialis*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Porphyridium cruentum*, and *Arthrospira platensis*). But the relationship between different cell disruption techniques and different microalgal species is typically not considered.

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Therefore, the purpose of this chapter was to:

(1) explore the feasibility of physical treatments (PEF, HVED and US) for extraction of hydrophilic (carbohydrates and proteins) and hydrophobic (chlorophyll *a*) bio-molecules from different microalgal species;

(2) compare the cell disintegration degrees of physical treatments (PEF, HVED and US) for different microalgal species;

(3) investigate the extraction behaviours and correlations of target molecules for different physical treatments (PEF, HVED and US);

(4) understand the impact of physical treatments on cell damage and the release of intracellular bio-molecules.

In this chapter, the first part (details are presented in article 1: ***Comparison of aqueous extraction assisted by pulsed electric energy and ultrasonication: Efficiencies for different microalgal species***) was published in the journal “Algal Research”. The second part (details are presented in article 2: ***Pulsed electric energy and ultrasonication assisted green solvent extraction of bio-molecules from different microalgal species***) was published in the journal “Innovative Food Science and Emerging Technologies”. These works were carried out under the direction of Dr. Nabil Grimi, Prof. Luc Marchal and in collaboration with Prof. Nikolai Lebovka and Prof. Eugène Vorobiev.

III.2 *Article 1: Comparison of aqueous extraction assisted by pulsed electric energy and ultrasonication: Efficiencies for different microalgal species*

(The article is presented on the following pages)

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## **Comparison of aqueous extraction assisted by pulsed electric energy and ultrasonication: Efficiencies for different microalgal species**

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## Abstract

Aqueous extraction assisted by pulsed electric energy (PEE) and ultrasonication (US) were tested for three microalgal species (*Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Parachlorella kessleri*). The PEE treatments were applied using pulsed electrical fields (PEF) and high voltage electrical discharges (HVED) modes. The extraction degrees of carbohydrates ( $Z_c$ ) and proteins ( $Z_p$ ) at different energy consumption ( $W$ ) were analyzed. For all tested species, HVED proved to be the most effective technique for the extraction of carbohydrates in comparison with PEF and US. The observed differences in extraction of carbohydrates for three microalgal species can reflect different cell morphologies and structures. However, PEE treatments (HVED and PEF) were less effective than US for the extraction of proteins. The selectivity indexes,  $S$  (the value of  $S \geq 1$  reflects a higher efficiency of selective extraction of carbohydrates when compared with that of proteins), were smaller with the application of US treatment and were depended on the microalgal species. The data evidenced that appropriate physical treatments can be used to tune the desired selectivity of extraction.

**Keywords:** Microalgae; Pulsed electrical fields; High voltage electrical discharges; Ultrasonication; Energy; Extraction selectivity

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## 1. Introduction

There is an increasing demand for sustainable food, feed, cosmetic, pharmaceutical and biofuel feedstocks as alternatives for traditional agricultural crops [1]. Microalgae have rapid growth rates and they can efficiently produce highly valued bio-molecules, over short periods of time by photosynthesis [2]. They also have the ability to live in existing earth ecosystems, such as marine, freshwater (ponds, puddles, canals, and lakes) and terrestrial habitats [2,3].

Microalgal biomass has been proven to be an important feedstock that was suitable for the production of proteins, sugars, dyes and other valuable bio-molecules, as well as lipids extraction for fuel purposes and biodiesel production [4]. These valuable bio-molecules are commonly located in the intracellular compartments, either in the cytoplasm, in internal organelles or bound to the cell wall of microalgae [1]. In most cases, microalgae have a rigid cell wall and complex intracellular structure (nucleus, chloroplast, mitochondria, Golgi apparatus, etc.) and each one of these organelles has a different composition and structure [5]. To facilitate the extraction process, the cells need to be disintegrated [4]. The popular cell disruption techniques include chemical hydrolysis [6–8], applications of freeze/thaw cycles [9], high pressure [10,11], bead milling [10,12], ultrasonication (US) [13,14], microwaves [15,16] and pulsed electrical fields (PEF) [17–19]. For example, the maximum total phenolic compounds and chlorophylls yields from *Nannochloropsis* sp. extracts obtained after US (400 W) treatments was about 5-fold and 9-fold higher compared to that found for the untreated samples and aqueous extraction [20]. Moreover, a increasing protein extraction rate was observed with increasing pulsed electric field strength, up to  $96.6 \pm 4.8\%$  of the free protein in *Chlorella vulgaris* (SAG 211-12) [21]. The combination of PEF and binary mixture of organic solvents and water allowed reaching of the high level of extraction pigments and non-degraded proteins from *Nannochloropsis* sp. [22]. Additionally, the PEF and US were also compared as pretreatment methods for extraction of proteins from *Nannochloropsis* sp. [23]. The results evidenced that PEF treatment allows selective extraction of a portion of pure proteins that are different from proteins extracted from US pretreated suspensions. However, the efficiency of these techniques can depend on the microalgal species. There are not a large number of studies conducted on the comparison of the disruption efficiencies for different algal species. The literature reviews only show one published work done to compare different techniques (grinding, ultrasonication, alkaline treatment, and high pressure homogenization)



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to assist aqueous extraction of proteins from five microalgal species (*Haematococcus pluvialis*, *Nannochloropsis oculata*, *Chlorella vulgaris*, *Porphyridium cruentum*, and *Arthrospira platensis*)[4].

In this work, the aqueous extraction of proteins and carbohydrates assisted by pulsed electrical fields (PEF), high voltage electrical discharges (HVED), and ultrasonication (US) treatments from three different microalgae (*Nannochloropsis* sp., *Phaeodactylum tricornutum* (*P. tricornutum*), and *Parachlorella kessleri* (*P. kessleri*)) were compared. The tested species have different cell shapes, structures and intracellular contents. Microalgae *Nannochloropsis* sp., and *P. tricornutum* are the marina microorganisms, and *P. kessleri* is the freshwater microorganism. The cells of *Nannochloropsis* sp. are spherical or slightly ovoid (2–4  $\mu\text{m}$  in diameter) , the cells of *P. tricornutum* are fusiform with a length of 20–30  $\mu\text{m}$  and a diameter of 1-3  $\mu\text{m}$ , and the cells of *P. kessleri* are near spherical (3–4  $\mu\text{m}$  in diameter) [24]. The *Nannochloropsis* sp. and *P. kessleri* cells have rigid cell walls mainly composed of cellulose and hemicelluloses [25], and cells walls of *P. tricornutum* are very poor in silica and composed of different organic compounds, particularly sulfated glucomannan [26]. Depending on the cultivation conditions these species may have rather different contents of carbohydrates and proteins [24]. This study also discusses the extraction efficiency relating it to the specific energy consumption for PEF, HVED, and US treatments, as well as the correlations between the extraction of carbohydrates and proteins.

## 2. Materials and methods

### 2.1 Microalgae

Microalgae *Nannochloropsis* sp., *P. tricornutum* and *P. kessleri* were provided by AlgoSolis (Saint-Nazaire, France). The details of the cultivation procedures were described previously for *Nannochloropsis* sp. [23], *P. tricornutum* [27] and *P. kessleri* [14]. The harvested biomasses were centrifuged and stored at -20 °C. The frozen microalgal pastes have moisture contents of  $\approx 80\%$  (*Nannochloropsis* sp.),  $\approx 70\%$  (*P. tricornutum*) and  $\approx 82\%$  (*P. kessleri*), respectively.

### 2.2 Chemicals

D-glucose, bovine serum albumin (BSA) standard were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bradford Dye Reagent was purchased from

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Thermo Fisher (Kandel, Germany). All other chemicals (sulfuric acid and phenol) with analytical grade were obtained from VWR (France).

### 2.3 Extraction procedures

The microalgal pastes were washed using deionized water. In the first washing, the biomass was diluted to 1% (w/v) dry matter (DM) (hereinafter %), agitated at 150 rpm for 10 min, and centrifuged for 10 min at 4600 g. The supernatant was removed, and the washing procedures were repeated 2 times. It has been previously demonstrated that application of washing for three times allows effective removal of salts, proteins and carbohydrates captured on the surface of algal cells [43]. The sediments were collected and freeze-dried for 64 h at -20 °C using a MUT 002A pilot freeze-drier (Cryotec, France). Finally, 1% of suspensions (250 g) were prepared and treated using pulsed electric energy (PEF and HVED) or US techniques (physical treatments). The treated suspensions were centrifuged, and the supernatants were analyzed for the content of carbohydrates and proteins (**Fig. 1**).

### 2.4 Physical treatments

The PEE and US treatments were applied as physical treatments.

#### 2.4.1 Pulsed electric energy (PEE)

The PEE-assisted extraction was done using the PEF or HVED mode. The treatments were applied using a high voltage pulsed power 40 kV-10 kA generator (Basis, Saint-Quentin, France). The generator provided exponential-shaped pulses with a repetition rate of 0.5 Hz. For the PEF treatment, a 1-L cylindrical batch treatment chamber with two parallel plate electrodes was used. The distance between the electrodes was fixed at 2 cm to produce a PEF electric strength of  $E = 20$  kV/cm. For the HVED treatment, a 1-L cylindrical batch treatment chamber with needle-plate geometry of electrode was used. The distance between the stainless steel needle and the grounded plate electrodes was fixed at 1 cm and the mean electric field strength was estimated as  $E = 40$  kV/cm. The PEE treatments consisted in the application of  $n$  successive pulses ( $n = 1-800$ ). The total treatment duration  $t_e$  was varied within 0.01-8 ms.

Disrupted microalgal suspension characteristics were measured between successive applications of the pulses or discharges.

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#### 2.4.2 Ultrasonication (US)

The US treatment was conducted using UP-400S ultrasound processor (Hielecher GmbH, Germany) at a constant frequency of 24 kHz. The ultrasound probe (diameter: 14 mm, length: 100 mm) was plunged into a beaker, containing the microalgal suspensions. The US duration,  $t$ , was varied within 1-880 s and the amplitude was fixed at 50%, which corresponded to the power,  $P_u = 200$  W.

#### 2.4.3 Specific energy consumption

For PEE-assisted extraction, the specific energy consumption,  $W$  (J/kg suspensions, hereinafter referred to as J/kg), was calculated using the following formula [28]:

$$W = (C \times U^2 \times n) / 2m \quad (1)$$

where  $C$  is the capacitance of the capacitor,  $U$  is the voltage of the generator,  $m$  is the mass of 1% suspension.

For US treatment, the specific energy consumption,  $W$  (J/kg), was calculated using the following formula [29]:

$$W = P_u \times t / m \quad (2)$$

where  $m$  is the mass 1% suspension.

The maximum energy consumptions for PEF, HVED and US treatments were chosen to be  $W_{max} \approx 704$  kJ/kg suspensions. These energy consumptions, in absence of cooling for 1% suspensions, correspond to the temperature increase of  $\Delta T \approx W_{max} / C_w \approx 168$  °C (here  $C_w = 4.186$  J/(g °C) is the heat capacity of water). Therefore, the cooling procedures are important for both PEE and US treatments. In our experiments, during PEE and US treatments the samples were cooled in order to prevent significant heating, and ensure that the elevation of temperature does not exceeded 5 °C above room temperature.

#### 2.5 Characterization

The suspensions were centrifuged using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 g for 10 min. The supernatants were collected and used for further characterization analysis. The analyses were based on colour reactions with reagents that were measured using a UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo

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Electron Corporation, MA). All the characterization measurements were done at room temperature.

### 2.5.1 Analysis of carbohydrates

The content of carbohydrates,  $C_c$ , was measured using a phenol-sulfuric acid method [30]. In brief, 1 mL of supernatant (diluted if required) was mixed with 100  $\mu$ L of phenol solution (5%, w/w) and 5 mL of concentrated sulfuric acid. The reaction mixture was kept at 20 °C for 20 min. The absorbance was measured at the wavelength  $\lambda = 490$  nm. D-glucose was used as a standard.

### 2.5.2 Analysis of proteins

The content of proteins,  $C_p$ , was measured using the method of Bradford [31]. In brief, 0.1 mL of supernatant was mixed with 1 mL of Bradford Dye Reagent. The mixture was vortexed for 10 s and kept for 5 min. The absorbance was measured at the wavelength  $\lambda = 595$  nm. BSA was used as a standard.

### 2.5.3 Extraction indexes

Based on the measured values of  $C_c$  and  $C_p$ , the following carbohydrate and protein extraction indexes were defined:

$$Z_c = (C_c - C_c^i)/(C_c^m - C_c^i), \quad (3a)$$

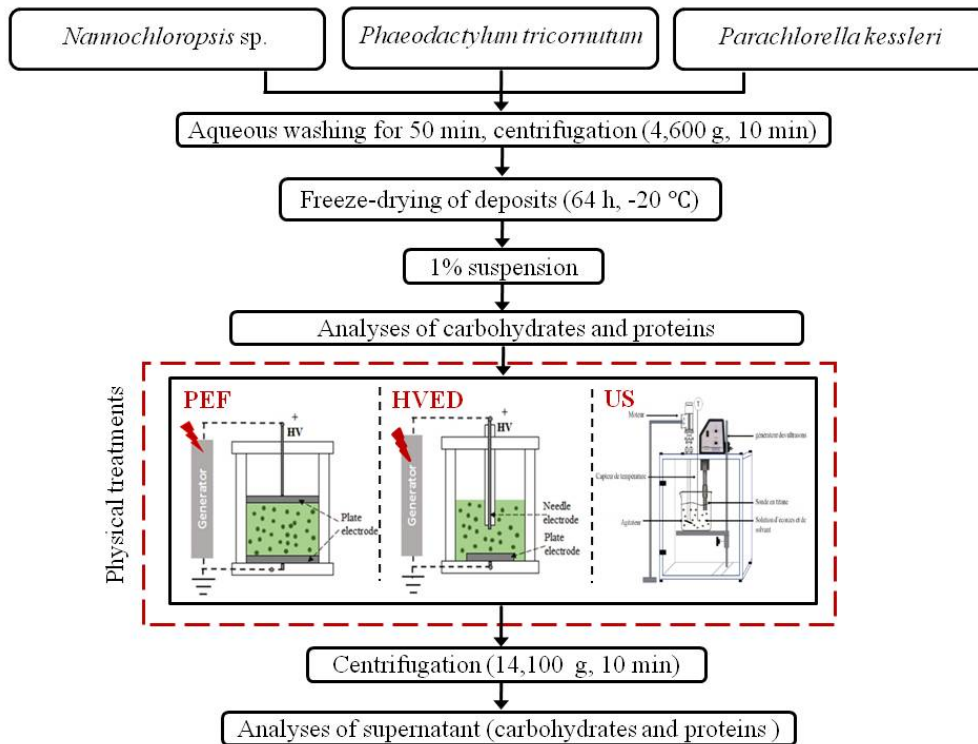
$$Z_p = (C_p - C_p^i)/(C_p^m - C_p^i), \quad (3b)$$

where the  $i$  and  $m$  refer to the initial and maximum values, respectively. The maximum contents  $C_c^m$  and  $C_p^m$  in raw microalgae (with washing, with freeze-drying) were measured according to the methods described by Phélippé et al. [33]. Briefly, total carbohydrates content of samples was measured using a phenol-sulfuric acid method [30], and total protein content was evaluated using the bicinchoninic acid method (Bicinchoninic Acid Protein Assay Kit, BCA1, Sigma Aldrich).

## 2.6 Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean  $\pm$  standard deviation. The error bars, presented on the figures, correspond to the standard deviations.

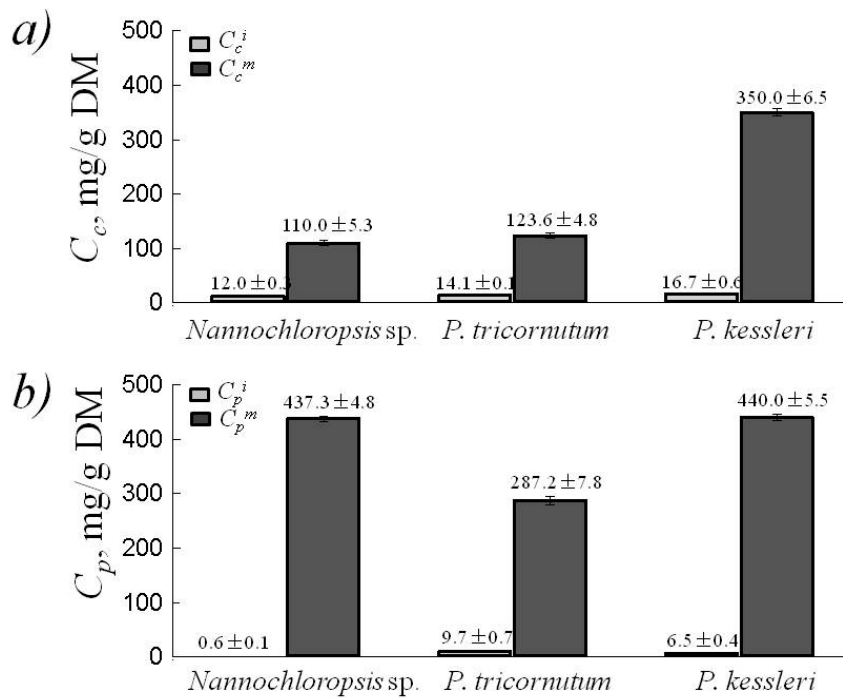
### 3. Results and discussion



**Fig. 1.** Schematic presentation of extraction procedures.

A schematic representation of the extraction procedures is presented in **Fig. 1**. **Fig. 2** presents the initial,  $C_c^i$ , and maximum contents,  $C_c^m$ , of carbohydrates (a), and the initial,  $C_p^i$ , and maximum contents,  $C_p^m$ , of proteins (b), for *Nannochloropsis* sp., *P. tricornutum* and *P. kessleri*. The initial extraction efficiency (before physical treatments) was characterized by the ratio  $I = C^i/C^m$  ( $I_c = C_c^i/C_c^m$  and  $I_p = C_p^i/C_p^m$  for carbohydrates and proteins, respectively).

The initial contents of carbohydrates and proteins correspond to the content of released components in 1% suspensions before physical treatments (**Fig. 1**). These contents can reflect a spontaneous cell lysis in distilled water [32] and they were rather small in all tested species ( $C_c^i \leq 17$  mg/g DM and  $C_p^i \leq 10$  mg/g DM) (**Fig. 2**). Besides, the initial carbohydrates content,  $C_c^i$ , was higher than proteins content,  $C_p^i$ , for all the species. This can be explained by a certain amount of extracellular polysaccharides present in the cell walls of microalgae [14].



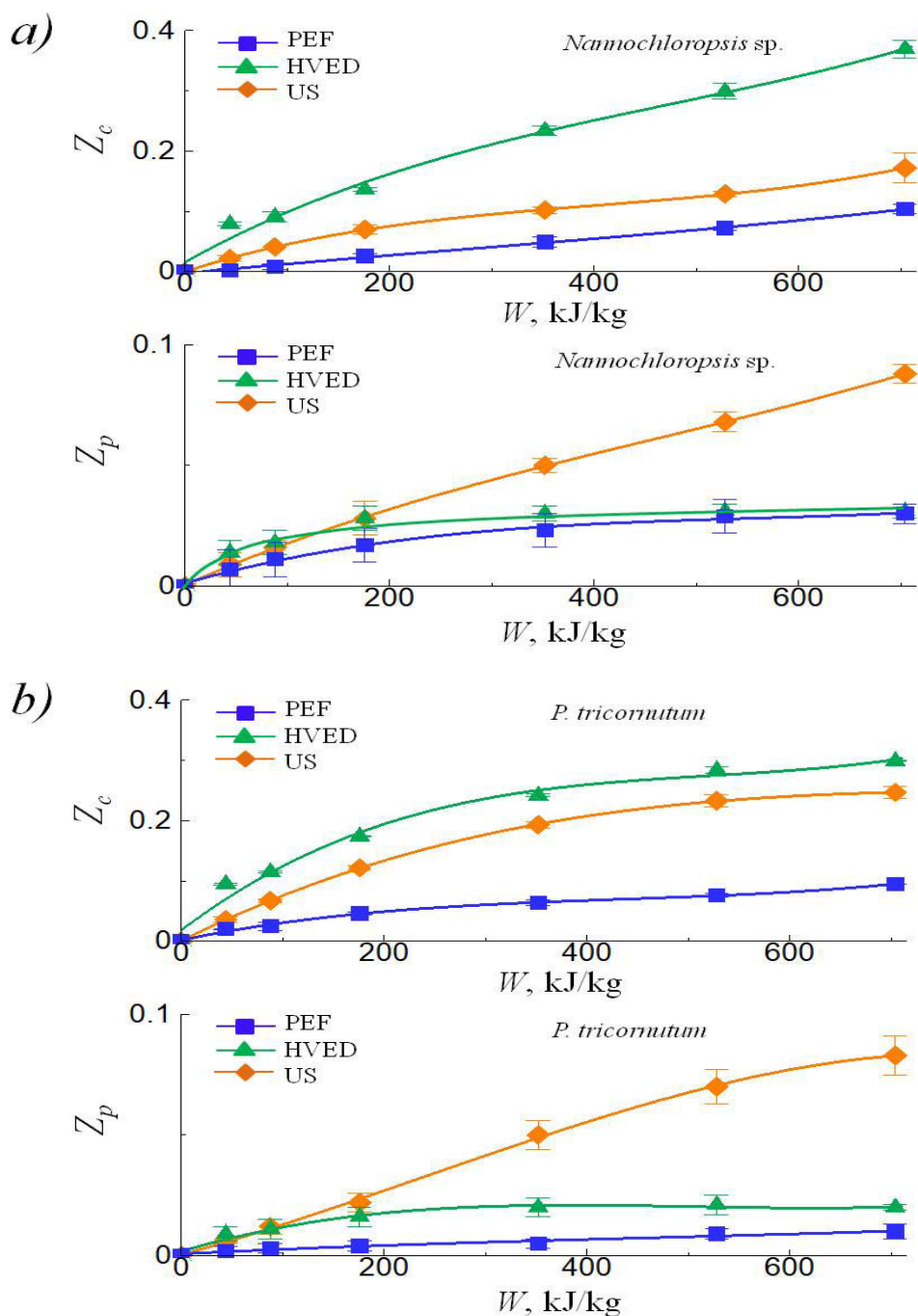
**Fig. 2.** The initial,  $C_c^i$ , and maximum content,  $C_c^m$ , of carbohydrates (a), and the initial,  $C_p^i$ , and maximum content,  $C_p^m$ , of proteins (b), for *Nannochloropsis* sp., *P. tricorutum*, and *P. kessleri*. The maximum contents of carbohydrates,  $C_c^m$ , and proteins,  $C_p^i$ , in raw microalgae.

The maximum contents in raw microalgae (with washing, with freeze-drying) were measured according to the methods described by Phélippé et al. [33]. These contents can be arranged in the order of *P. kessleri* > *P. tricorutum* > *Nannochloropsis* sp. for the carbohydrates, and in the order of *P. kessleri*  $\approx$  *Nannochloropsis* sp. > *P. tricorutum* for the proteins. The maximum contents of carbohydrates,  $C_c^m$ , and proteins,  $C_p^m$ , were obtained for *P. kessleri*.

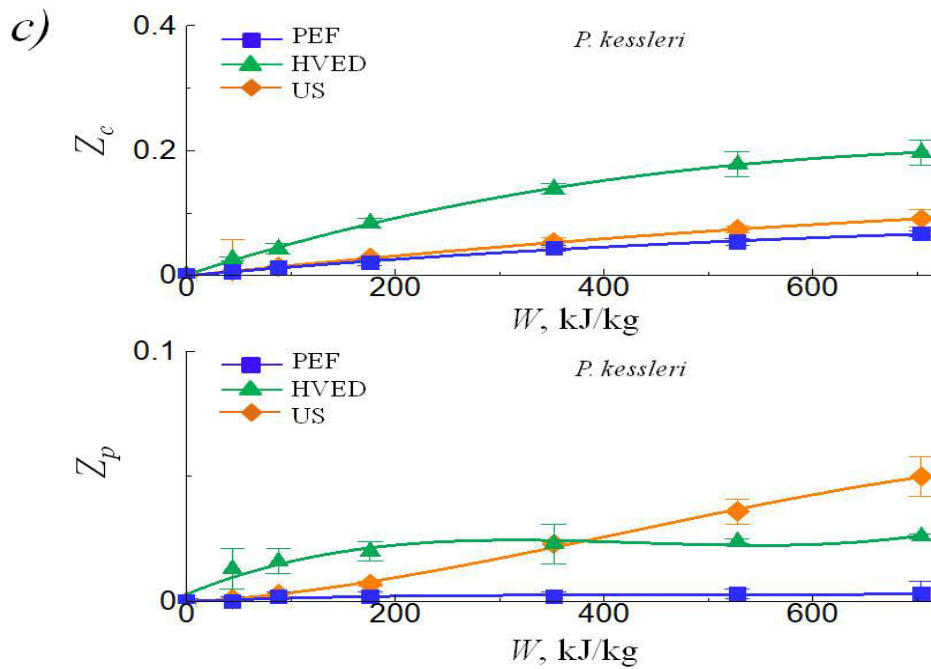
The initial extraction efficiencies (before physical treatments) were rather different among the tested species. For example, for *P. tricorutum*, the values of  $I_c = 11.4\%$  and  $I_p = 3.3\%$  were higher than those obtained from *Nannochloropsis* sp. ( $I_c = 10.9\%$ ,  $I_p = 0.1\%$ ), and *P. kessleri* ( $I_c = 4.8\%$ ,  $I_p = 1.4\%$ ). These observations can reflect either the differences in the cell wall structure of all the tested species or the residue degree of bio-molecules on the cell walls after the washing procedure.

The extraction kinetics of carbohydrates and proteins were represented as carbohydrates extraction index,  $Z_c$  (Eq. 3a), and proteins extraction index,  $Z_p$  (Eq. 3b), versus the specific energy consumptions,  $W$ , for PEF, HVED and US treatments. **Fig. 3** illustrates

these dependencies for *Nannochloropsis* sp. (a), *P. tricornutum* (b) and *P. kessleri* (c). The values of  $Z_c$  and  $Z_p$  continuously increased with the increase of  $W$ , and even at a maximal energy consumption level (in this work  $W_{max} \approx 704$  kJ/kg), some values of  $Z_c$  or  $Z_p$  were still far from the saturated values.

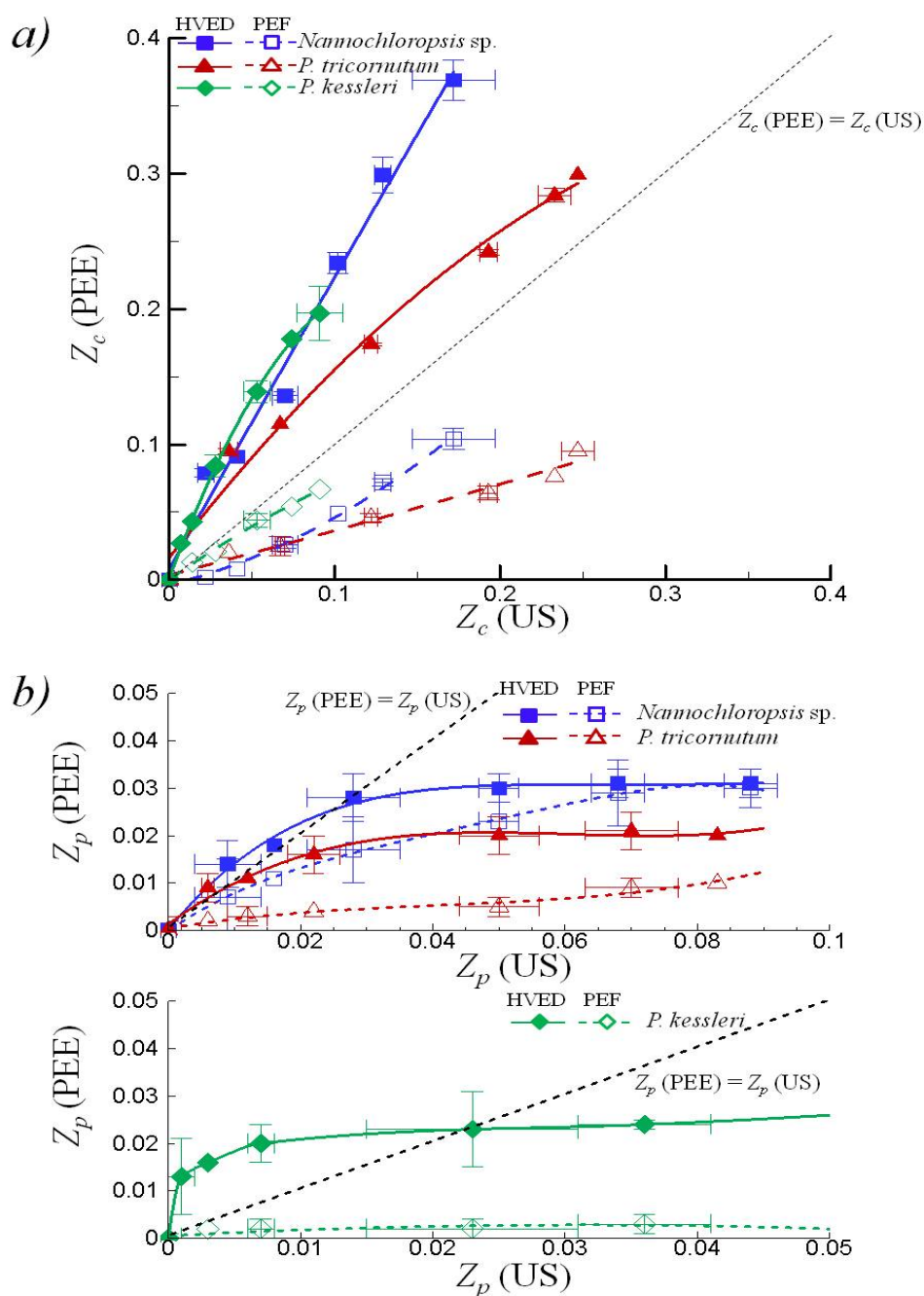






**Fig. 3.** Extraction indexes of carbohydrates,  $Z_c$ , and proteins,  $Z_p$ , versus specific energy consumption,  $W$ , after applying pulsed electrical fields (PEF), high voltage electrical discharges (HVED) and ultrasonication (US) treatments for *Nannochloropsis* sp. (a), *P. tricornutum* (b) and *P. kessleri* (c).

At the fixed values of  $W$ , the levels  $Z_c$  and  $Z_p$  can be used for comparing the efficiency of different applied physical treatments. Therefore, for a better understanding of the extraction efficiencies, the correlations between extraction indexes obtained for the same levels of energy consumptions,  $W$ , using PEE treatments,  $Z(\text{PEE})$ , and US treatment,  $Z(\text{US})$ , were analyzed. **Fig. 4** shows these correlations of carbohydrates extraction index,  $Z_c$ , (a), and proteins extraction index,  $Z_p$ , (b) between PEE (HVED: solid lines, filled symbols; PEF: dashed lines, open symbols) and US treatments. The correlations with  $Z(\text{PEE}) = Z(\text{US})$  (gray dashed lines in **Fig. 4**) correspond to the equivalence of the extraction efficiencies for PEE and US treatments.



**Fig. 4.** Correlation of carbohydrates extraction index,  $Z_c$ , (a), or proteins extraction index,  $Z_p$ , (b) between pulsed electric energy (PEE) (high voltage electrical discharges (HVED): solid lines, filled symbols; pulsed electrical fields (PEF): dashed lines, open symbols) and ultrasonication (US) treatments for *Nannochloropsis sp.*, *P. tricornutum*, and *P. kessleri*.

In general, the PEF treatment was less effective for extraction of carbohydrates and proteins for all microalgal species when compared with the HVED or US treatments. This can

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reflect the weakness of the electroporation mechanism itself for the extraction of these bio-molecules from microalgal cells with rigid walls.

For the extraction of carbohydrates (**Fig. 4a**), the HVED treatment was more efficient than the US treatment. For all the tested species, we have observed that  $Z_c(\text{HVED}) > Z_c(\text{US})$  at the equivalent applied energy. This effect can be attributed to the partial fragmentation of cell walls, the release of cell-wall-linked polysaccharides, and the enhanced diffusion of carbohydrate bio-molecules from the interior to the exterior of the cells [34,35]. The enhanced mass transfer phenomena induced by HVED can also reflect the highly turbulent conditions that accelerate the convection of intracellular components from particles to the surrounding medium [36,37].

More complicated correlations of  $Z_p(\text{PEE})$  vs  $Z_p(\text{US})$  were observed for the extraction of proteins (**Fig. 4b**). These dependencies were significantly non-linear. For *Nannochloropsis* sp. and *P. tricornutum*, the most efficient technique was US treatment, and HVED treatment resulted in a better extraction in comparison with PEF. Better extraction was observed for *Nannochloropsis* sp. in comparison with *P. tricornutum*. For *P. kessleri* at a small value of  $Z_p \leq 0.02$ , the most efficient technique was HVED treatment, but at larger extraction levels ( $Z_p > 0.02$ ), the extraction efficiencies were arranged in the ascending order of  $\text{US} > \text{HVED} > \text{PEF}$ . The better functionality of US for extraction of proteins can reflect the formation of cavitation bubbles, their collapse on the cell surface, inside the cell walls, and in the cytoplasm, induced damage of cells and the phenomena of micro-streaming. Additionally, US can produce small cavities in cell walls and organelles (e.g. chloroplast), allowing some proteins in the form of water-soluble pigment-protein complexes to penetrate through the cell membrane [38].

Therefore, the different correlations observed for carbohydrates and proteins can reflect the different extraction mechanisms of these bio-molecules from microalgae. The disintegration of microalgal cells, breakage of organelles and internal structures to release water-soluble bio-molecules can depend on the mechanisms of cell disruption processes and location of bio-molecules in different parts of the cells, including cell wall, cytoplasm, chloroplast and all the organelles inside the barrier of the cell wall. The differences in release for microalgal species can reflect the variations of natural cell sizes, morphology and different places of accumulation of bio-molecules inside the cells. For example, *Nannochloropsis* sp. and *P. kessleri* exhibit several structural similarities, including cell size ( $\approx 2\text{-}4 \mu\text{m}$  in average

diameter) and shape (spherical). Yet, *P. tricornutum* cell is known to be fusiform with a length of 20-30  $\mu\text{m}$  and a diameter of 1-3  $\mu\text{m}$ . The *P. kessleri* can accumulate significant amounts carbohydrates in the form of starch granules [39], which are hardly soluble. However, in *Nannochloropsis* sp., a starch synthesis pathway is absent, hence it is not able to accumulate starch [40]. The microalgal proteins can be also located in different parts of the cells [38]. The protein release directly correlates with the degree of cell disintegration [1]. For example, the PEE treatments were effective for the release of water-soluble proteins from cytoplasm or from the inside of weak organelles, but a more complete extraction of proteins required the application of US, bead milling or high pressure homogenization [41].

**Table 1** Overview of the final extraction efficiency (after physical treatments (pulsed electric energy (PEE) and ultrasonication (US)) at the same maximum energy consumptions,  $W_{\max} \approx 704$  kJ/kg) of carbohydrates,  $F_c = C/C^m$ , and proteins,  $F_p = C_p/C_p^m$ , and selectivity index,  $S = F_c/F_p$ , for *Nannochloropsis* sp., *P. tricornutum*, and *P. kessleri*.

Microalgae	Methods	Final extraction efficiency, $F$ (%)		Selectivity index, $S$
		Carbohydrates	Proteins	
<i>Nannochloropsis</i> sp.	PEF	20.2 $\pm$ 0.8	3.1 $\pm$ 0.4	6.5 $\pm$ 1.3
	HVED	43.7 $\pm$ 1.6	3.2 $\pm$ 0.3	13.7 $\pm$ 2.0
	US	26.3 $\pm$ 6.5	8.9 $\pm$ 0.4	2.9 $\pm$ 0.6
	$C^m$	110.0 $\pm$ 5.3 mg/g	437.3 $\pm$ 4.8 mg/g	
<i>P. tricornutum</i>	PEF	19.7 $\pm$ 0.3	4.1 $\pm$ 0.3	4.8 $\pm$ 0.5
	HVED	37.8 $\pm$ 1.0	5.3 $\pm$ 1.0	7.1 $\pm$ 1.9
	US	33.3 $\pm$ 1.0	11.4 $\pm$ 1.1	2.9 $\pm$ 0.2
	$C^m$	123.6 $\pm$ 4.8 mg/g	287.2 $\pm$ 7.8 mg/g	
<i>P. kessleri</i>	PEF	11.1 $\pm$ 0.5	1.8 $\pm$ 0.2	6.2 $\pm$ 1.0
	HVED	23.5 $\pm$ 1.8	4.0 $\pm$ 0.1	5.9 $\pm$ 0.6
	US	13.4 $\pm$ 4.2	6.4 $\pm$ 1.3	2.1 $\pm$ 0.7
	$C^m$	350.0 $\pm$ 6.5 mg/g	440.0 $\pm$ 5.5 mg/g	

The extraction levels of carbohydrates and proteins for *Nannochloropsis* sp., *P. tricornutum* and *P. kessleri* are compared in **Table 1**. The final extraction efficiency (after physical treatments PEE and US at the same maximum energy consumptions,  $W_{\max} \approx 704$  kJ/kg) was characterized by the ratio  $F = C/C^m$  ( $F_c = C_c/C_c^m$  and  $F_p = C_p/C_p^m$  for carbohydrates and proteins, respectively). The investigated microalgal species accumulated

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more proteins than carbohydrates. The highest extraction efficiency of carbohydrates ( $\approx 44\%$  using the HVED treatment) was obtained for *Nannochloropsis* sp., while the highest extraction efficiency of proteins ( $\approx 11\%$  using the US treatment) was obtained for *P. tricornutum*.

For characterization of relative selectivity of carbohydrate and protein extraction, the selectivity index,  $S = F_c/F_p$ , was used. For non-selective extraction, the value of  $S = 1$  is expected. The analysis of selectivity was useful for the estimation of the purity of the extracted species, and it has been demonstrated that bead milling is selective towards proteins regardless of the tested species [1,42].

In our case, for all treatments and all microalgal species, the values of  $S$  were higher than 1. It reflects the higher efficiency of selective extraction of carbohydrates as compared with proteins. The selectivity indexes were smaller with the application of US treatment ( $S = 2.1-2.9$ ) as compared with PEE treatments ( $S = 4.8-13.7$ ). This means that the smallest selectivity was obtained for extraction assisted with US. The highest selectivity indexes were observed with application of HVED for *Nannochloropsis* sp. ( $S \approx 13.7$ ) and *P. tricornutum* ( $S \approx 7.1$ ), and with the application of PEF for *P. kessleri* ( $S \approx 6.2$ ). Therefore, for carbohydrates' selective release, the relatively mild cell disruption technique is required. However, for the maximum release of bio-molecules (including the small- and large sized), more intensive disruption techniques are required. Therefore, physical treatment can be chosen in function of the desired selectivity of extraction of bio-molecules.

#### 4. Conclusions

This work highlights the aqueous extraction of carbohydrates (relatively small molecules) and proteins (larger molecules) assisted by PEE (PEF or HVED) and US techniques. The results showed that extraction efficiency of target molecules depends on both the applied physical treatments and kind of microalgal species. In general, the smallest efficiency was observed for the PEF treatment. However, the smallest selectivity indexes,  $S$  (the value of  $S$  reflects the relative selectivity of carbohydrate extraction in comparison with proteins) were obtained for US treatment. The values of  $S$  depend on the microalgal species. It can be speculated that the highest selectivity with large values of  $S$  can be obtained using the mild PEE techniques (PEF, or HVED). However, maximum output of all bio-molecules requires the application of more intensive disruption techniques and they can result in a

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smaller selectivity. Therefore, the appropriate physical treatments could be used to tune the desired selectivity of extraction.

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### **Conflict of interest statement**

We declare that this manuscript has not any potential financial or other interests that could be perceived to influence the outcomes of the research.

### **Statement of informed consent, human/animal rights**

No conflicts, informed consent, human or animal rights applicable

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III.3 *Article 2*: Pulsed electric energy and ultrasonication assisted green solvent extraction of bio-molecules from different microalgal species

(The article is presented on the following pages)

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**Pulsed electric energy and ultrasonication assisted green solvent extraction  
of bio-molecules from different microalgal species**

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## Abstract

The effects of physical treatments (pulsed electrical fields (PEF), high voltage electrical discharges (HVED) and ultrasonication (US)) on aqueous extraction of carbohydrates and proteins, and ethanolic extraction of chlorophyll *a* from three microalgal species (*Nannochloropsis* sp., *P. tricornutum* and *P. kessleri*) have been studied. The total energy consumption of 530 kJ/kg suspension was applied for each treatment. For studied species, HVED was the most effective for extraction of carbohydrates, while US was the most effective for extraction of proteins and chlorophyll *a*. The observed differences for studied species can reflect the more fragile cell wall structure for *P. tricornutum* as compared with *Nannochloropsis* sp. or *P. kessleri*. The applied PEE of US treatments along with combinations of aqueous extraction of carbohydrates and proteins, and ethanolic extraction of pigments can be used in future implementations of selective extraction of valuable biomolecules from microalgae.

**Keywords:** Carbohydrates; Microalgae; Pigments; Proteins; Pulsed electric energy; Ultrasonication

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## 1. Introduction

Microalgae are microscopic unicellular organisms capable to convert solar energy to chemical energy via photosynthesis (Hosikian, Lim, Halim, & Danquah, 2010) and they have the advantage of capturing CO<sub>2</sub> from the environment and combustion processes, thereby reducing greenhouse gases (Gerde, Montalbo-Lomboy, Yao, Grewell, & Wang, 2012). Microalgae can accumulate large amounts of metabolites over short periods of time, including carbohydrates, proteins, and lipids, as well as pigments (Khili, 2013; Sankaran et al., 2018). For example, for cultivation of *Chlorella vulgaris* under illumination with red light, the highest yield of chlorophyll *a* was achieved, corresponding to 1.29% of cell biomass (da Silva Ferreira & Sant'Anna, 2017). The chlorophylls content can be also greatly affected by the cultivation temperature, stirring the microalgal culture and content of nutrients (nitrogen, phosphorus) and micronutrients (iron, zinc, manganese, copper).

Natural pigments have an important role in the photosynthetic metabolism and pigmentation of microalgae (D'Alessandro & Antoniosi Filho, 2016). The major photosynthetic pigments are represented by chlorophylls, violaxanthin, and vaucheraxanthin in microalgae (Reboloso-Fuentes, Navarro-Pérez, Garcia-Camacho, Ramos-Miras, & Guil-Guerrero, 2001). Commonly, there exists a directly proportional relationship between content of chlorophyll *a* and the amount of algal biomass (Henriques, Silva, & Rocha, 2007).

However, the extraction of valuable bio-molecules from the microalgae is not an easy task. These bio-molecules are commonly located in the intracellular compartments, protected by the rigid cell walls, and membranes surrounding the cytoplasm and the internal organelles (Postma et al., 2017). For example, the proteins are commonly located in the cell walls, cytoplasm and chloroplasts, whereas the pigments (chlorophylls and some carotenoids) are located in the thylakoids of the chloroplasts. Different techniques to release water-soluble bio-molecules from microalgae by chemical hydrolysis (Duongbia, Chaiwongsar, Chaichana, & Chaiklangmuang, 2019; Lorenzo-Hernando, Ruiz-Vegas, Vega-Alegre, & Bolado-Rodriguez, 2019; Sedighi, Jalili, Darvish, Sadeghi, & Ranaei-Siadat, 2019), bead milling (Garcia, Lo, Eppink, Wijffels, & van den Berg, 2019; Rivera et al., 2018), high pressure homogenization (Pataro et al., 2017), ultrasonication (US) (Gonzalez-Balderas, Velasquez-Orta, Valdez-Vazquez, & Ledesma, 2020; Skorupskaite, Makareviciene, Sendzikiene, & Gumbyte, 2019; Zhang, Grimi, Marchal, Lebovka, & Vorobiev, 2019), pulsed electrical fields (PEF) (Parniakov et al., 2015b, 2015a; Pataro et al., 2017), high voltage electrical discharges



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(HVED) (Grimi et al., 2014) have been applied. Note that pigments are poorly soluble in water (practically insoluble), the classical organic solvent extraction or supercritical fluid extraction are commonly used for their extraction (Hosikian et al., 2010; Macias-Sánchez et al., 2008). However, as far as we know the PEF and HVED techniques were still rarely applied for assistance of extraction of insoluble pigments.

The main aim of this work is to explore the feasibility of physical treatments (PEF, HVED and US) to recovery of water-soluble (proteins and carbohydrates) and -insoluble (chlorophyll *a*) bio-molecules from different microalgal species. The data were compared for three different microalgal species (*Nannochloropsis* sp., *Phaeodactylum tricornutum* (*P. tricornutum*), and *Parachlorella kessleri* (*P. kessleri*)). These species have different cell shapes, structures and biomass composition. The cells of *Nannochloropsis* sp. (marina green algae), and *P. kessleri* (freshwater green algae) are approximately spherical (2–4 µm in diameter), while the cells of *P. tricornutum* (marina diatom) have the fusiform shape (similar to the lemon-shape) with a length of 20–30 µm and a diameter of 1-3 µm (Alhattab, Kermanshahi-Pour, & Brooks, 2019). Besides, *Nannochloropsis* sp. and *P. kessleri* cells have strong and rigid cell walls mainly composed of cellulose and hemicelluloses (Payne & Ripplingale, 2000), while the cell walls of *P. tricornutum* are poor in silica and composed of sulfated glucomannan (Francius, Tesson, Dague, Martin-Jézéquel, & Dufrêne, 2008). The studied microalgal species have different contents of carbohydrates, proteins, total pigments and lipids. For example, the *Nannochloropsis* sp. contains maximum content of lipids ( $\approx 9.0\%$  (wt. DW biomass) as compared with *P. tricornutum* ( $\approx 3.9\%$ ) and *P. kessleri* ( $\approx 3.8\%$ ), whereas the *P. kessleri* contains maximum contents of carbohydrates ( $\approx 35\%$ ) as compared with *Nannochloropsis* sp. ( $\approx 11\%$ ) and *P. tricornutum* ( $\approx 12.4\%$ ). The impact of different physical treatments on bio-molecules extractability was discussed at equivalent energy consumption. Attention was also focused on the effects of physical treatments on extraction kinetics of chlorophyll *a*.

## **2. Materials and methods**

### *2.1 Chemicals*

D-glucose, bovine serum albumin (BSA) and chlorophyll *a* standard (#C5753) were provided from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bradford Dye Reagent and

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ethanol (EtOH, 95%, v/v) were obtained from Thermo Fisher (Kandel, Germany). Sulfuric acid and phenol were purchased from VWR (France).

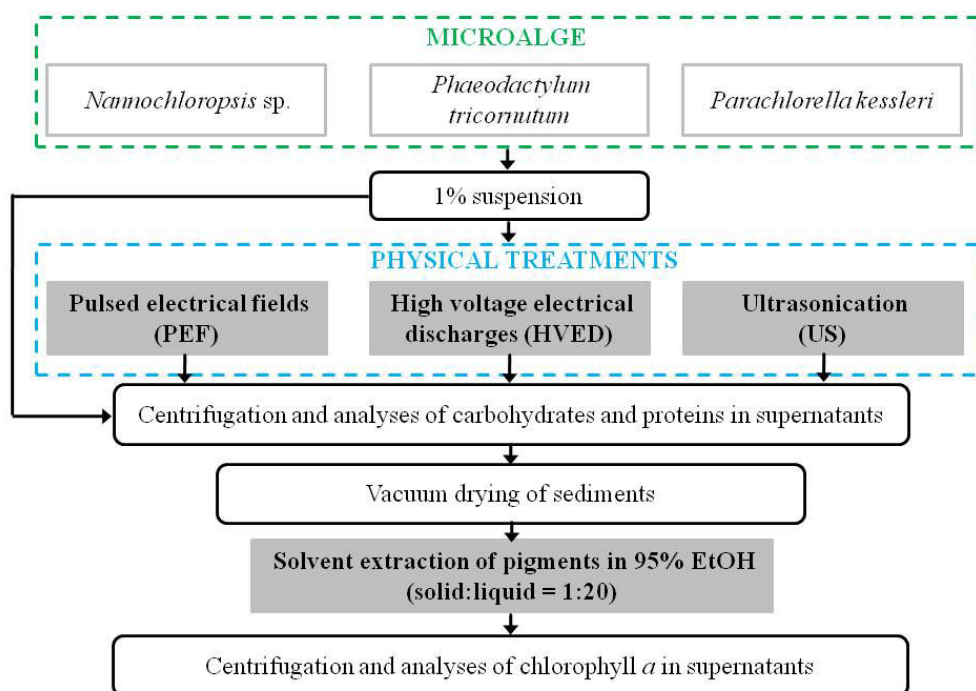
## 2.2 Microalgae

Three microalgal species (*Nannochloropsis* sp, *P. tricornutum* and *P. kessleri*) were provided by AlgoSolis (Saint-Nazaire, France). The details of cultivation procedures were described previously for *Nannochloropsis* sp. (Parniakov et al., 2015a), *P. tricornutum* (Guihéneuf et al., 2011) and *P. kessleri* (Zhang et al., 2019).

The samples were obtained as frozen microalgal pastes with  $\approx 80\%$  (*Nannochloropsis* sp.),  $\approx 70\%$  (*P. tricornutum*) and  $\approx 82\%$  (*P. kessleri*) of moisture content, respectively. The pastes were preliminary washed 3 times using deionized water. Briefly, in the applied washing procedure, the biomass was diluted to 1% dry matter (DM) (hereinafter %), agitated at 150 rpm for 10 min, and centrifuged for 10 min at 4,600 g. Then supernatant was removed, and the sediment was freeze-dried using a MUT 002A pilot freeze-drier (Cryotec, France) for 64 h at  $-20\text{ }^{\circ}\text{C}$ . The composition of the biomass was determined according to the previously described methods (Macias-Sánchez et al., 2008; Phelippe, Goncalves, Thouand, Cogne, & Laroche, 2019; Ritchie, 2006). The carbohydrates content was determined after two passes in high pressure disrupter (CellD, Constant System) at 270 MPa and centrifugation at 3,000g for 5 minutes. The proteins content was determined in the total high-pressure lysate. The total pigments content was measured after centrifugation of intact cells and methanol extraction. The analysis' data gave that *Nannochloropsis* sp. contains  $\approx 11\%$  (wt. DW biomass) of carbohydrates,  $\approx 43.7\%$  of proteins,  $\approx 2.0\%$  of total pigments and  $\approx 9.0\%$  of lipids; *P. tricornutum* contains  $\approx 12.4\%$  of carbohydrates,  $\approx 28.7\%$  of proteins,  $\approx 1.0\%$  of total pigments and  $\approx 3.9\%$  of lipids; *P. kessleri* contains  $\approx 35\%$  of carbohydrates,  $\approx 44\%$  of proteins,  $\approx 4.0\%$  of total pigments and  $\approx 3.8\%$  of lipids.

## 2.3 Extraction procedures

**Fig. 1** presents the schema of the applied experimental procedures. The applied procedure include cell disintegration by physical treatments (leaching in pure water), and common extraction using green solvent for recovery of chlorophyll *a*. In control experiments, untreated (U) suspensions were also analyzed.



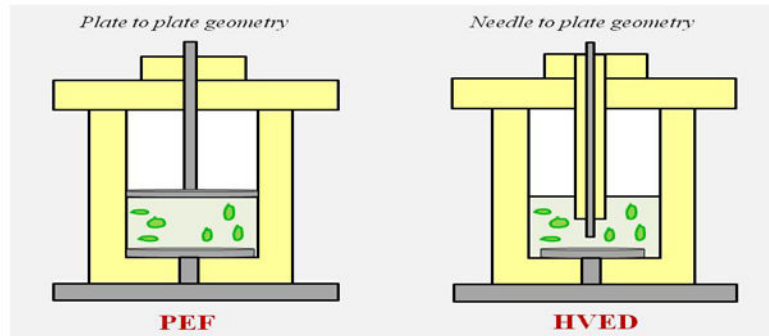
**Fig. 1.** Schema of the applied experimental procedures.

### 2.3.1 Physical treatments

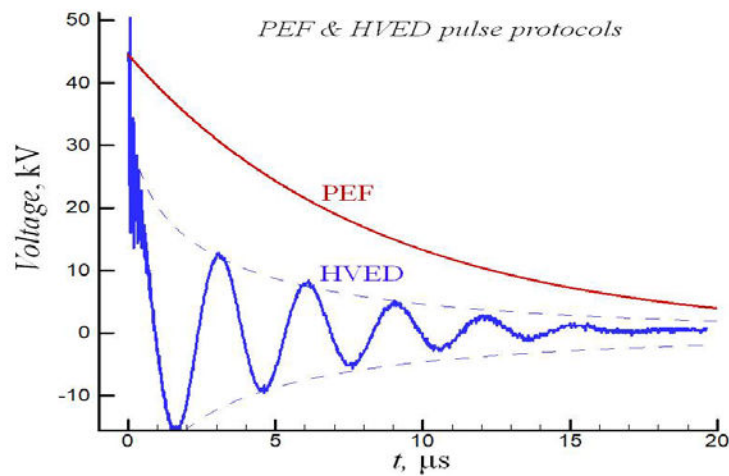
For physical treatments, the suspensions with the biomass concentrations of 1% were prepared. All treatments (PEF, HVED and US) were performed using 250 g of the suspension.

Treatments by pulsed electric energy (PEF or HVED) were done using a high voltage pulsed power generator (40 kV-10 kA, Basis, Saint-Quentin, France) in cylindrical batch treatment chamber with different types of electrodes (**Fig. 2a**). The PEF treatment was done between two parallel plate electrodes. The distance between the electrodes was fixed at 2 cm to produce the electric field strength of  $E = 20$  kV/cm. The HVED treatment was performed using electrodes in needle-plate geometry. The distance between the stainless steel needle and the grounded plate electrodes was fixed at 1 cm, the corresponding electric field strength of  $E = 40$  kV/cm. The protocols of pulsed electric energy (PEF or HVED) treatments comprised application of  $n = 600$  successive pulses with a frequency of 0.5 Hz, the total electrical treatment duration was  $t_e = 0.01$ -6 ms. The exponential decay of voltage  $U \propto \exp(-t/t_p)$  with effective decay time  $t_p \approx 10 \pm 0.1$   $\mu$ s were observed for applied modes of treatment (**Fig. 2b**).

a)



b)



**Fig. 2.** Schematic representation of pulsed electric energy (PEF and HVED) treatment chambers (a), and pulsed protocols (b) used for treatment of microalgal suspensions.

Specific energy consumption,  $W$  (J/kg suspension), was calculated for PEF and HVED treatment as following formula (Yu, Gouyo, Grimi, Bals, & Vorobiev, 2016):

$$W = (C \times U^2 \times n) / 2m \quad (1)$$

where  $C$  is the capacitance of the capacitor;  $U$  is the voltage of the generator;  $n$  is number of pulses;  $m$  is the mass of 1% suspension (kg).

Treatment by US, a UP-400S ultrasound processor (Hielscher GmbH, Germany) with a constant frequency of 24 kHz was applied. The ultrasound probe (diameter: 14 mm, length: 100 mm) was plunged into a beaker, containing of suspension. The total treatment time of US was  $t_u = 660$  s, and the amplitude was fixed at 50%, which corresponded to the power,  $W_u = 200$  W.

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Specific energy consumption,  $W$  (J/kg suspension), was calculated for US treatment as following formula:

$$E = W_u \times t_u / m \quad (2)$$

where  $m$  is the mass of 1% suspension (kg).

For all treatments (PEF, HVED and US), the samples were cooled in order to prevent overheating, the temperature was maintained approximately at ambient temperature, and elevation of temperature not exceeded 5 °C.

### 2.3.2 Solvent extraction

After physical treatments, the microalgal cells were dried by vacuum in a vacuum chamber (Cole-Parmer, USA) at pressure of 30 kPa, and temperature of 50 °C for 24 h. Then dried biomass was mixed with EtOH (95%, v/v) with solid-liquid ratio of 1: 20 (w/w) and solvent extraction under the stirring at 150 rpm was done for the time of  $t = 1440$  min (24 h). To avoid any evaporation, the extraction cells were covered with aluminum foil during the solvent extraction process. The pigments content was measured continuously during extraction.

### *2.4 Characterization*

The supernatant was collected by centrifugation using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 g for 10 min, and then used for analysis. All the characterization measurements were done at ambient temperature.

#### 2.4.1 Carbohydrates analysis

The carbohydrates content,  $C_c$ , was determined by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). D-glucose was used as a standard. Briefly, 1 mL of supernatant was mixed with 0.1 mL of phenol solution (5%, w/w) and 5 mL of concentrated sulfuric acid. The mixture was stored at 20 °C for 20 min. The absorbance was measured at the wavelength  $\lambda = 490$  nm using a UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The extraction yield of carbohydrates,  $Y_c$  (%), was calculated by as following formula:

$$Y_c = C_c / C_c^{max} \times 100 \quad (3)$$

where  $C_c^{max}$  the total carbohydrate content in microalgae.

#### 2.4.2 Proteins analysis

The proteins content,  $C_p$ , was determined using the method of Bradford (Bradford, 1976). BSA was used as a standard. Briefly, 0.1 mL of supernatant was mixed with 1 mL of Bradford Dye Reagent. The mixture was vortex for 10 s and kept for 5 min. The absorbance was measured at the wavelength  $\lambda = 595$  nm using a UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The extraction yield of proteins,  $Y_p$  (%), was calculated by as following formula:

$$Y_p = C_p / C_p^{max} \times 100 \quad (4)$$

where  $C_p^{max}$  the total proteins content in microalgae.

#### 2.4.3 Pigments analysis

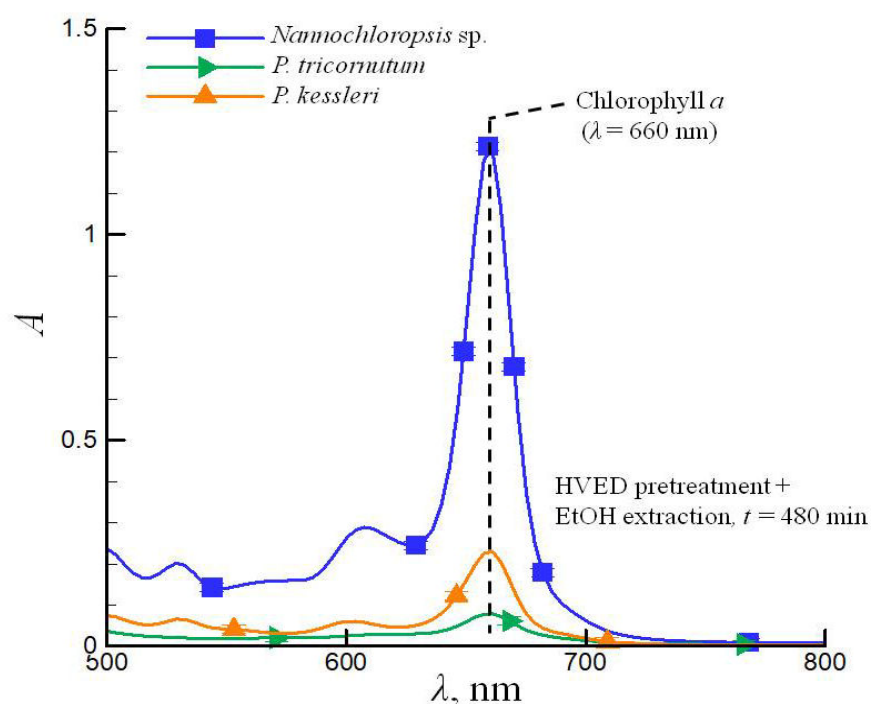


Fig. S1. Examples of the UV absorption spectra of supernatants, obtained after solvent extraction ( $t = 480$  min) from HVED treated samples.

Absorption spectra of supernatants (diluted if required) obtained from solvent extraction procedure was measured in the wavelength range of 300-900 nm against the blank (with the precision of  $\pm 1$  nm) (See, Supplementary materials Fig. S1). The maximum

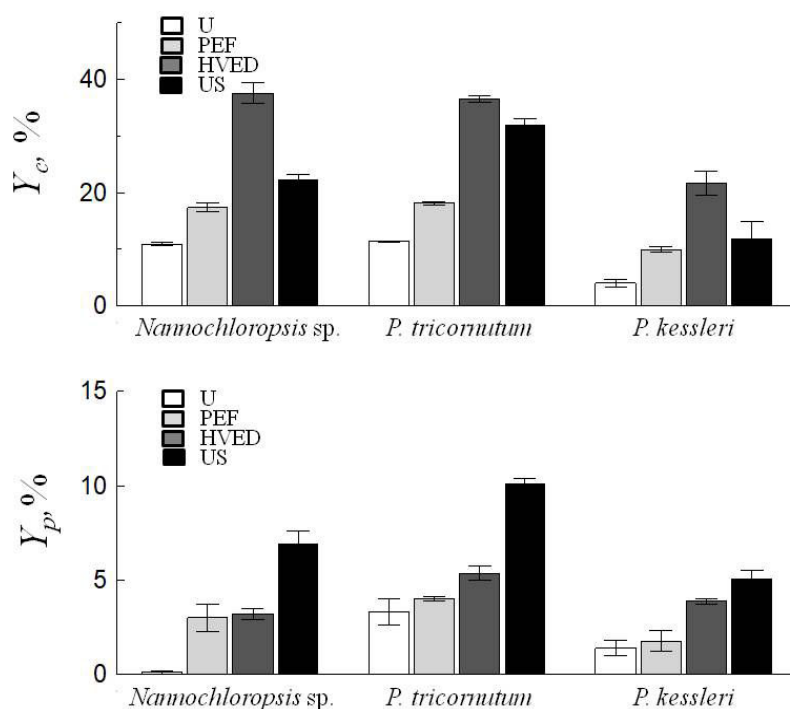
absorbance of chlorophyll *a* of all microalgal species was at the wavelength of  $\lambda = 660$  nm. The content of chlorophyll *a*,  $C_{chl\ a}$ , in the extracts were estimated by chlorophyll *a* calibration curve ( $A=87.86\times C+0.0055$ ,  $R^2=0.9998$ ).

## 2.5 Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean  $\pm$  standard deviation. The error bars, presented on the figures, correspond to the standard deviations.

## 3. Results and discussion

### 3.1 Extractability of carbohydrates and proteins



**Fig. 3.** The extraction yields of carbohydrates,  $Y_c$ , and proteins,  $Y_p$ , in the supernatants, obtained from untreated (U) and physically (PEF, HVED, US) treated samples for different microalgal species. The total energy consumption of physical treatments was the same,  $W \approx 530$  kJ/kg suspension.

The cell disintegration efficiency of tested physical treatments at equivalent energy consumption were evaluated by monitoring the extractability of water-soluble carbohydrates (small-size molecules) and proteins (large-size molecules), respectively. **Fig. 3** presents the



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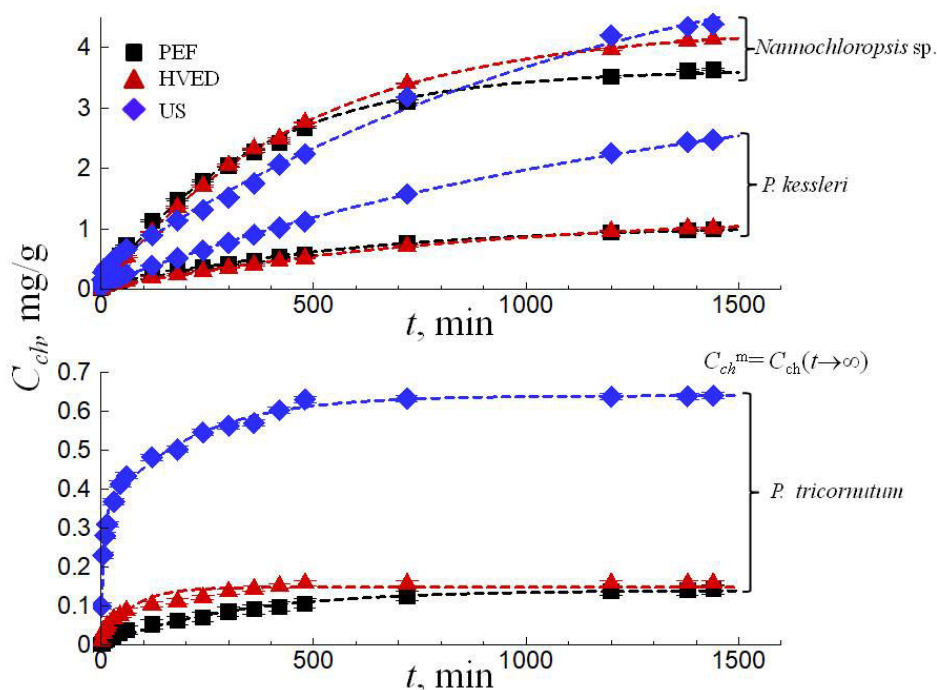
extraction yields of carbohydrates,  $Y_c$ , and proteins,  $Y_p$ , in the supernatants, obtained from three microalgal species after application of different physical treatments. The total energy consumption for all treatments was the same,  $W \approx 530$  kJ/kg suspension. At this energy consumption, the pulsed electric energy treatments were sufficient for extraction of water-soluble bio-molecules (e.g. proteins) and further increase in  $W$  resulted in insignificant supplementary effects (Grimi et al., 2014; Parniakov et al., 2015b).

The lowest extraction yields of carbohydrates ( $Y_c = 4\text{-}11.5\%$ ) and proteins ( $Y_p = 0.1\text{-}3.5\%$ ) were obtained for U samples for each tested specie. The application of physical treatments improved the extraction yields for both carbohydrates and proteins, as compared to the U samples. The higher extraction yields for carbohydrates as compare to that for proteins were observed. The highest extraction yield of carbohydrates ( $Y_c \approx 37.5 \pm 1.8$  % using the HVED treatment) was obtained for *Nannochloropsis* sp., while the highest extraction yield of proteins ( $Y_p \approx 10.1 \pm 0.3$  % using the US treatment) was obtained for *P. tricornutum*. For all tested species, the extraction yields of carbohydrates and proteins can be arranged in the rows of  $U < \text{PEF} < \text{US} < \text{HVED}$  and  $U < \text{PEF} < \text{HVED} < \text{US}$ , respectively. Therefore, the HVED treatment was the most efficient technique to extract carbohydrates, while US treatment was the most efficient technique to extract proteins. However, at once the PEF treatment obtained the smallest extraction efficiencies of water-soluble bio-molecules for all tested species. This reflects that the electroporation mechanism itself was not very effective for extraction of intracellular molecules as compared with efficiency of HVED and US treatments (Pataro et al., 2017). The observed data are in agreement with previously published results (Grimi et al., 2014). The poor yield of proteins for PEE and US assisted extractions can be explained by the following arguments. The microalgal proteins are located within different parts of the cells, including the cell wall, cytoplasm, chloroplast and all organelles inside the barrier of the cell wall (Safi et al., 2015). The “gentle” treatments, like PEE or US, can partially assist a release of the proteins present in cytoplasm or inside weak organelles. The complete extraction proteins require more serious disintegration of cells with applications of stronger techniques like bead milling or high pressure homogenization (Pataro et al., 2017). However, the application of severe disruption techniques may induce significant to biological macromolecules (Günerken et al., 2015).

Among the tested species, *P. tricornutum* demonstrated the highest extraction yields of carbohydrates and proteins, while the lowest extraction yields were observed for *P. kessleri*.

This possibly reflects the differences in resistance of cell walls against physical damages for these species. For PEF and US treatments, the values of  $Y_c$  and  $Y_p$  were arranged the row *P. tricornutum* > *Nannochloropsis* sp. > *P. kessleri*, whereas for HVED treatment they were arranged the row *Nannochloropsis* sp. > *P. tricornutum* > *P. kessleri* for carbohydrates and in the row *P. tricornutum* > *P. kessleri* > *Nannochloropsis* sp. for proteins. This extraction sensibility for different physical treatment techniques can reflect that the differences in cell structure (e.g. size, shape, cell-wall structure and location of bio-molecules) in the tested microalgal species.

### 3.2 Extraction kinetic of pigments



**Fig. 4.** The extraction kinetics of chlorophyll *a*,  $C_{ch}$ , for different microalgal species for physically (PEF, HVED, US) treated samples. The dashed lines in the Fig. 4 correspond to the fittings of the experimental data (symbols) using one-exponential (Eq. (5) for PEE) and two-exponential (Eq. (6) for US) laws.

**Fig. 4** presents kinetics of chlorophyll *a*,  $C_{ch}$ , extraction in the EtOH (95%, v/v) for different microalgae with application of different physical pretreatments. The value of  $C_{ch}$  increased with the increase of extraction time,  $t$ . For all species, at relatively long time ( $t \approx 24$  h), the US treatment was most efficient. HVED resulted in the approximately extraction

efficiency as compared with PEF. The similar tendencies were observed for all times of extraction for *P. tricornutum*, and *P. kessleri*, but for *Nannochloropsis* sp. at  $t < 700$  min, the extraction efficiencies for pulsed electric energy treatments were more effective than for US treatment (**Fig. 4**).

The analysis of the experimental data showed that the extraction behavior of chlorophyll *a* after PEF, HVED, and US treatments followed the different kinetics. The extraction assisted by pulsed electric energy (PEF and HVED) can be fitted using the first-order exponential equation:

$$C_{ch} = C_{ch}^m [1 - \exp(-t/\tau)] \quad (5)$$

where  $C_{ch}$  is the content of chlorophyll *a* in the course of extraction;  $C_{ch}^m$  is the maximum value of  $C_{ch}$  for long extraction times;  $t$  is the time of extraction, and  $\tau$  is the effective extraction time.

The extraction of chlorophyll *a* assisted by US occurred in two stages and can be fitted by the following two-exponential law:

$$C_{ch} = C_{ch}^m [C_f^* (1 - \exp(-t/\tau_f)) + (1 - C_f^*) (1 - \exp(-t/\tau))] \quad (6)$$

where  $\tau_f$  and  $\tau$  correspond to the effective extraction times for the first (fast) and second (slow) stages, respectively. Here,  $C_f^* = C_f/C_{ch}^m$ ,  $C_f$  is the maximum concentration extracted during the first (fast) stage.

The dashed lines in the Fig. 4 correspond to the fittings of the experimental data (symbols) using one-exponential (Eq. (5) for PEE) and two-exponential (Eq. (6) for US) laws. In all cases, the determination coefficients of fitting were rather high (in the interval  $R^2 = 0.96-0.99$ ).

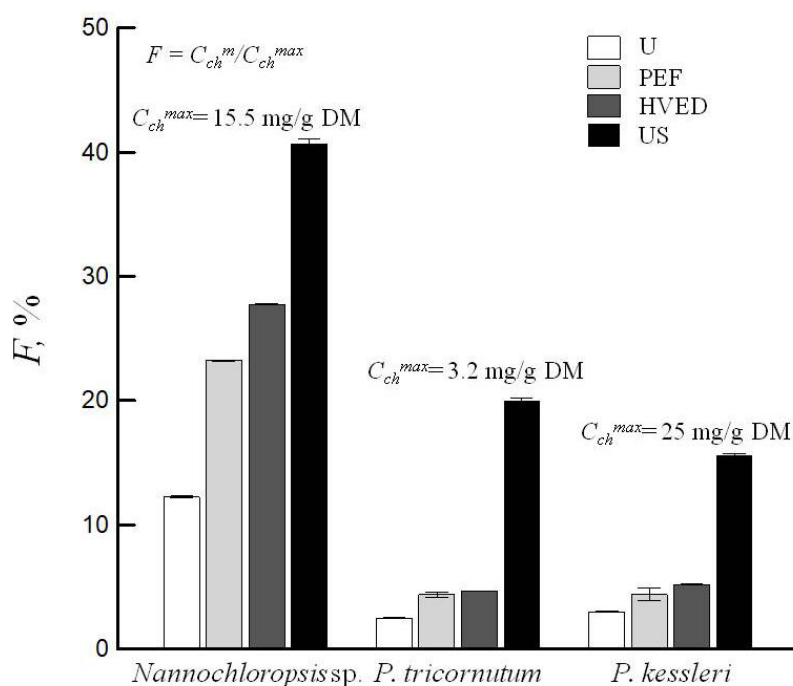
The corresponding parameters evaluated for one-exponential (PEE) and two-exponential (US) laws for different species are presented in Supplementary materials (Table S1). For US assisted extraction, the duration of the second (slow) stage was significantly higher of the first one ( $\tau \gg \tau_f$ ). For *Nannochloropsis* sp. and *P. kessleri*, the relative fraction of extracted chlorophyll *a* during the fast stage was rather small ( $C_f = 0.02-0.05$ ) and it was  $C_f^* = 0.52 \pm 0.04$  for *P. tricornutum*.

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The obtained data clearly demonstrated the quite different behaviors of extraction kinetic of chlorophyll *a* with assistance of pulsed electric energy (PEF and HVED) or US treatments. The similar two-exponential behavior was previously observed for aqueous extraction assisted by US fennel tissue (Moubarik, El-Belghiti, & Vorobiev, 2011). The two stages in US assisted extraction can reflect the presence of pigments with different binding inside the cells and to the cell walls of the microalgal species. The first (fast) stage can be attributed to the release of some portion of weakly coupled pigments (possibly coupled with cell walls), while the second (slow) stage can be related with extraction of remained pigments from interior cell organelles. The values of relative concentration extracted during the first (fast) stage,  $C_1^*$ , can be arranged in the rows of *P. tricornutum* > *Nannochloropsis* sp. > *P. kessleri*. This order was in line with the order of extraction efficiencies of carbohydrates and proteins by US treatment. It possibly reflects the more fragile cell wall structure of *P. tricornutum* as compared to that for *Nannochloropsis* sp. or *P. kessleri*. The cell walls of *P. tricornutum* are composed of sulfated glucomannan (Francius et al., 2008) and they have more fragile structure as compare with cell walls of *Nannochloropsis* sp. and *P. kessleri* mainly composed of cellulose and hemicelluloses (Payne & Rippingale, 2000).

To compare the efficiencies of extraction for untreated (U), physically treated (by PEF, HVED, and US) samples, the ratios  $F = C_{ch}^m / C_{ch}^{max}$  ( $C_{ch}^{max}$  is the total chlorophyll *a* content in microalgae) have been evaluated. **Fig. 5** presents the values of *F* for studied microalgal species.

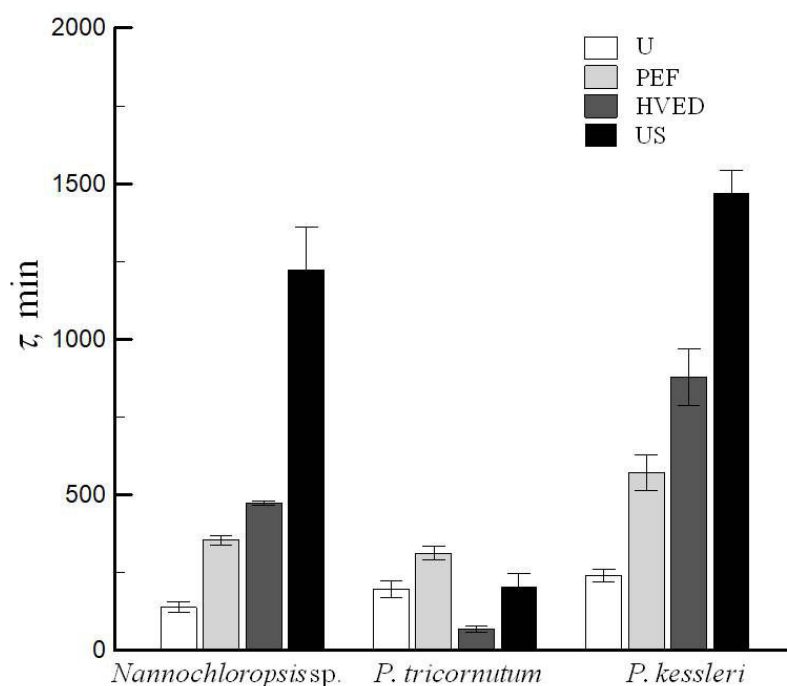
For all studied microalgal species, the values of *F* can be arranged in the same row as for extraction yields of proteins (U < PEF < HVED < US). The highest extraction efficiency (18-40%) was observed for US assisted extraction. The similar effect of US treatment on extraction of proteins and pigments can reflect formation of protein-pigment complexes. The increased aqueous extraction of proteins supplemented with extraction of pigments was previously observed for application of US treatment of microalgal suspension (Parniakov et al., 2015).



**Fig. 5.** Maximum extraction efficiency of chlorophyll *a*, *F*, for different microalgal species for untreated (*U*) and physically (*PEF*, *HVED*, *US*) treated samples.

Note that the highest content of chlorophyll *a* was observed in the raw *P. kessleri*, and it was smallest in the raw *P. tricornutum* (**Fig. 5**). However, for each tested physical method, the better extraction efficiency was observed from *Nannochloropsis* sp. as compared with *P. tricornutum* and *P. kessleri*. For example, the highest value of *F* ( $\approx 40\%$ ) obtained from *Nannochloropsis* sp. after *US* pretreatment was almost 2-fold and 2.6-fold higher than those obtained from *P. tricornutum* and *P. kessleri*.

**Fig. 6** compares the extraction time of slow stage,  $\tau$ , for different microalgal species for untreated (*U*), and *PEF*, *HVED*, and *US* (slow stage) treated samples. For green microalgae (*Nannochloropsis* sp. and *P. kessleri*), the value of  $\tau$  can be arranged in the row of  $U < PEF < US < HVED$ . The similar order was observed for both the values of *F* (**Fig. 5**) and  $\tau$  (**Fig. 6**). However, for *P. tricornutum*, the more complicated behaviour was observed. Here, the extraction efficiency was highest for *US* treatment whereas the longest extraction time was observed for *PEF* treatment. The observed behavior reflect the sensibility of extraction efficiency upon the physical treatment and type of microalgal species.



**Fig. 6.** Effective extraction time,  $\tau$ , for different microalgal species for untreated (U) and PEF, HVED, US (slow stage) treated samples.

#### 4. Conclusions

This study compares the extraction efficiencies of water-soluble (carbohydrates and proteins) and -insoluble (chlorophyll *a*) bio-molecules assisted by PEF, HVED and US techniques. The extraction efficiency arranged in the rows of U < PEF < US < HVED (for carbohydrates) and U < PEF < HVED < US (for proteins) was observed for all tested microalgal species. PEF treatment demonstrated the smallest efficiency for extraction of bio-molecules. The kinetics of extraction of chlorophyll *a* in EtOH solution was described using one-exponential (PEF and HVED) and two-exponential (US) equations. Significant differences in extraction behavior were observed for green microalgae (*Nannochloropsis* sp. and *P. kessleri*) and diatom (*P. tricornutum*). For *Nannochloropsis* sp. and *P. kessleri*, the US treatment was the most effective for extraction of chlorophyll *a*, but the longest extraction times were required with application of this technique. The observed differences for studied species can reflect the more fragile cell wall structure for *P. tricornutum* as compared with *Nannochloropsis* sp. or *P. kessleri*.

#### Declaration of competing Interest

None.

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### III.4 Chapter conclusion

Chapter III is focused on the effect of three physical pre-treatments (PEF, HVED and US) on cell disruption and release of intracellular bio-molecules from different microalgal species. The feasibility of three physical treatments to assist extraction of hydrophilic (carbohydrates and proteins) and hydrophobic (chlorophyll a) molecules were respectively confirmed in two articles. By comparing the obtained data, it was evidenced that the extraction efficiency depends on the mechanism of applied physical treatments and extracted target molecules. At equivalent energy consumption, the extraction efficiency arranged in the rows of HVED > US > PEF (for carbohydrates) and US > HVED > PEF (for proteins) was observed for all tested microalgal species. Among them, the PEF treatment demonstrated the smallest efficiency for extraction of carbohydrates and proteins due to its mechanism only cause cell membrane damage. However, for all tested physical treatments, the extraction degree of carbohydrates was  $\leq 40\%$ , while the extraction degree of proteins was  $\leq 10\%$ . They allowed selective extraction more carbohydrates than proteins. The relative mild PEE technologies have the higher extraction selectivity than US technology.

Three physical technologies assisted extraction of hydrophobic molecule (chlorophyll a) presented different extraction behaviours. The extraction of chlorophyll a using PEE technology occurs in one stage (diffusion). By contrast, the extraction using US treatment occurs in two stages (convection and diffusion), the first stage with a fast chlorophyll a transfer from the inside of microalgal cell, and the second stage corresponds to the prolonged chlorophyll a transfer by molecular diffusion from interior of the microalgal cell. Moreover, based on the observation of the different behavior between green microalgae and diatom, the cell wall of *P. tricornutum* was more fragile than *Nannochloropsis* sp. or *P. kessleri*. These obtained results will help for understanding the correlations between selected methods and efficiency of extracted target bio-molecules. The appropriated cell disruption methods should be selected and used to tune the desired target molecules. However, in order to obtain higher extraction efficiencies of intracellular molecules, combined process will be needed.

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## **Chapter IV Effect of combination process for selective and energy efficient extraction of bio-molecules from microalga *Parachlorella kessleri***

### IV.1 Chapter introduction

The effectiveness and selective extractability of alternative physical treatments (PEF, HVED and US) have been evidenced in chapter III. However, they are relative mild cell disruption methods. The extraction of larger size molecules or more bounded to the intracellular organelles, still requires the application of more intensive cell disruption technique. The HPH treatment is a purely mechanical process, which is one of the most commonly employed methods for the large-scale cell disruption (Grimi et al., 2014). It has been considered as a promising method for complete disruption of biological cells. However, HPH causes the non-selective release of bio-molecules, produces large amounts of cell debris (Norton and Sun, 2008), complicate the downstream separation processes (Balasundaram et al., 2009); as well as high energy consumption (Lee et al., 2013).

Although several studies have already highlighted the potential of PEF, US or HPH in the microalgae biorefineries, to date, there is no studies focus on the effect of their combination on the microalgae biorefineries. For these reasons, the combination of physical and mechanical cell disruption methods may considerably promote the implementation of biorefinery concept on microalgae, enabling a faster and more efficient extraction of bio-molecules. This also contributes to promote the reduction of energy costs, and the extraction time. Therefore, the combination of alternative physical treatments (PEF, HVED and US) and mechanical HPH treatment is interesting to be carried out for realize this proposes. Moreover, the effects of different concentrations used on extraction efficiencies and energy consumption were also discussed.

In this chapter, the first part (details are presented in article 3: *Effect of ultrasonication, high pressure homogenization and their combination on efficiency of extraction of bio-molecules from microalga Parachlorella kessleri*) was published in the journal “Algal Research”. This work was carried out under the direction of Dr. Nabil Grimi, Prof. Luc Marchal and in collaboration with Prof. Nikolai Lebovka and Prof. Eugène Vorobiev. The second part (details are presented in article 4: *Effect of combined pulsed electric energy and high pressure homogenization on selective and energy efficient*

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*extraction of bio-molecules from microalga Parachlorella kessleri*) was submitted to the journal “LWT”. This work was carried out under the direction of Dr. Nabil Grimi, Prof. Luc Marchal and in collaboration with Prof. Eugène Vorobiev.

IV.2 *Article 3: Effect of ultrasonication, high pressure homogenization and their combination on efficiency of extraction of bio-molecules from microalga Parachlorella kessleri*

(The article is presented on the following pages)

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**Effect of ultrasonication, high pressure homogenization and their combination on efficiency of extraction of bio-molecules from microalga**

***Parachlorella kessleri***

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## Abstract

The efficiencies of disintegration of microalgal *Parachlorella kessleri* cells by ultrasonication (US) and high pressure homogenization (HPH) treatments, and extraction of ionics, proteins, carbohydrates, and pigments were investigated. The applied procedures included individual US treatment, individual HPH treatment or combined US treatment followed by HPH treatment. The test concentrations of cells were 1 % and 10 % dry matter. The microstructures of cells and suspensions were analyzed using scanning electron microscopy, light microscopy and light scattering techniques. Extraction was characterized by the ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , and pigment (dyes),  $Z_d$ , extraction indexes. Application of US treatment (400 W, 30 min, 1 % dry matter) gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$ , and application of HPH treatment (400 bar, 4 passes, 1 % dry matter) gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.20$ , and  $Z_p \approx 0.11$ . In both cases, the efficiency of extraction can be arranged in the row  $Z_i < Z_p < Z_c$ . Application of preliminary US treatment with 10 % dry matter and final HPH treatment with 1 % dry matter allows increasing the extraction efficiency and decreasing the energy consumptions. For example, US (400 W, 30 min, 1 % dry matter) + HPH (1200 bar, 4 passes, 1 % dry matter) treatment ( $\approx 106$  kJ/g dry matter) gave  $Z_i \approx 0.49$ ,  $Z_c \approx 0.69$ , and  $Z_p \approx 0.32$ , whereas US (400 W, 30 min, 10 % dry matter) + HPH (1200 bar, 4 passes, 1 % dry matter) treatment ( $\approx 53.8$  kJ/g dry matter) gave  $Z_i \approx 0.76$ ,  $Z_c \approx 0.83$ ,  $Z_p \approx 0.74$ .

**Keywords:** Microalgae; *Parachlorella kessleri*; Ultrasonication; High pressure homogenization; Selective extraction; Bio-molecules

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## Nomenclature

$A$	absorbance of pigment peaks
$c$	specific heat capacity of suspension, J/g°C
$C$	concentration of carbohydrate, mg/g DM
$C_m$	concentration of suspension, % DM
$C_p$	concentration of protein, mg/g DM
$d$	diameter of particles, $\mu\text{m}$
$E$	specific energy consumption for HPH and US, kJ/g DM
$m$	mass of suspension, g
$N$	number of HPH passes
$p$	homogenizing pressure, bar
$P_a$	actual power of US, W
$P_g$	generator power of US, W
$t$	time of US, min
$\Delta T$	temperature elevation, °C
$V$	relative volume, %
$Z_c$	carbohydrate extraction index
$Z_d$	pigment (dye) extraction index
$Z_i$	ionic extraction index
$Z_p$	protein extraction index

### Abbreviations

DM	dry matter
HPH	high pressure homogenization
P	HPH treatment
PSD	particle size distribution
S	US treatment
SEM	scanning electron microscopy
U	Untreated
US	Ultrasonication

### Greek symbols

$\rho$	density of suspension, kg/m <sup>3</sup>
$\sigma$	electrical conductivity (mS/cm)
$\lambda$	wavelength of absorbance

### Subscripts or superscripts

$i$	Initial
$f$	Final
$v$	violet absorbance
$r$	red absorbance

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## 1. Introduction

Nowadays, increased interest has been focused on development of emerging technologies for the total recovery of bio-molecules from marine substrates like microalgae. Microalgae have high growth rate, photosynthetic efficiency. They are rich in valuable bioactive components, such as proteins, lipids, polyunsaturated fatty acids, carbohydrates, pigments and polyphenols [1–3], that can be used in food, feed, cosmetics, pharmaceutical and biofuel industries [4–6].

The microalga *Parachlorella kessleri* (*P. kessleri*) is a unicellular freshwater organism (Chlorophyta). It has near spherical cells with diameter of 2.5-10  $\mu\text{m}$  and 60-80-nm-thick rigid cell walls [7,8]. *P. kessleri* can rapidly accumulate biomass, starch, proteins and lipids [9–11]. It was demonstrated that under unfavourable growth conditions (lack of light, nutrient stress, nitrogen starvation), the culture can accumulate large amounts of energy-rich compounds, such as triglycerides and starch [12]. However, these valuable compounds are enclosed in intracellular vacuoles and chloroplast, protected by the rigid cell walls and membranes [13], thus greatly limit their recovery during the process of extraction. From the oldest techniques (e.g. decoction and maceration) to conventional extraction techniques (e.g. Soxhlet), these techniques are often use larger volume of organic solvents or aqueous, depending on the polarity of the target compounds [14]. In addition, these methods are suffer from some disadvantages, like small scale, long extraction time and low process efficiency, and may lead to the co-extraction of undesirable components, with greater complexity in downstream separation steps [15].

The effective methods for recovery of intracellular contents from microalgae using chemical, enzymatic and different physical treatments such as ultrasonication (US), application of microwaves, pulsed electric fields, and mechanical stresses (high pressure homogenization (HPH), bead milling (BM) etc...) have been reported [13,16–19]. The efficacy of *P. kessleri* cell disintegration by US treatment was evaluated by laser light scattering methods [20]. In the US treatment, the cell walls can be damaged by the bursting of cavitation bubbles outside the cells and developments of the extreme high pressures. The effective time of sonication was dependent on the growth phase of *P. kessleri* cells. In stationary-phase, the cell walls were more resistant to the US treatment and the disruption effect was decreased with the increasing cell concentration. For the nitrogen-starved *P. kessleri* cultures, the effects of disruption by HPH and BM on the profile of fatty acids and

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lipids composition have been investigated [21,22]. The efficiency of HPH and BM can be explained by high-pressure shears, elongations, turbulences, and cavitations. For the proportion of amphiphilic free fatty acids and lysophosphatidylcholine was more marked in HPH than in BM. HPH disruption techniques applied at a pressure of 1750 bar (4 cycles) to the strain *P. kessleri* UTEX2229 allowed extraction of the 65% of the total lipids and 46% of the TAG [22]. However, the mechanical techniques are highly energy consuming and require a specific energy consumption of at least 33 kJ/g DM [23]. In addition, growing number of studies have been conducted combined methods to achieve synergy thereby increasing yield in recent years. For example, the use of US in combination with microwave irradiation enhanced oil production from *Chlorella vulgaris* was studied [24]. According to Tavanandi et al [25], combined US and freezing/thawing method obtained the highest yield (91.62%) of *C-Phycocyanin* from *Arthospira platensis*, while US (43.05%) or freezing and thawing alone (62.56%). Cho et al. [26] in their work used a combined conventional Floch method with HPH treatment (1200 psi, 35 °C), which can be easily destruct the rigid cell walls of microalgae and release the intact lipids with minimized extraction time and temperature.

The efficiency of extraction of bio-molecules from *P. kessleri* cultures can be improved with using of advanced protocols based on US and HPH treatments and their combinations. However, to the best of our knowledge, the studies on such for *P. kessleri* cultures are scarce at the present. The aim of this work was to study the impact of different individual and combined US and HPH protocols on extraction efficiencies of ionic components, proteins, carbohydrates, and pigments from microalga *P. kessleri*. The behaviors of ionics, carbohydrates, proteins and pigments recovery were investigated in dependence of extraction protocols. The microstructure of cells and suspensions was observed. The distribution functions of cells were determined. Finally, the extraction efficiency in dependence of specific energy consumption and concentration of suspension was discussed.

## **2. Materials and methods**

### **2.1 Chemicals**

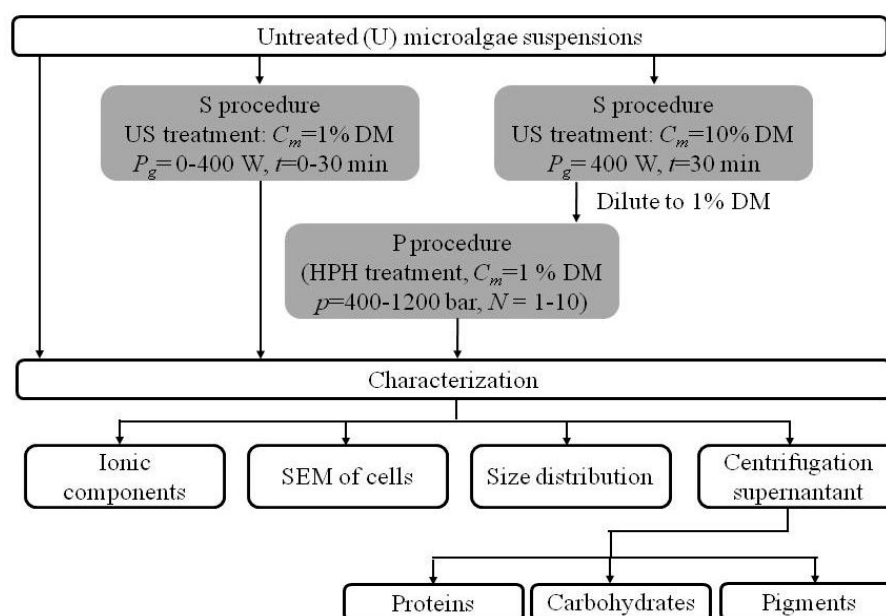
Sulfuric acid, D-glucose, phenol and bovine serum albumin (BSA) standard were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The other chemicals used were analytical grade.

### **2.2 Microalgae**

Microalga *P. kessleri* used in this study were kindly provided by AlgoSolis (Saint-Nazaire, France). They were produced in one step and batch mode in a flat panel airlift photobioreactor (PBR) (6 L) at  $25 \pm 0.5$  °C. Culture homogenization was achieved by sterile air injection at the bottom of the PBR. The pH and temperature were measured using Mettler probe (Mettler Toledo SG 3253 sensor). The value of pH 7 was adjusted with CO<sub>2</sub> bubbling, and constant light was provided by a LED array panel. The harvested culture was centrifuged and stored at -20 °C until use.

The moisture content of *P. kessleri* was  $\approx 87\%$ . The biomass pastes were first thawed at ambient temperature and then diluted with deionized water in order to prepare different suspensions with a final biomass concentration,  $C_m$ , of 1 and 10% dry matter (DM) (hereinafter %), respectively. All extractions were performed using 500 g of suspensions.

### 2.3 Extraction procedures



**Fig. 1.** Schema of applied extraction procedures for bio-molecules recovery from *P. kessleri* biomass.

**Fig. 1** presents the schema of applied extraction techniques for bio-molecules recovery from *P. kessleri* biomass. The applied procedures included the US treatment (S procedure), HPH treatment (P procedure) and US treatment followed by HPH treatment (S + P procedure). In control experiments, untreated (U procedure) suspensions were also analyzed.

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For S procedure, 1% or 10% suspensions were used. For US, the UP-400S ultrasound processor (Hielecher GmbH, Germany) was used. It was operated at a constant frequency of 24 kHz. The ultrasound probe with a diameter of 14 mm and a length of 100 mm was plunged into a beaker, containing 500 g of suspensions. The time of US,  $t$ , and generator power (declared),  $P_g$ , were varied within the ranges 0-30 min (0, 5, 10, 20, 25 and 30 min), and 0-400 W (0, 100, 200 and 400 W) respectively. The actual ultrasonic power,  $P_a$ , was estimated from the temperature elevation,  $\Delta T$ , in sample using following equation:

$$P_a = mc\Delta T/t \quad (1)$$

where  $c$  ( $\approx 4.18$  J/g K) is the specific heat capacity of suspensions. For the applied procedures, it was  $P_a \approx 27.9 \pm 0.5$  W ( $P_g = 100$  W),  $P_a \approx 76.6 \pm 0.6$  W ( $P_g = 200$  W), and  $P_a \approx 160.2 \pm 0.5$  W ( $P_g = 400$  W).

Specific energy consumption  $E$  (kJ/g DM) was calculated for S procedure as follows:

$$E = P_a t / (C_m m). \quad (2)$$

For example, for  $C_m = 1\%$  suspension and  $t = 30$  min, the values of  $E$  were  $\approx 10$  kJ/g DM ( $P_g = 100$  W),  $\approx 27.6$  kJ/g DM ( $P_g = 200$  W), and  $\approx 57.7$  kJ/g DM ( $P_g = 400$  W). Note that heating of the same suspension from 20 to 100 °C requires  $\approx 33.4$  kJ/g DM. Therefore, the suspensions were immersed in a cooling bath to avoid overheating. The maximum temperature increase during the US at 400 W for 30 min is not exceeded 45 °C. The moderate temperatures were used to avoid thermal degradation of organic compounds as well as provide an efficient application of US [27,28]. In principle, the prolonged US can cause degradation of targeted compounds. Note that no specific reaction products after sonication (5 to 55 min) applied to the isolated phenolic compounds of apple pomace were previously observed [27].

For the P procedure, 500 g of suspensions were homogenized in a NS 100L-PANDA 2K two-stage high pressure homogenizer (Niro Soavi S.p.A., Parma, Italy). The instrument operating conditions restrict the maximum concentration and the suspensions were always diluted to  $C_m = 1\%$ . The average throughput of the equipment was 10 L/h. The homogenizing pressure,  $p$ , was fixed in range of 400 to 1200 bar (1 bar =  $10^5$  Pa). The number of passes ( $N$ ) was varied from 1 to 10. The initial temperature of suspensions before P procedure was 22 °C

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and the temperature elevation after HPH treatment never exceeded 30 °C. Before each next pass through the homogenizer the suspension was cooled to 22 °C.

Specific energy consumption,  $E$ , for P procedure was estimated as follows [29]:

$$E = pN/C_m\rho \quad (3)$$

where  $p$  is the pressure of treatment (Pa),  $N$  is the number of passes, and  $\rho$  ( $\approx 10^6$  g/m<sup>3</sup>) is the density of the suspensions. For example, at  $C_m = 1\%$  suspension and  $N = 4$ , we have  $E \approx 16$  kJ/g DM (400 bar),  $E \approx 32$  kJ/g DM (800 bar), and  $E \approx 48$  kJ/g DM (1200 bar).

## 2.4 Characterization

The characterization measurements were done at the same temperature (22 °C). In this work, for maximum disintegration of suspension, the P procedure with  $p = 1500$  bar,  $N = 10$ , and  $W \approx 150$  kJ/g DM was always applied.

### 2.4.1 Ionic components

The degree of extraction of ionic components was characterized using electrical conductivity disintegration index  $Z_i$  (ionic extraction index) [30]:

$$Z_i = (\sigma - \sigma_i)/(\sigma_f - \sigma_i), \quad (4a)$$

where  $\sigma$  is the electrical conductivity of suspensions and the subscripts  $i$  and  $f$  refer to the initial and final (maximum) values, respectively. In experiments with maximally disintegrated 1% suspension, we have obtained the value of  $\sigma_f$  equal to  $0.91 \pm 0.01$  mS/cm.

The above equation gives  $Z_i = 0$  for the untreated and  $Z_i = 1$  for the maximally disintegrated suspensions.

The electrical conductivity was measured using a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany).

### 2.4.2 Microstructure of cells and suspensions

Scanning electron microscopy (SEM) images of cells were obtained at 2500 folds magnification using a QUANTA 250 FEG equipment (FEI Company, France) at 20 kV accelerating voltage. For SEM investigations, the microalgal cells were fixed with buffered aldehyde and in osmium tetroxide, then dehydrated in ethanol, drying with air dryer,

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mounted on a specimen stub, coated with carbon [31]. Optical microscopy images of suspensions were obtained at 40 folds magnification using a VisiScope light microscope (VWR, Italy). In each experiment, 18 images from three different samples were analyzed.

#### 2.4.3 Particle size disruption

The particle size distribution (PSD) was measured in the diapason 0.01-3000  $\mu\text{m}$  using laser diffraction Malvern Mastersizer 2000 instrument (Malvern, Orsay, France). Before injection into Malvern cells the suspensions were carefully stirred. The PSD was calculated using the original Malvern software.

#### 2.4.4 Analyses of supernatant

The suspensions were centrifuged using a mySPIN6 Mini Centrifuge (Thermo Fisher Scientific, China) at 6,000 rpm (2,000 g) for 10 min. The supernatants were used for further chemical analysis.

The content of water-soluble carbohydrates was determined using a phenol-sulfuric acid method [34]. D-glucose was used as a standard. The color reaction was initiated by mixing 1 mL of supernatants (diluted if required) with 0.1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid (Sigma-Aldrich, France). The reaction mixture was kept at 20 °C for 20 min. Absorbance was measured at 490 nm and concentration of carbohydrates,  $C$ , was evaluated.

The concentration of proteins,  $C_p$ , was determined by means of Bradford method [35]. The diluted supernatant (0.1 mL) was mixed with 1 mL of Bradford Dye Reagent (Thermo Fisher, Kandel, Germany) using the vortex mixer VX-200 (Labnet International, France) and kept at 22 °C for 5 min. The absorbance was measured at 595 nm by the UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). For calibration of instrument, the BSA was used.

Absorption spectra were measured in the wavelength range of 350-900 nm against the blank (with the precision of  $\pm 1$  nm) by UV/VIS spectrophotometer Spectronic Genesys 20 (Thermo Electron Corporation, MA). The content of pigments was estimated spectrophotometrically by analysis of absorbance of peaks,  $A$ , at the wavelengths of  $\lambda_v \approx 400$  nm (violet) and  $\lambda_r \approx 680$  nm (red) (Fig. S1). These peaks can be attributed to the absorbance of carotene and chlorophylls dyes [16].



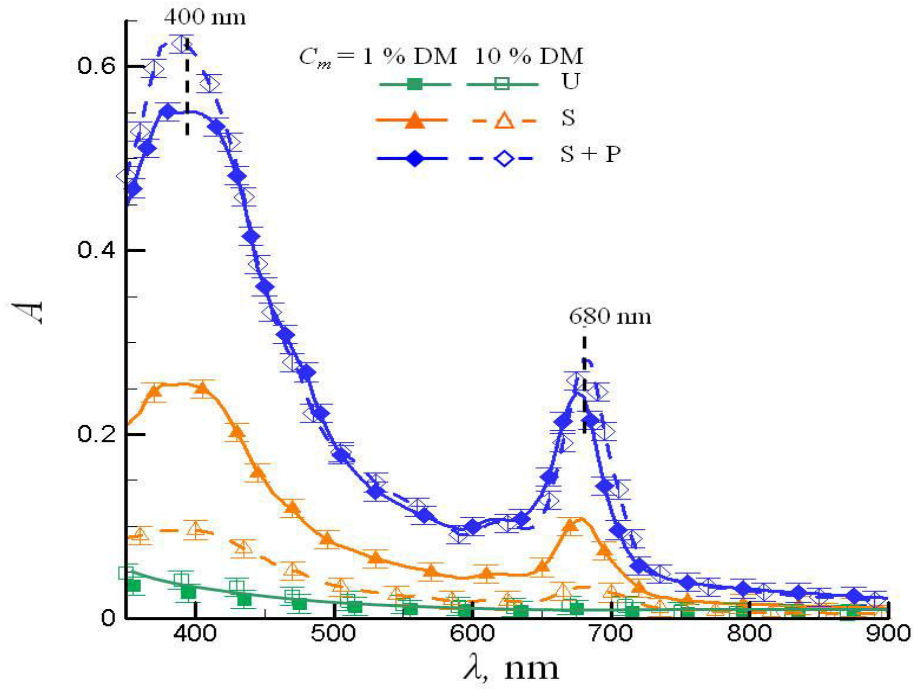


Fig. S1. Examples of the UV absorption spectra of supernatants, obtained after treatment of microalgae suspensions ( $C_m = 1, 10\%$ ) by US treatment (S procedure) (400 W, 30 min), and S + HPH treatment (P procedure) (1200 bar, 4 passes) procedures. For 10% supernatant, 1: 10 dilution was applied. Here, the peaks at  $\lambda_v = 400$  nm (violet) and  $\lambda_r = 680$  nm (red).

Based on the measured values of  $C$ ,  $C_p$  and  $A$ , the following carbohydrate, protein and pigment (dye) extraction indexes were defined:

$$Z_c = (C - C_i)/(C_f - C_i), \quad (4b)$$

$$Z_p = (C_p - C_p^i)/(C_p^f - C_p^i), \quad (4c)$$

$$Z_d = (A - A_i)/(A_f - A_i), \quad (4d)$$

where the  $i$  and  $f$  refer to the initial and final (maximum) values, respectively.

In experiments with maximally disintegrated 1% suspension (for the P procedure with  $p = 1500$  bar and  $N = 10$ ), we have obtained the following maximum values:  $C_f = 1335.5 \pm 10.6$  mg/g,  $C_p^f = 834.1 \pm 8.2$  mg/g,  $A_f = 0.777 \pm 0.01$  ( $\lambda_v = 400$  nm), and  $A_f = 0.356 \pm 0.01$  ( $\lambda_r = 680$  nm).

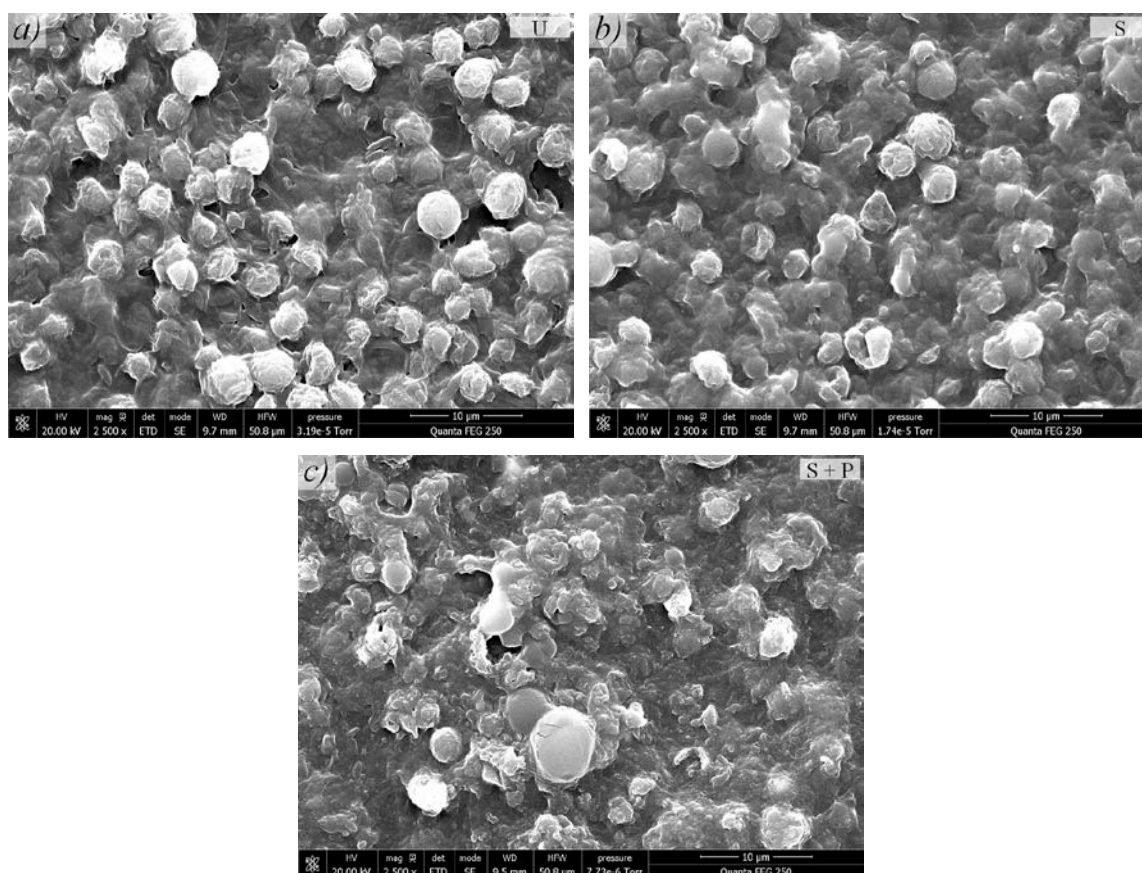
## 2.5 Statistical analysis

Each experiment was repeated at least three times. The error bars, presented on the figures, correspond to the standard deviations.

### 3. Results and discussion

#### 3.1 Cell structure and distribution of particle sizes in suspensions

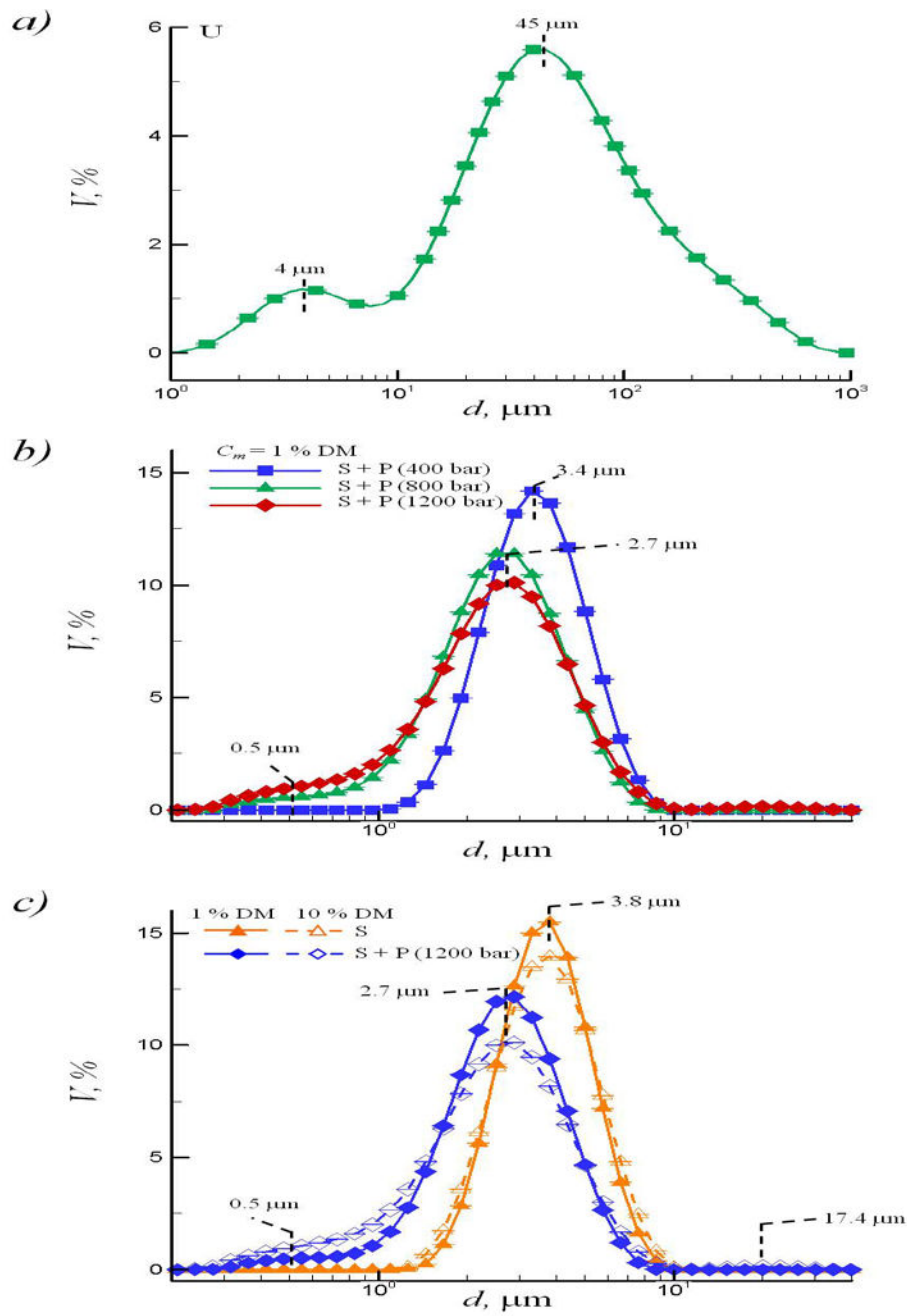
**Fig. 2** presents SEM images of untreated (U) microalgae cells (a), and cells obtained after treatment of suspensions ( $C_m = 1\%$ ) using S (400 W, 30 min) (b), and S + P (1200 bar,  $N = 4$ ) (c) procedures. The SEM images for U and S samples were rather similar.



**Fig. 2.** Scanning electron microscopy (SEM) images of untreated cells (U) (a), and the cells obtained after treatment using S procedure (400 W, 30 min) (b), and S + P procedure (1200 bar, 4 passes) (c) procedures. All test concentration of cells was 1 % dry matter.

The small interspaces and holes were observed and some of cells were damaged. For the U samples, it can reflect effects of harvesting (**Fig. 2a**). For the S samples, shear stress released externally by cavitations during US treatment can introduce interspaces, holes and micro-fractures in cells and produce shrinkage (**Fig. 2b**). However, these effects were not

clearly visible in SEM images. For S + P samples, most of cells were damaged and the cell walls almost completely lost their integrity (Fig. 2c).



**Fig. 3.** Particle size distributions (PSD) of untreated suspensions (U) (a), and the suspensions obtained after treatment using S procedure (400 W, 30 min, 1% dry matter) + P procedure (400-1200 bar,  $N = 4$ , 1% dry matter) (b), and S (400 W, 30 min) + P (1200 bar,  $N = 4$ ) procedures (S procedure was applied at 1% and 10% suspensions, respectively; P procedure was applied at 1% suspensions) (c).

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The effects of U, S and S + P procedures on the microstructure of suspensions were elucidated using the data on the PSD (**Fig. 3**). The PSD revealed the presence of a bimodal distribution of untreated (U) cells with the small and large peaks located at  $\approx 4 \mu\text{m}$  and  $\approx 45 \mu\text{m}$ , respectively (**Fig. 3a**). We assume that the smaller peak corresponds to the individual cells of *P. kessleri*, whereas larger peak corresponds to the agglomerated cells forming clusters. The bimodal distributions that reflect agglomeration were also earlier observed for microalgae *Nannochloris oculata* [32] and yeast cells [33]. As was previously conjectured the aggregates can represent microalgae flocks formed by the harvesting mechanism used (flocculation) [32].

**Fig. 3b** presents data on the PSD for the 1% suspension treated using S (400 W, 30 min) + P (400, 800, and 1200 bar,  $N=4$ ) procedure. For S + P (400 bar) sample, a single peak with median diameter located at  $\approx 3.4 \pm 0.1 \mu\text{m}$  was observed. Therefore, it can be concluded that such treatment procedure allows complete disaggregation of the agglomerated cells in untreated suspension (**Fig. 3a**). For S + P (800 bar) sample, a single peak with median diameter located at  $\approx 2.7 \pm 0.1 \mu\text{m}$  was observed and the further increase of  $p$  (S + P (1200 bar) sample) resulted in appearance the noticeable increase of the content of cell debris with size  $\leq 1 \mu\text{m}$ .

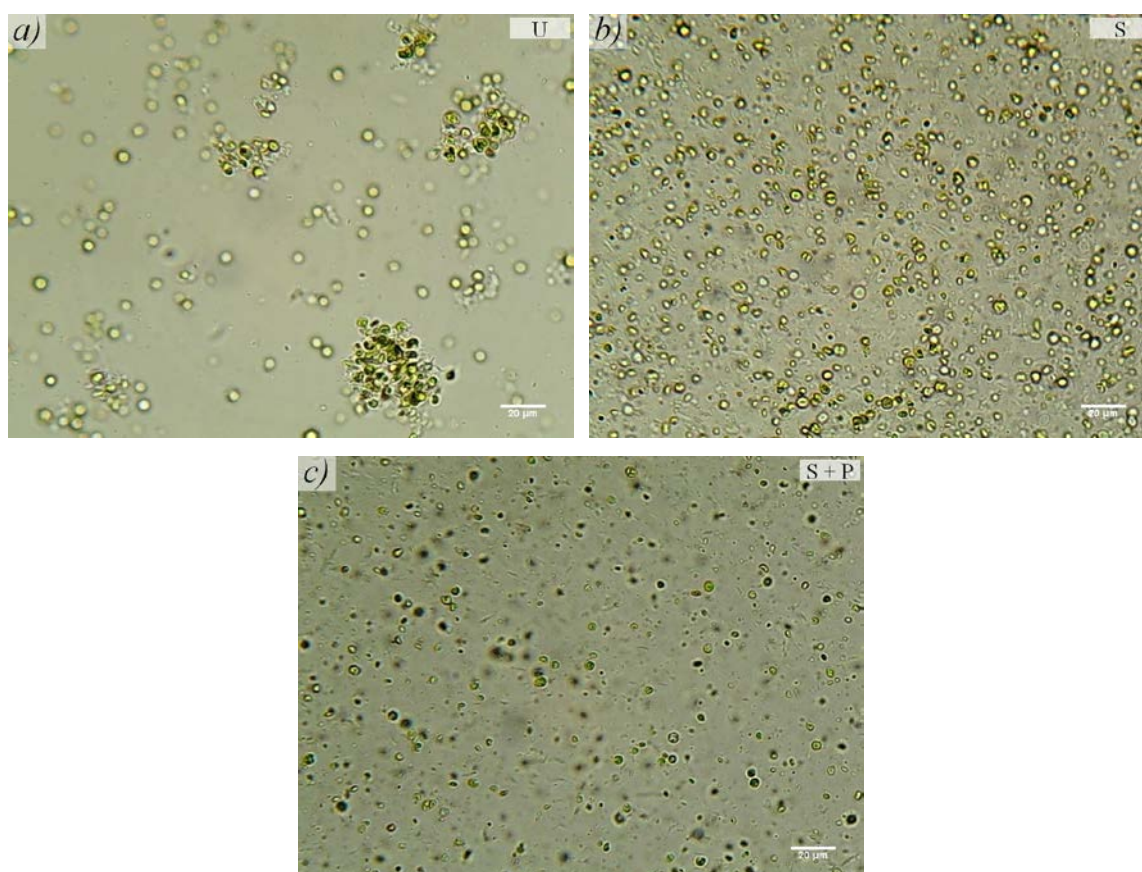
**Fig. 3c** compares data on the PSD for 1% and 10% suspensions treated using S (400 W, 30 min) and S (400 W, 30 min) + P (1200 bar,  $N=4$ ) procedures. Note that for individual US (S samples) for both 1% and 10%, the single peaks with median diameters located at  $\approx 3.8 \pm 0.1 \mu\text{m}$  were observed. These values are smaller than value of  $4.0 \pm 0.1 \mu\text{m}$  for intact cell, that can reflect effects of US on the structure of cell walls. Increase of suspension concentration resulted in decrease of peak height and minor broadening of peak.

For combined procedure (S + P samples), the PSD revealed the presence of the multimodal distributions with peaks located at  $\approx 0.5$ ,  $2.7$  and  $17.4 \mu\text{m}$  that correspond to the formation of cell debris, damaged cells, and conglomerates of cell, respectively. The observed re-aggregation of cells can be result of cell-cell walls adhesion at high pressures during the HPH treatment. Note that for the S and S + P samples, the observed effects were more prominent for more concentrated suspensions (**Fig. 3c**).

**Fig. 4** compares optical microscopy images of untreated (U) suspensions (a), and suspensions after treatment using S (400 W, 30 min) (b), and S + P (1200 bar,  $N=4$ ) (c)



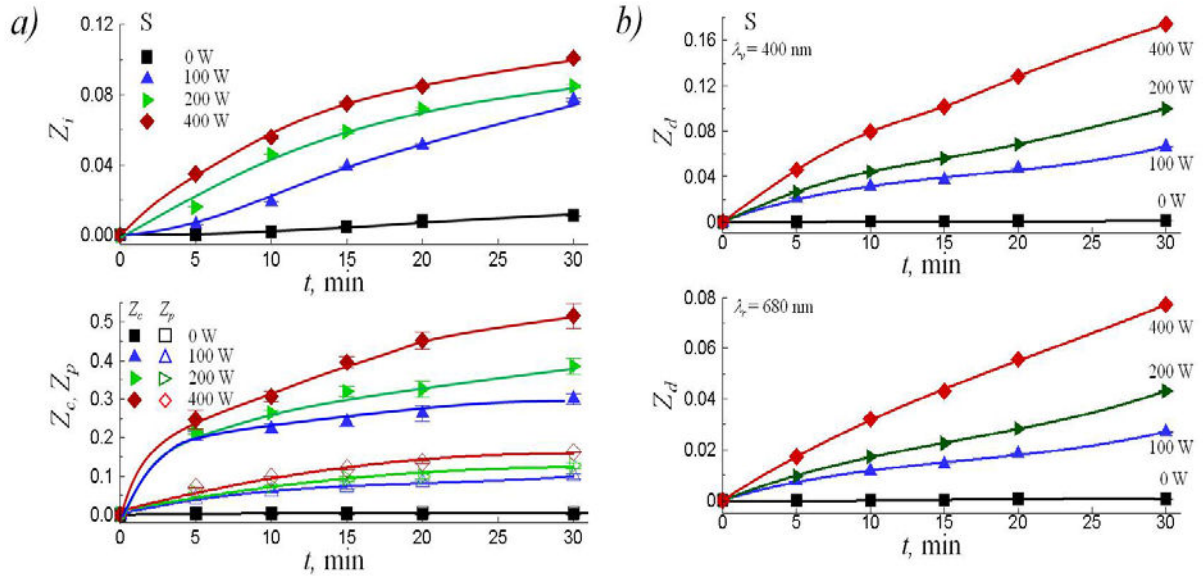
procedures. The concentration of suspensions was 1%. The obtained images supported data of PSD on presence of agglomerated cells in the untreated suspension (**Fig. 4a**) and their complete disaggregation after application of S procedure (**Fig. 4b**). The application of the combined S + P procedure resulted in appearance of cell debris, small aggregates, and some most resistant cells remained undamaged (**Fig. 4c**).



**Fig. 4.** Optical microscopy images of untreated suspensions (U) (a), and the suspensions obtained after treatment using S procedure (400 W, 30 min) (b), and S + P procedure (1200 bar,  $N = 4$ ) (c) procedures. All test concentration of suspensions was 1 % dry matter.

### 3.2 Extraction of bio-molecules

**Fig. 5** shows changes of ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , (a), and pigment,  $Z_d$ , (b) extraction indexes in the course of the US treatment (S procedure) at different applied US powers (0-400 W). The values of all extraction indexes increased with increasing of US power and extraction time,  $t$ , and the maximum degrees of extraction were obtained by using the highest power and the longest applied extraction time,  $t = 30$  min.



**Fig. 5.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ), protein ( $Z_p$ ) (a), and pigment ( $Z_d$ ) (b) extraction indexes versus the time ( $t$ ), for US treatment at different powers (0-400 W). All test concentration of suspensions was 1 % dry matter and the value of  $Z_d$  was measured at two different wavelength  $\lambda_v = 400$  nm (violet) and  $\lambda_r = 680$  nm (red).

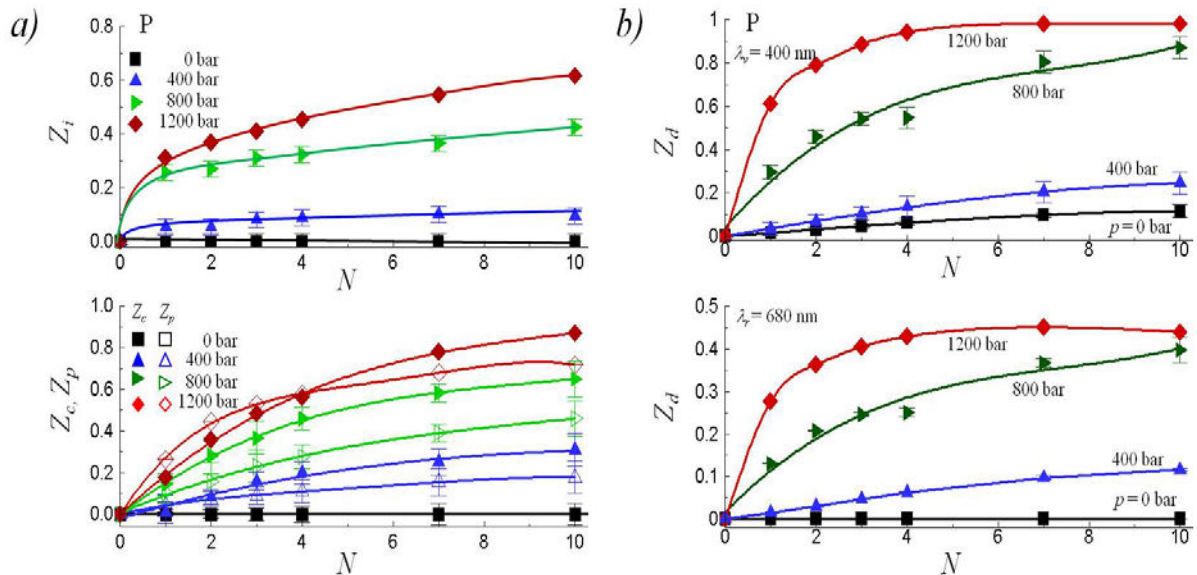
The obtained data evidenced that even at  $t = 30$  min all the measured parameters were still far from the saturated values. The time changes of  $Z_d$  at wavelength of  $\lambda_v = 400$  nm and  $\lambda_r = 680$  nm were rather similar, but they were more pronounced at  $\lambda_v = 400$  nm (**Fig. 5b**). Note that extracted dyes (carotene and chlorophylls) are practically insoluble in water. These changes can reflect increase of concentration of soluble in water dye binding molecules that support the presence of dyes in water. It is known that stabilization of dye in water can be supported, for example, dye-macromolecular water-soluble complexes [36].

Extraction for 30 min at 400 W resulted in  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$  (**Fig. 5a**). The obtained values can be arranged in the following row:

$$Z_i < Z_p < Z_c, \quad (5)$$

The data evidenced the highest efficiency for carbohydrates and smallest efficiency for extraction of ionic components. It can be explained by release of a certain amount of extracellular polysaccharides present in the cell walls of microalgae [37,38].

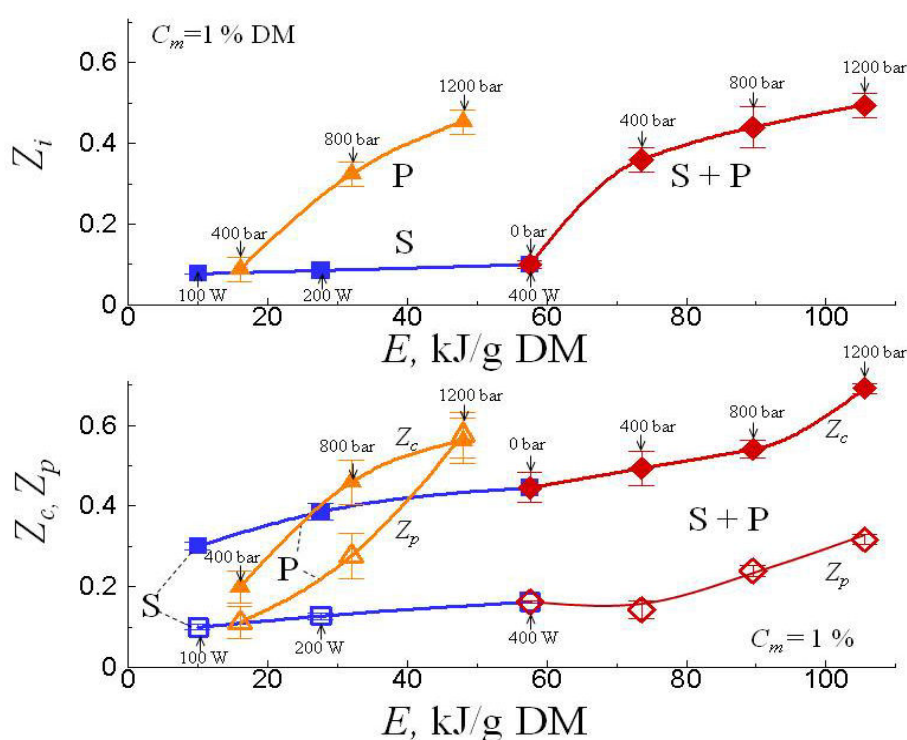
The estimated indexes for pigments are  $Z_d \approx 0.174 (\lambda_v)$ , and  $Z_d \approx 0.077 (\lambda_r)$  and their ratio  $r = Z_d(\lambda_v)/Z_d(\lambda_r)$  was  $\approx 2.2$ . Note that value of  $Z_d(\lambda_v)$  was comparable with value of  $Z_p$ . Therefore, we can speculate that stabilization of dye in water reflects release of water-soluble proteins.



**Fig. 6.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ), protein ( $Z_p$ ) (a), and pigment ( $Z_d$ ) (b) extraction indexes versus the number of passes ( $N$ ) in the course of the P procedure at different applied pressures (400-1200 bar). All test concentration of suspensions was 1 % dry matter, and the value of  $Z_d$  was measured at two different wavelength  $\lambda_v = 400$  nm (violet) and  $\lambda_r = 680$  nm (red).

**Fig. 6** shows changes of ionic,  $Z_i$ , carbohydrate,  $Z_c$ , protein,  $Z_p$ , (a), and pigment,  $Z_d$ , (b) extraction indexes for P procedure at different applied pressures (400, 800, and 1200 bar). The maximum degrees of extraction were obtained by using the highest values of  $p$  and  $N$ . Extraction for  $N = 10$  at  $p = 1200$  bar resulted in  $Z_i \approx 0.62$ ,  $Z_c \approx 0.87$ ,  $Z_p \approx 0.71$  (**Fig. 6a**). Note that for P procedure, the obtained values can be arranged in the same rows as for S procedure (Eq. 5). The estimated indexes for pigments are  $Z_d \approx 0.99 (\lambda_v)$ , and  $Z_d = 0.45 (\lambda_r)$  and their ratio  $r = Z_d(\lambda_v)/Z_d(\lambda_r)$  was also  $\approx 2.2$ , the same as for S procedure. Note that application of first passes ( $N \leq 4$ ) allowed obtaining the noticeable degree of extraction and further passes resulted in insignificant supplementary effects at high specific energy consumption. Therefore in further experiments the protocols with  $N = 4$  were applied.

The obtained data evidenced that both individual S and P procedures allow increase of extraction of bio-molecules with efficiencies arranged in the row represented by Eq. (5). However, on the one hand, individual S procedure is not very efficient for damage of cells and it results in moderate extraction yields. On the other hand, the individual P procedure can lead to the intensive generation of cell debris's and degradation of bio-molecules with increased energy consumption and elevation of temperature [37]. Application of procedures that combines the important qualities of US and HPH treatment can be attractive in terms of extraction yield, selectivity and energy consumption.



**Fig. 7.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ) and protein ( $Z_p$ ) extraction indexes versus the specific energy consumption ( $E$ ), for treatment using individual S procedure (100-400 W, 30 min), P procedure (400-1200 bar,  $N = 4$ ) and combined S (400 W, 30 min) + P (400-1200 bar,  $N = 4$ ) procedures. All test concentration of suspension was 1 % dry matter.

**Fig. 7** compares the ionic,  $Z_i$ , carbohydrate,  $Z_c$ , and protein,  $Z_p$ , extraction indexes versus the specific energy consumption,  $E$ , using S, P and S + P procedures for diluted suspensions with  $C_m = 1\%$ . The time of US was  $t = 30$  min, and number of passes of HPH was  $N = 4$ . Efficiency of ionics and proteins extraction is higher for HPH (P procedure) than for US (S procedure) treatment at the same energy consumption. However, for the



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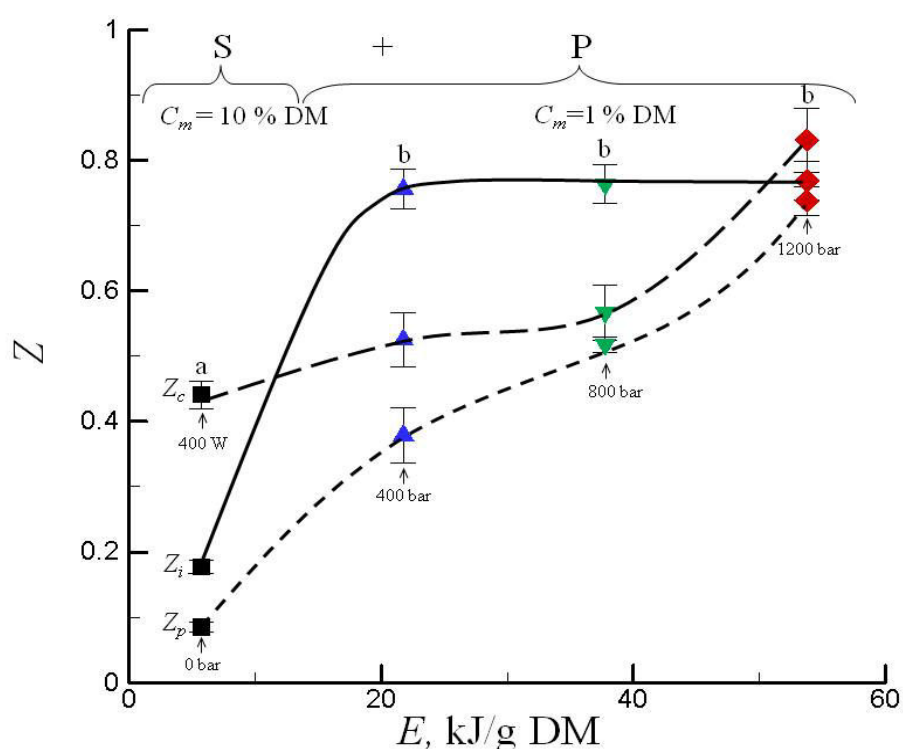
carbohydrates, the effect depends upon energy. For example, for the same energy consumption of  $E = 15$  kJ/g DM, the carbohydrates extraction with US was higher ( $Z_c = 0.3$ ) than with HPH ( $Z_c = 0.2$ ) (**Fig. 7**). The extraction of bio-molecules for combined S + P procedure can display synergetic behaviour. For example, individual S (400 W) and P (400 bar) procedures were ineffective for extraction of ionic components, and gave  $Z_i \leq 0.10$ . However, combined S (400 W) + P (400 bar) procedure gave  $Z_i \approx 0.37$  (**Fig. 7**). The similar behaviour was also observed for combined S (400 W) + P (800, 1200 bar) procedures.

For the extraction of carbohydrates, the combined S + P procedure was less effective. For example, the individual S (400 W, 56 kJ/g DM) and P (400 bar, 16 kJ/g DM) procedures for extraction of carbohydrate gave  $Z_c \approx 0.45$  and  $Z_c \approx 0.20$ , respectively, whereas the combined S + P procedure (400 W, 400 bar, 73.5 kJ/g DM) gave  $Z_c \approx 0.49$ . However, the combined S + P procedure at higher pressure (400 W, 1200 bar, 105.6 kJ/g DM) gave  $Z_c \approx 0.69$ .

Moreover, the extraction of proteins for combined S + P procedure was ineffective as compare with individual P (800, 1200 bar,  $N = 4$ ) procedure. For example, extraction of proteins using P (1200 bar,  $N = 4$ ) procedure gave  $Z_p \approx 0.57$  at  $E \approx 48$  kJ/g DM (**Fig. 6a**), and extraction using S (400 W) + P (1200 bar) gave  $Z_p \approx 0.32$  at  $E \approx 106$  kJ/g DM (**Fig. 7**). Possibly it reflected a degradation of proteins or their irreversible binding to the cell wall provoked by US. Therefore, application of individual S, P or combined S + P procedure requires thorough adaptation of extraction protocol accounting for the selectivity of extraction, purity of extract and energy consumptions.

Note, that considerable specific energy consumptions were obtained for diluted suspensions ( $C_m = 1\%$ ). For concentrated suspensions, the extraction can be more effective in terms of energy consumption per g DM. **Fig. 8** compares extraction behaviour with application of combined S + P procedure. In these experiments, the concentration was  $C_m = 10\%$  in the preliminary S ( $W = 400$  W,  $t = 30$  min,  $E \approx 5.6$  kJ/g DM) procedure and it was  $C_m = 1\%$  in the final P ( $p = 400, 800, 1200$  bar,  $N = 4$ ) procedure. After the S procedure the extraction indexes were  $Z_i \approx 0.18$ ,  $Z_c \approx 0.44$ ,  $Z_p \approx 0.09$ . Note that for concentration of  $C_m = 1\%$ , the similar preliminary S ( $W = 400$  W,  $t = 30$  min,  $E \approx 56$  kJ/g DM) procedure gave  $Z_i \approx 0.10$ ,  $Z_c \approx 0.45$ ,  $Z_p \approx 0.16$ . Application of the final P procedure at  $p = 400$  bar ( $E \approx 21.8$  kJ/g DM) increased the level of  $Z_i$  up to  $\approx 0.76$  ( $p < 0.05$ ) and no further increase in  $Z_i$  was observed

with increase of  $p$  or  $E$  (**Fig. 8**). However, for carbohydrates and proteins the extraction indexes  $Z_c$  and  $Z_p$  continuously increased with increase of  $p$  or  $E$ . Finally at the pressure of 1200 bar ( $E \approx 53.8$  kJ/g DM), they reached values of  $Z_c \approx 0.83$  and  $Z_p \approx 0.74$ . These values can be compared with maximum values of extraction indexes  $Z_i \approx 0.49$ ,  $Z_c \approx 0.69$ , and  $Z_p \approx 0.32$  obtained using S (1%, 400 W, 30 min) + P (1%, 1200 bar, 4 passes) procedure ( $\approx 106$  kJ/g DM). Therefore, the preliminary sonication of more concentrated suspensions allowed increasing the extraction efficiency and decreasing the energy consumptions.



**Fig. 8.** Ionic ( $Z_i$ ), carbohydrate ( $Z_c$ ) and protein ( $Z_p$ ) extraction indexes versus the specific energy consumption ( $E$ ), using individual S procedure (400 W, 30 min, 10 % dry matter), and combined S (400 W, 30 min, 10 % dry matter) + P procedure (400-1200 bar,  $N = 4$ , 1 % dry matter) procedures.

#### 4. Conclusions

Application of individual S, P or combined S + P procedure requires thorough adaptation of extraction protocols accounting for the required selectivity of extraction, purity of extracts and energy consumptions. The S procedure allowed disaggregation of cells and it can affect the structure of cell walls. The P procedure was always applied to the 1%

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suspension and it has also supplementary effects on damage of cells. This procedure can produce impurities, cell debris and provoke re-aggregation of cells. The concentration of the treated suspensions is also important. For diluted suspension ( $C_m = 1\%$ ), the application of individual S or P procedure allowed extraction of components that can be arranged in the row  $Z_i < Z_p < Z_c$ . For combined S + P procedure, the synergetic behaviour was observed for extraction of ionic components, and absent for extraction of carbohydrates. Moreover, it was negative for extraction of proteins. It can reflect formation of cell wall protein complexes induced by changes cell walls during preliminary sonication. Obtained data also allowed speculation that stabilization of dyes in water can reflect release of water-soluble proteins. The preliminary sonication of more concentrated suspensions (10 %) followed by HPH of 1% suspensions allowed increasing the extraction efficiency and decreasing the energy consumptions.

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### **Declaration of contributions**

All authors have worked in the conception and design of the study. RZ performed experiments, preliminary analyzes of the results. NG designed the protocol and supervised the work. LM has provided the studied materials and discussed the results. RZ, NG, NL, EV have realized the interpretation of data, drafted and the revised the manuscript.

### **Conflict of interest statement**

We declare that this manuscript has not any potential financial or other interests that could be perceived to influence the outcomes of the research.

### **Statement of informed consent, human/animal rights**

No conflicts, informed consent, human or animal rights applicable

### **Declaration of authors**

All authors have approved the manuscript and agree with peer review process and its submission to Algal Research

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IV.3 *Article 4*: Effect of combined pulsed electric energy and high pressure homogenization on selective and energy efficient extraction of bio-molecules from microalga *Parachlorella kessleri*

(The article is presented on the following pages)

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**Effect of combined pulsed electric energy and high pressure  
homogenization on selective and energy efficient extraction of bio-molecules  
from microalga *Parachlorella kessleri***

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## Abstract

This work investigates the potential of pulsed electric energy (E procedure; pulsed electric fields (PEF) and high voltage electrical discharges (HVED)) and high pressure homogenization (P procedure) for extraction of bio-molecules from *Parachlorella kessleri*. The applied procedures included E, P, and their combination (E + P). The cell concentrations of suspension applied in E procedure were 0.5-2.5% dry matter, while the 0.5 % suspension was always applied for P procedure. The effects of applied procedures on the extraction of ionics, carbohydrates, proteins, and pigments were evaluated. The data evidenced that the E procedure allowed selective extraction of ionics and carbohydrates. However, the P procedure was most efficient for simultaneous release all the bio-molecules. The P procedure (1200 bar, 10 passes) gave 4-fold higher content for pigments, 1.2-fold higher content for carbohydrates and 6.5-fold higher content for proteins than E procedure (HVED, 40 kV/cm, 8 ms). For combined procedure, the application of preliminary E procedure with 0.5% suspension allowed increasing the extraction of carbohydrates at high energy consumption. By contrast, the application of preliminary E procedure with 1.5% and 2.5% suspensions allowed enhancing extraction efficiencies of carbohydrates and proteins, and reducing total energy consumption.

**Keywords:** Microalgae; Pulsed electric energy; High pressure homogenization; Selective extraction; Bio-molecules

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## 1. Introduction

Microalgae have been considered as a renewable feedstock for the food, feed and biofuel industries due to their rich composition, superior areal productivities compared to traditional crops and no dependence on fresh water and arable land (Garcia et al., 2018). They can also rapidly accumulate biomass and produce desired bio-molecules by change culture growth conditions (like light, nutrient stress and nitrogen starvation, etc) and control their metabolism (Li et al., 2013).

The green microalga *Parachlorella kessleri* (*P. kessleri*) is a unicellular freshwater organism (Chlorophyta) with a 2.5-10  $\mu\text{m}$  mean diameter. Their cell wall was electron-transparent ( $\approx$  60-80 nm). However, these interesting bio-molecules are commonly located either inside the cell cytoplasm or are bound to cell membrane and require disintegration before extraction (Garcia et al., 2018). Some efforts have been done to break the microalgal cell wall by chemical hydrolysis (Sedighi et al., 2019), high pressure disruption (Bernaerts et al., 2019), ultrasound (Zhang et al., 2019), microwave (Chew et al., 2019), or bead milling (Garcia et al., 2019), etc. However, these technologies refer to the use of severe processing conditions that negatively affect the quality and purity of the extracts and complicating downstream purification processes (Martinez et al., 2017). For example, mechanical disruption was considered as highly energy inefficient, when they conducted under laboratory conditions and required a specific energy consumption of at least 33 MJ/kg dry biomass (Lee, Lewis, & Ashman, 2012). These limitations have inspired numerous investigations of alternative methods for recovery of bio-molecules from microalgae.

Recently, increasing interest in the use of pulsed electric fields (PEF) to improve the extraction efficiencies of algal bio-molecules (Jaeschke et al., 2019; Juan Manuel Martinez et al., 2019; Parniakov et al., 2015a). PEF treatment can cause the increment of cell membrane permeability (electroporation) by applying high-intensity electric field pulses of short duration (from  $\mu\text{s}$  to ms) (Puertolas & Barba, 2016). However, some research groups have also found that PEF was less effective for extraction of proteins. More efficient extraction of proteins from microalgae required more powerful disintegration of the cell walls, which can be provided by high voltage electrical discharges (HVED) (Grimi et al., 2014). HVED treatment combines electrical and mechanical effects for cell permeabilisation, can cause cell structure damage and accelerate the extraction efficiencies of bio-molecules.

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However, to the best of our knowledge, the implementation of energy efficient cell disintegration and selective extraction of microalgal bio-molecules remains largely unexplored. This study investigated the effect of individual pulsed electric energy (PEF, HVED) and HPH treatments, and their combination on the selective extraction of bio-molecules from *P. kessleri*. The dependence of recovery behaviors of ionics, pigments, carbohydrates and proteins on the different extraction procedures was discussed. The distribution functions of microalgal cells were also observed. Finally, the extraction efficiency in dependence of specific energy consumption and concentration of suspension were evaluated.

## 2. Materials and methods

### 2.1 Chemicals

D-glucose and bovine serum albumin (BSA) standard were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Bradford Dye Reagent was purchased from Thermo Fisher (Kandel, Germany). Other chemicals of analytical grade were obtained from VWR (France).

### 2.2 Microalgae

Microalga *P. kessleri* was provided by AlgoSolis, Saint-Nazaire, France. The microalga was obtained as a frozen paste with  $\approx 82\%$  of moisture content. The composition of biomass was  $\approx 44\%$  (w/w dry matter biomass) of proteins,  $\approx 35\%$  of carbohydrates, and  $\approx 3.8\%$  of total lipids. The pastes were first thawed at ambient temperature, and were washed 3 times by deionized water.

### 2.3 Extraction procedures

The applied extraction procedures included pulsed electric energy (PEE) treatments (E procedure: PEF/HVED treatment), HPH treatment (P procedure), and PEE followed by HPH treatment (E + P procedure). The untreated (U procedure) suspension was also analyzed as the control experiment.

For the individual E or P procedure, the suspension with the biomass concentration,  $C_m$ , of 0.5% dry matter (hereinafter %) was always used (i.e.  $C_m = 0.5\%$ ). The combined E + P procedure involved using different concentrations of suspension in preliminary E procedure as follows:

- i) PEF ( $C_m = 0.5\%$ ) + HPH ( $C_m = 0.5\%$ );
- ii) HVED ( $C_m = 0.5\%$ ) + HPH ( $C_m = 0.5\%$ );
- iii) HVED ( $C_m = 1.5\%$  or  $2.5\%$ ) + HPH ( $C_m = 0.5\%$ );

For all the applied E + P procedure, a centrifugation step was carried out after the E procedure. Then the residue was re-suspended to 0.5% suspension by deionized water, and then subjected to P procedure.

### 2.3.1. Pulsed electric energy (PEE) treatments

The PEE treatments (E procedure) were made in PEF and HVED modes using high voltage pulsed power 40 kV-10 kA generator (Basis, Saint-Quentin, France). PEF treatment was performed in a batch one-liter cylindrical treatment chamber between two plate electrodes. The distance between the electrodes was fixed at 2 cm to produce a high PEF intensity of 20 kV/cm. HVED treatment was performed in the same treatment chamber with a needle-plate geometry of electrode. The distance between the stainless steel needle and the grounded plate electrode was fixed at 1 cm and the corresponding electric field strength of 40 kV/cm. The suspension with a total mass of 250 g was introduced between the electrodes. The generator provided pulses of an exponential form with a pulse repetition rate of 0.5 Hz (2 s between pulses:  $\Delta t = 2$  s). Treatments comprised application of  $n$  successive pulses. The total treatment duration of PEE,  $t_e$ , was varied within 0.01–8 ms ( $n = 1$ –800). Note that for extraction time of PEE was calculated as  $t = n \times \Delta t$ . Disrupted microalgal suspension characteristics were measured between successive discharges or pulses. The temperature was maintained approximately at ambient temperature, and elevation of temperature not exceeded 5 °C.

Specific energy consumption,  $W$  (J/kg dry matter), was calculated for E procedure using the following formula (Yu, Gouyo, Grimi, Bals, & Vorobiev, 2016):

$$W = (n \times P_i)/m \quad (1)$$

where  $m$  is the mass of the dry weight of biomass in suspension (kg), and  $P_i$  refers to the energy consumption of one electric pulse or discharge calculated from the following formula:

$$P_i = (C \times U^2)/2 \quad (2)$$

where  $C$  is the capacity of the capacitor, and  $U$  is the voltage of the generator. In this study,  $P_i = 220$  J was applied for all the PEE treatments.

### 2.3.2. High pressure homogenization (HPH) treatment

The HPH treatment (P procedure) was done using a two-stage high pressure homogenizer (Niro Soavi S.p.A., Parma, Italy). The average throughput of the homogenizer was 10 L/h. The homogenizing pressure,  $p$ , and number of passes,  $N$ , were varied within the ranges 400-1200 bar (1 bar =  $10^5$  Pa), and 1-10 passes, respectively. The suspensions with a total mass of 250 g were processed. The initial temperature of suspensions before P procedure was approximately at ambient temperature and the cooling system to maintain the temperature elevation after P procedure never exceeded 5 °C.

Specific energy consumption,  $W$  (J/kg dry matter), was calculated for P procedure using the following formula (Anand, Balasundaram, Pandit, & Harrison, 2007):

$$W = pN/C_m\rho \quad (3)$$

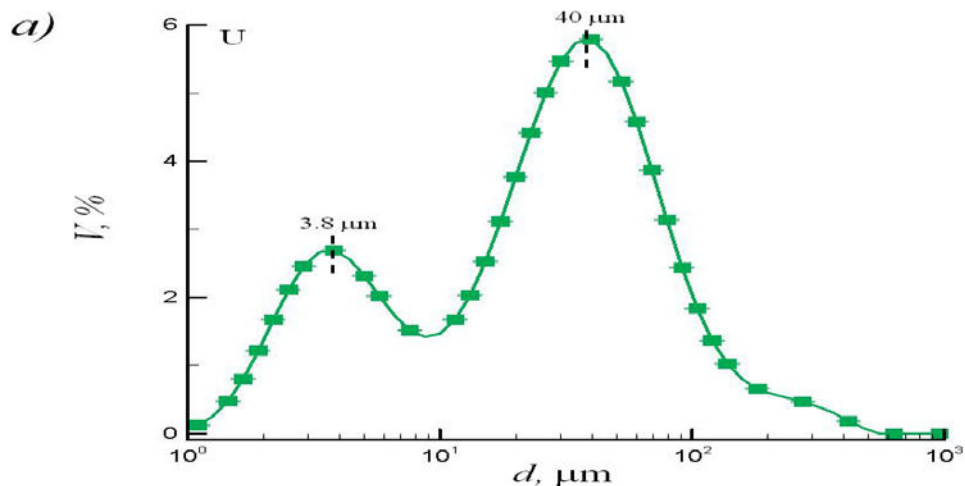
where  $\rho$  ( $\approx 10^6$  g/m<sup>3</sup>) is the density of the suspension.

## 2.4 Characterization

All the characterization measurements were done at room temperature.

### 2.4.1 Particle size disruption

The particle size distribution (PSD) of cells was measured in the range from 0.01 to 3000  $\mu\text{m}$  by Malvern Mastersizer 2000 (Orsay, France). The calculated median meter ( $d_{(50)}$ ) was used to monitor the PSD of cells (See, Supplementary materials Fig. S1).





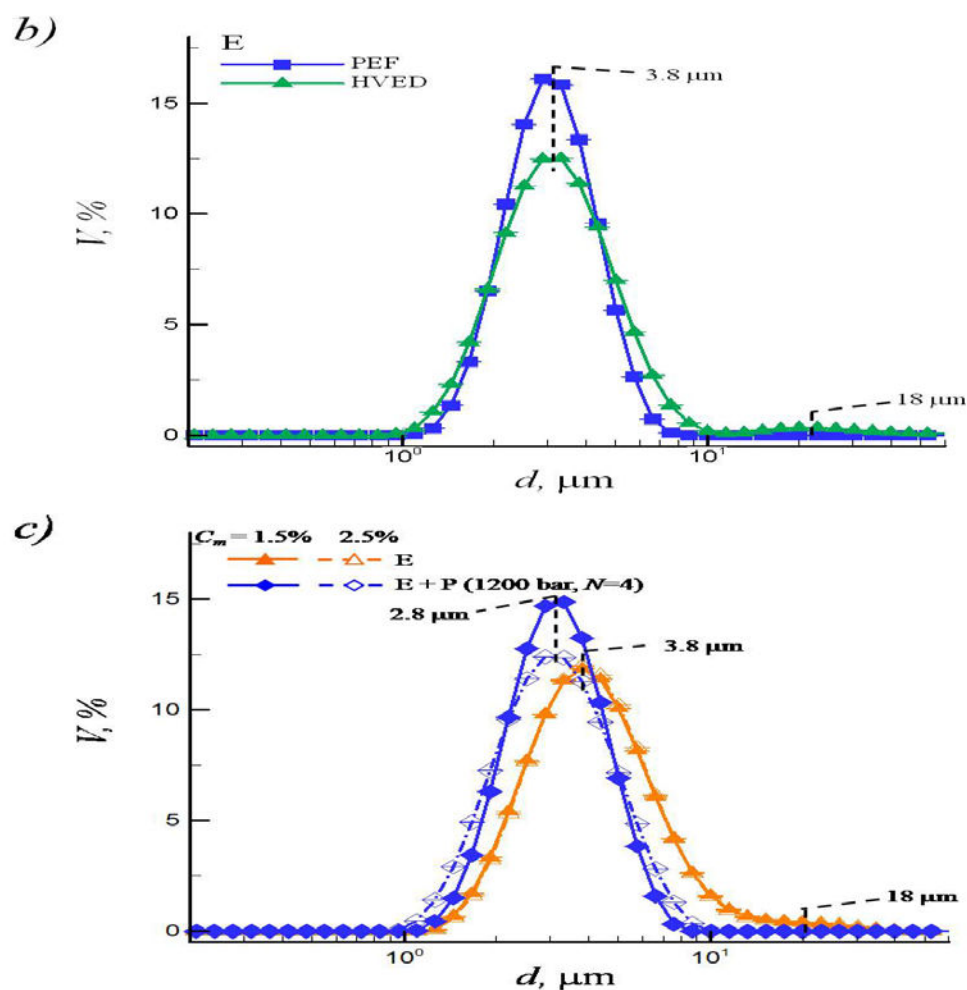


Fig. S1. Particle size distributions (PSD) of untreated (U) suspensions (a), and the suspensions treated by PEE treatments (E procedure, 0.5% dry matter, 8 ms) (b), and combined E (HVED treatment, 1.5% or 2.5% dry matter, 6 ms) + P (HPH treatment, 0.5% dry matter, 1200 bar, 4 passes) procedures (c).

#### 2.4.2 Ionic components

The electrical conductivity of suspensions was measured using a hand-held Conductivity/TDS meter (Thermo Fisher Scientific, France).

#### 2.4.3 Analyses of supernatant

The suspensions were centrifuged using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France) at 14,100 g for 10 min. The supernatants were collected for characterization analysis.

Absorption spectra were measured by a UV/VIS spectrophotometer (Thermo Electron Corporation, MA) within 350-900 nm against the blank (with the precision of  $\pm 1$  nm). The

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pigments content was evaluated by analysis of the absorbance of peaks,  $A$ , at the wavelength of  $\lambda \approx 680$  nm. This peak can be attributed to the absorbance of chlorophylls (Parniakov et al., 2015a).

The carbohydrates content,  $C_c$ , was measured using a phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The proteins content,  $C_p$ , was measured using the method of Bradford (Bradford, 1976).

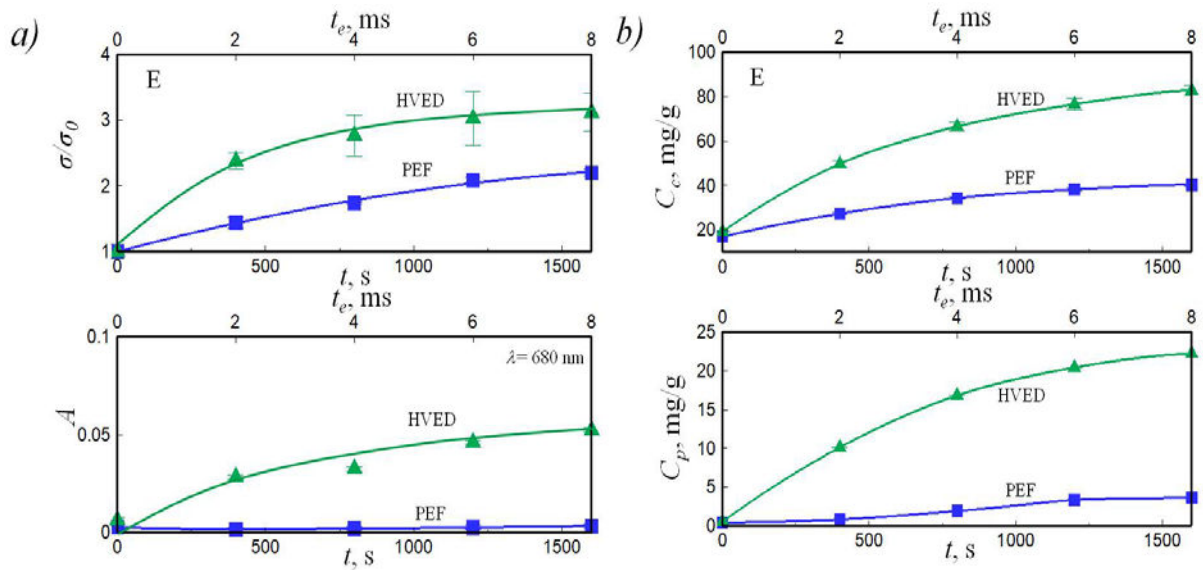
### 2.5 Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean  $\pm$  standard deviation. The error bars, presented on the figures, correspond to the standard deviations.

## 3. Results and discussion

### 3.1 Extraction of bio-molecules

**Fig. 1** presents the changes of relative electrical conductivity,  $\sigma/\sigma_0$ , and absorbance of pigments,  $A$ , (a), and the contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , (b), in the course of individual E procedure. The data were compared between PEF and HVED treatments. The application of PEF can significantly increase the relative electrical conductivity and the carbohydrates content as compared to the U samples ( $t_e = 0$  ms). The maximum values of  $\sigma/\sigma_0 \approx 2.2$  and  $C_c \approx 40.4$  mg/g were obtained by using the longest electrical treatment duration,  $t_e = 8$  ms. However, PEF treatment at  $t_e = 8$  ms only gave  $A \approx 0.003$ , and  $C_p \approx 4$  mg/g, respectively. The data evidenced that the PEF treatment was ineffective for extraction of pigments, and less effective for extraction of proteins. This possibly reflects that PEF treatment was opening pores on cell membranes, allowing release small-sized cytoplasmic proteins, but most proteins are larger and more bonded to the cell structure (Carullo et al., 2018). Moreover, some other studies claimed to obtain higher amounts of proteins and pigments from *Nannochloropsis* sp., but PEF treatment used in their studies was assisted with organic solvent extraction (Parniakov et al., 2015b, 2015a).

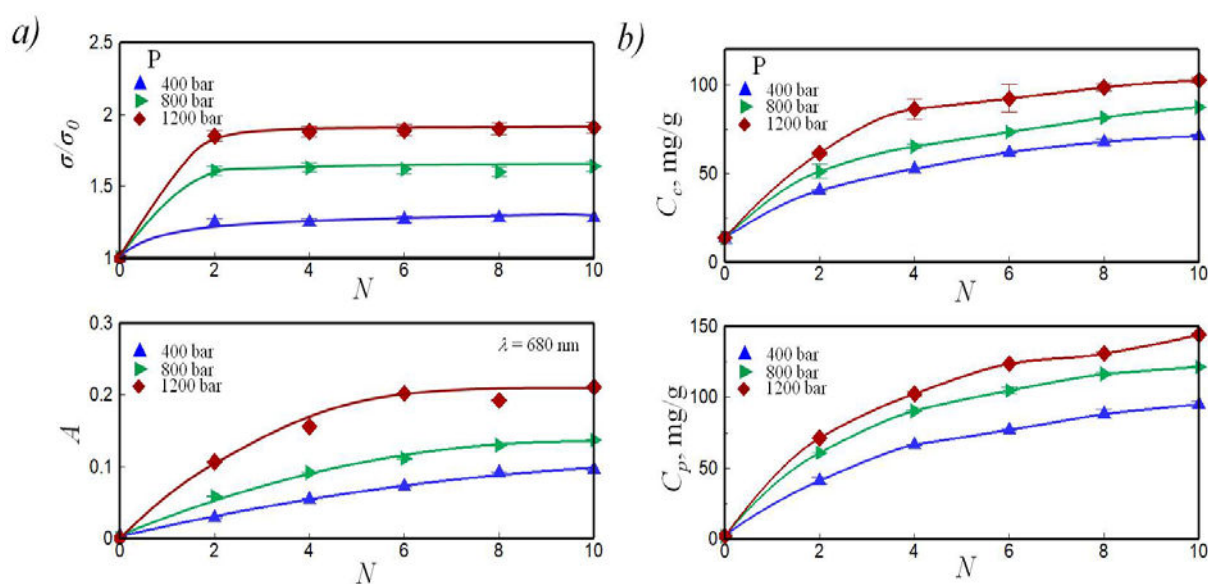


**Fig. 1.** Relative electrical conductivity,  $\sigma/\sigma_0$ , after and before treatment, and absorbance of pigments,  $A$ , at the wavelength of  $\lambda = 680$  nm (a), and the contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , (b), versus extraction time of PEE treatments (E procedure),  $t$ . The suspension's concentration was 0.5% dry matter.

In contrast, HVED treatment allowed enhance all measured values as compared to the U samples ( $t_e = 0$  ms). The similar extraction behaviors were observed for ionics, pigments, carbohydrates and proteins. All the measured values increased with the increase of  $t_e$ . HVED treatment at  $t_e = 8$  ms resulted in the highest values of  $\sigma/\sigma_0 \approx 3.1$ ,  $A \approx 0.05$ ,  $C_c \approx 83$  mg/g and  $C_p \approx 22$  mg/g. Moreover, note that at  $t_e = 6$  ms the measured values were reached the saturated level. Therefore, it can be concluded that HVED treatment was more effective than PEF treatment for the recovery of ionics, pigments, carbohydrates and proteins. However, the spectral data evidenced that pigments extraction was practically absent after both HVED and PEF treatments ( $A < 0.1$ ) since extracted chlorophylls are practically insoluble in water. Their extraction required application of adapted solvents and more intensive cell disruption technologies in the form of pigments-macromolecular water-soluble complexes.

**Fig. 2** presents the changes of relative electrical conductivity,  $\sigma/\sigma_0$ , and absorbance of pigments,  $A$ , (a), and the content of carbohydrates,  $C_c$ , and proteins,  $C_p$ , (b) versus the number of passes,  $N$ , for P procedure at different applied pressures,  $p = 400$ -1200 bar. All the measured values increased with the increase of applied pressure,  $p$ , and number of passes,  $N$ . The extraction for P procedure (1200 bar,  $N = 10$ ) gave the highest values of  $\sigma/\sigma_0 \approx 1.9$ ,  $A \approx$

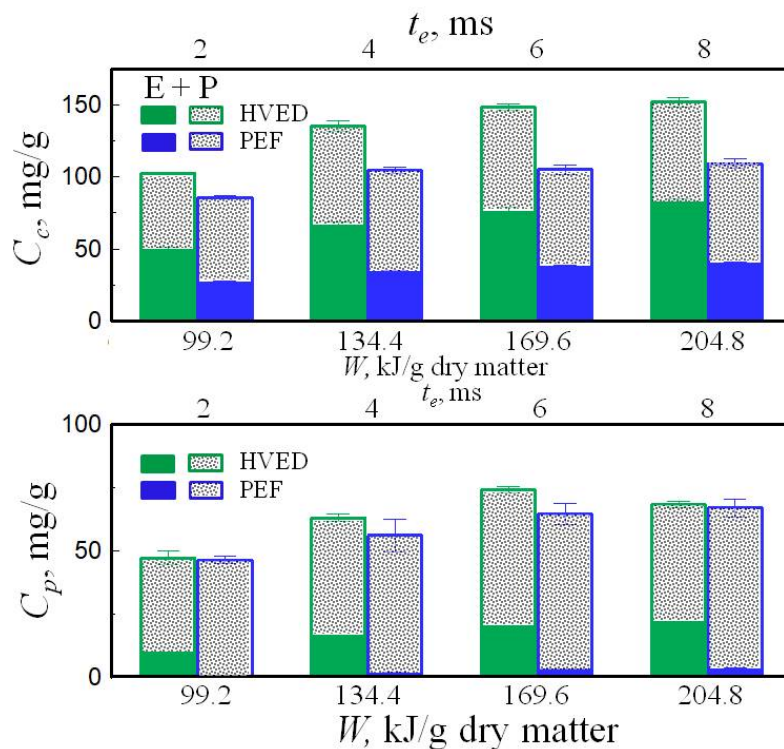
0.2,  $C_c \approx 102.6$  mg/g and  $C_p \approx 144.2$  mg/g. Note that the application of P procedure for  $N = 2$  increased the value of  $\sigma/\sigma_0$  up to  $\approx 1.3$ , 1.6, and 1.9 for  $p = 400$ , 800 and 1200 bar, respectively and no further increase in  $\sigma/\sigma_0$  was observed with an increase of  $N$  (**Fig. 2a**). However, for pigments, carbohydrates and proteins, the first four passes ( $N = 4$ ) allowed obtaining a noticeable extraction efficiencies for all applied pressures and further passes resulted in insignificant supplementary effects at high specific energy consumption. Therefore, in further combination protocols  $N = 4$  for P procedure was applied.



**Fig. 2.** Relative electrical conductivity,  $\sigma/\sigma_0$ , after and before treatment, and absorbance of pigments,  $A$ , at the wavelength of  $\lambda = 680$  nm (a), and the contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , (b), versus the number of HPH passes,  $N$ , at different pressures (400–1200 bar) (P procedure). The suspension's concentration was 0.5% dry matter.

The obtained data showed that the extraction efficiencies of bio-molecules depend dramatically on the used cell disruption techniques. The E procedure is more effective for recovery of ionics than P procedure. For both HVED and PEF treatments at  $t_e = 8$  ms gave higher value of  $\sigma/\sigma_0$  than HPH treatment at 1200 bar for  $N = 10$ . Note that HVED is also very efficient for the recovery of carbohydrates. HVED treatment at  $t_e = 8$  ms give the similar value of  $C_c$  with P procedure for  $N = 4$  at 1200 bar. However, P procedure can lead to the intensive generation of cell debris and release of all the bio-molecules with increased energy consumption. The application of P procedure (1200 bar,  $N = 10$ ) gave 4-fold higher content for pigments and 6.5-fold higher content for proteins as compared to E procedure (HVED

treatment). The application of procedures that combine the important qualities of E and P procedure can be attractive in terms of extraction yield, selectivity and energy consumption.



**Fig. 3.** The contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , versus the specific energy consumption,  $W$ , for combined E (HVED or PEF treatment, 2-8ms) + P (HPH treatment, 800 bar, 4 passes) procedure. The suspension's concentration was 0.5% dry matter.

**Fig. 3** compares the contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , versus the specific energy consumption,  $W$ , for treatment using combined E + P procedure for diluted suspensions with  $C_m = 0.5\%$ . The treatment duration of E procedure was  $t_e = 2-8$  ms, the applied pressure and number of HPH passes were 800 bar and  $N = 4$ , respectively. The used E + P procedure consisted in applying  $\approx 99-205$  kJ/g dry matter of specific energy consumption. It was observed that the E (by HVED treatment) + P procedure showed higher values when compared to the E (by PEF treatment) + P procedure at the equivalent energy. The carbohydrates content in the E + P procedure increased with the energy consumption, the maximum value of  $C_c \approx 151.9$  mg/g was observed for E (HVED, 8 ms) + P procedure with an energy input of  $\approx 204.8$  kJ/g dry matter. However, the maximum value of  $C_p \approx 74.1$  mg/g was observed for E (HVED, 6 ms) + P procedure ( $\approx 169.6$  kJ/g dry matter), further discharges

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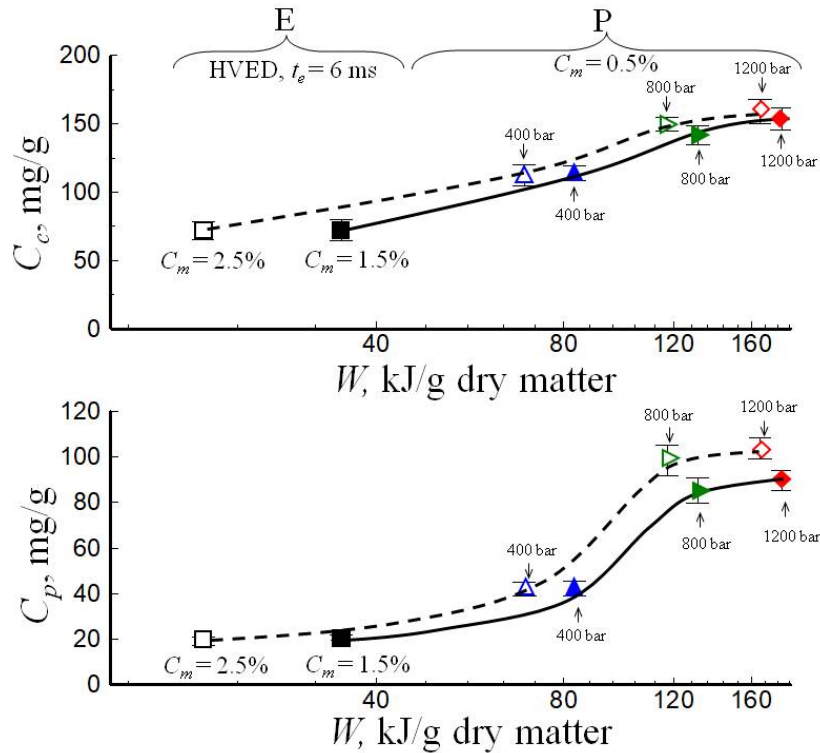
duration in the preliminary HVED treatment resulted in the slight decrease of proteins content in final P procedure at high specific energy consumption.

Note that for extraction of carbohydrates, the E + P procedure can display synergetic behaviour. For example, individual E ( $t_e = 4$  ms) or P (800 bar,  $N = 4$ ) procedure gave  $C_c \approx 34.4$  mg/g (for PEF treatment),  $\approx 66.6$  mg/g (for HVED treatment) (**Fig. 1b**), and  $\approx 65.2$  mg/g (for HPH treatment) (**Fig. 2b**). However, the combined E ( $t_e = 4$  ms) + P (800 bar,  $N = 4$ ) procedure gave  $C_c \approx 104.8$  mg/g (for preliminary PEF treatment) and  $\approx 135.2$  mg/g (for preliminary HVED treatment) (**Fig. 3**). Similar behaviour was also observed for combined E (6 or 8 ms) + P (800 bar,  $N = 4$ ) procedures. However, the extraction of proteins for combined E + P procedure was ineffective when compared with individual P (800 bar,  $N = 4$ ) procedure. For example, extraction of proteins using P (800 bar,  $N = 4$ ) procedure gave  $C_p \approx 90.4$  mg/g ( $\approx 64$  kJ/g dry matter) (**Fig. 2b**), whereas the combined E + P (4 ms, 800 bar,  $N = 4$ ) procedure gave  $C_p \approx 56$  mg/g (for preliminary PEF treatment) and  $\approx 63$  mg/g (for preliminary HVED treatment) at  $\approx 134.4$  kJ/g dry matter (**Fig. 3**). Therefore, the application of individual E, P, or E + P procedure should be done based on the most suitable extraction selectivity, purity of extract and energy consumptions.

It was observed that the considerable specific energy consumption was needed for diluted suspensions ( $C_m = 0.5\%$ ). For concentrated suspensions, the extraction can be more effective in terms of energy consumption per g dry matter. **Fig. 4** compares extraction behaviours for carbohydrates and proteins after application of E + P procedure for concentrated suspensions. In these experiments, 1.5% or 2.5% suspensions was used in the preliminary E (HVED, 6 ms;  $\approx 35.2$  and 21.2 kJ/g dry matter, respectively) procedure, and then 0.5% re-suspensions were used in the final P (400-1200 bar,  $N = 4$ ) procedure. After the preliminary E procedure, the values of  $C_c \approx 72.2$  mg/g and  $C_p \approx 20.6$  mg/g were obtained for 1.5% suspensions. For 2.5% suspensions, the values were observed for carbohydrates ( $C_c \approx 71.8$  mg/g) and proteins ( $C_p \approx 19.0$  mg/g) (**Fig. 4**). Note that for 0.5% suspensions, the similar preliminary E (HVED, 6 ms,  $\approx 105.6$  kJ/g dry matter) procedure gave  $C_c \approx 76.6$  mg/g,  $C_p \approx 20.4$  mg/g (**Fig. 3**). The extracted contents of carbohydrates and proteins were not significant different among different cell concentration of suspensions. This phenomenon was also observed in previously report (Safi et al., 2017), the authors investigated extraction of water-soluble proteins from *Nannochloropsis gaditana* using PEF treatment. They reported that the



yield of proteins was statistically the same for used concentration of suspension ranging from 15 g/L to 45 g/L (i.e. 1.5% ~ 4.5% suspensions).

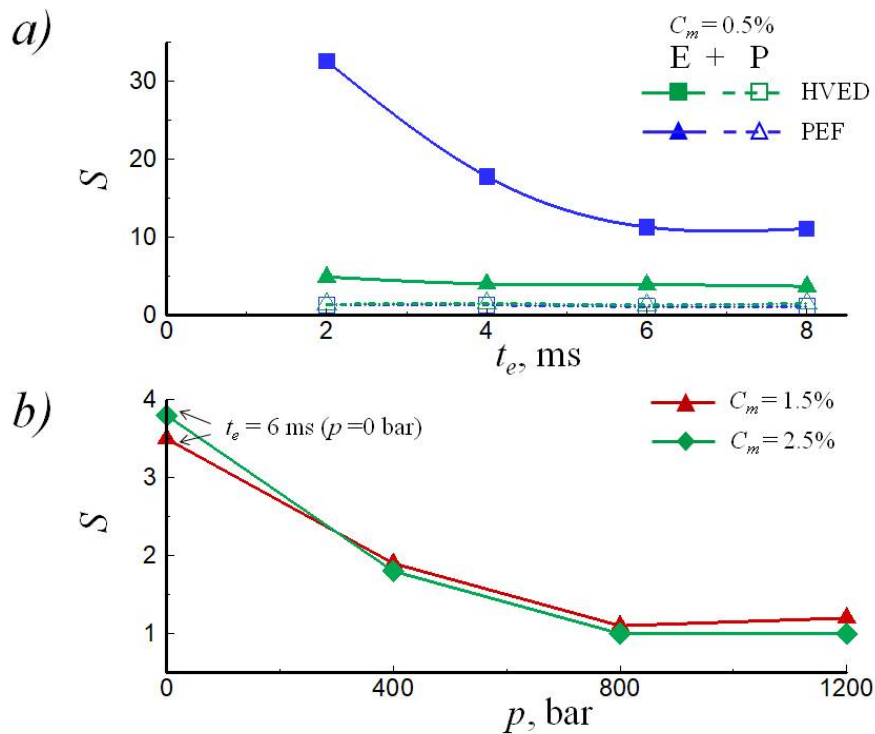


**Fig. 4.** The content of carbohydrates,  $C_c$ , and proteins,  $C_p$ , versus the specific energy consumption,  $W$ , for combined E (1.5% or 2.5% dry matter, HVED treatment, 6ms) + P (0.5% dry matter, 400-1200 bar, 4 passes) procedure.

Moreover, the final P procedure at 400 bar ( $\approx 83.2$  kJ/g dry matter) increased the values of  $C_c$  up to 113.7 mg/g and  $C_p$  up to 42.3 mg/g for 1.5% suspensions (**Fig. 4**). Similar behaviours were observed for 2.5% suspensions using lower energy consumption ( $\approx 69.1$  kJ/g dry matter). Furthermore, the values of  $C_c$  and  $C_p$  continuously increased with the increase of  $p$  or  $E$  for both 1.5% and 2.5% suspensions. For example, at  $p = 800$  bar, they reached the values of  $C_c \approx 141.9$  mg/g and  $C_p \approx 85.3$  mg/g for 1.5% suspensions ( $\approx 131.2$  kJ/g dry matter); and the values of  $C_c \approx 149.6$  mg/g and  $C_p \approx 98.5$  mg/g for 2.5% suspensions ( $\approx 117.1$  kJ/g dry matter). These values can be compared with the values of  $C_c \approx 148.5$  mg/g, and  $C_p \approx 74.1$  mg/g, obtained after using E (HVED, 6 ms,  $C_m = 0.5\%$ ) + P (800 bar,  $N = 4$ ) procedure ( $\approx 169.6$  kJ/g dry matter) (**Fig. 3**). Therefore, the preliminary P procedure of more concentrated suspensions allowed increasing the extraction efficiency and decreasing the energy consumptions.

### 3.2 Selective carbohydrate release

For characterization of relative selectivity of carbohydrates and proteins extraction, the selectivity ratio,  $S = C_d/C_p$ , was used. For non-selective extraction, the value of  $S = 1$  is expected.  $S$  can be regarded as a quality parameter for cell disruption processes, i.e., a higher selectivity for the desired product makes further fraction/purification processing easier (e.g., less impurities) (Postma, Suarez-Garcia, et al., 2017).



**Fig. 5.** The selectivity ratio,  $S$ , versus electrical treatment time,  $t_e$ , for individual E (0.5% dry matter, HVED or PEF treatment, 2-8 ms) and combined E (0.5% dry matter, HVED or PEF treatment, 2-8 ms) + P (800 bar, 4 passes) procedure (a), and homogenizing pressure,  $p$ , for combined E (1.5% or 2.5% dry matter, HVED treatment, 6 ms) + P procedure (0.5% dry matter, 400-1200 bar, 4 passes) (b).

**Fig. 5** presents the selectivity ratio,  $S$ , versus electrical treatment time,  $t_e$ , for individual E and combined E (2-8 ms) + P (800 bar, 4 passes) procedure for diluted suspensions ( $C_m = 0.5\%$ ) (a), and homogenizing pressure,  $p$ , for combined E (HVED, 6 ms) + P (400-1200 bar,  $N = 4$ ) procedure for concentrated suspensions ( $C_m = 1.5\%$  or  $2.5\%$ ) (b). In our case, the applied procedures are selective extraction towards carbohydrates ( $S > 1$ ). The selectivity ratio were smaller with the application of P procedure ( $S = 1-1.8$ ) as compared with



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E procedure ( $S \geq 3.5$ ). This finding means that the smallest selectivity was obtained for extraction assisted with HPH treatment. The PEF treatment gives on average the highest selectivity ratio, followed by the HVED treatment. Therefore, for carbohydrates' selective release, the relatively mild cell disruption technique is required.

Moreover, for the E procedure with diluted suspension ( $C_m = 0.5\%$ ), the values of  $S$  decrease with the increase of  $t_e$  (**Fig. 5a**). Note that the application of E procedure for  $t_e = 6$  ms decreased the values to  $S \approx 11$  and  $S \approx 4$  for PEF and HVED treatment, respectively and no further decrease in  $S$  was observed with an increase of  $t_e$ . This finding can be explained by a smaller quantity of small-sized water-soluble proteins were released with the longer treatment time, resulted in the decrease of  $S$ . However, the selectivity ratio in the final P procedure was almost stable ( $S = 1-1.5$ ) regardless of applied  $t_e$  in preliminary E procedure (**Fig. 5a**).

Note that for the E procedure (HVED, 6 ms), the similar values were observed ( $S \approx 4$ ) regardless of applied cell concentration (**Fig. 5a and b**). However, the values of  $S$  in final P procedure applied  $p = 400$  bar ( $S = 1.8$ ) were higher than that obtained from final P procedure applied  $p = 800$  or  $1200$  bar ( $S = 1$ ) (**Fig. 5b**). This possibly reflects that applied lower homogenizing pressure was less effective for microalgae cell damage and release larger-size proteins. The finding agree with the previously study (Geciova, Bury, & Jelen, 2002), who reported that pressures of HPH ranging from 550-2000 bar are appropriate for the disruption of microbial cell. Therefore, we can speculate that E procedure allow selective release small-sized bio-molecules (e.g. ionics, carbohydrates) resulting in relative pure fractions without negatively harming the other molecules.

#### 4. Conclusion

This study provides insights into the effects of individual E, P, and their combination for extraction of bio-molecules from *P. kessleri* in terms of extraction efficiency, selectivity and energy efficient. The E procedure allowed selective extraction of smaller-sized molecules, while it was ineffective for extraction of pigments. For the E procedure, HVED was more efficient than PEF. The P procedure, instead, was the most effective for extraction of proteins and pigments. The combined E + P procedure exhibited a synergetic behavior for carbohydrates' extraction for 0.5% suspension and allowed increasing the carbohydrates and proteins contents with reduced the energy consumption for concentrated suspensions.

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#### IV.4 Chapter conclusion

Chapter IV is focused on selective and energy efficient extraction of intracellular bio-molecules from microalgae by combined treatment. Based on the results of Chapter III, physical treatments allowed selective extraction of carbohydrates, while their extraction efficiency is relative low. HPH treatment is the most effective technology in terms of extraction, but also the least selectivity and the most energy-consuming. Therefore, with regard to the choice of the appropriate method, the combined treatment (physical treatment + HHP) seems to be a very promising extraction strategy both for the efficiency and the selectivity of the extraction, but also in terms of energy. In Chapter IV Therefore, selective extraction of more carbohydrates by the preliminary physical treatments (PEE/US), following by the more intensive HPH treatment for supplemental extraction of reserved proteins from microalgal biomass were carried out. The synergetic behaviour for extraction of water-soluble components in dependence of specific energy consumption and cell concentration of suspension were discussed.

The results evidenced that the concentration of the preliminary physically treated suspensions is important for extraction efficiencies and total process energy consumption. For diluted suspension ( $\leq 1\%$ ), the combined procedures are less effective or negative for extraction of bio-molecules. The higher extraction efficiency are usually obtained with the higher energy consumption. However, the preliminary physical treatments of more concentration suspensions ( $\geq 1\%$ ) followed by HPH of diluted suspension ( $\leq 1\%$ ) allowed increasing the extraction efficiency, and decreasing the energy consumption. Because the use of more concentrated suspensions during the process of preliminary physical treatments can be obtain the same extraction efficiency with the lower energy consumption. Therefore, the application of combined process for intracellular bio-molecules recovery requires taking into account the selectivity of extraction, purity of extracts and energy consumption. However, except extraction of hydrophilic compounds, some remained hydrophobic components (e.g. pigments and lipids) are important composition of microalgae. In order to obtain maximum valorisation of microalgal biomass, a solvent extraction procedure following aqueous extraction procedure will be needed.

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## Chapter V Effect of multistage extraction procedure on extraction and fractionation of bio-molecules from microalgae

### V.1 Chapter introduction

Microalgae can serve as raw material for biofuels or agricultural biostimulants, but at the same time are a promising source for food and feed production due to their high proportion of proteins and micronutrients. For example, the lipids obtained from microalgal biomass has been considered as a most promising feedstock to produce biodiesel (Hernández-Pérez et al., 2019; Veillette et al., 2017). The microalgal proteins can be used instead of conventional food supplements due to their nutritional values and amino acid profiles (Becker, 2007), and polysaccharides can be hydrolyzed to reduced sugars which have potential application in the production of bioethanol (Fu et al., 2010). Therefore, for maximal valorisation of microalgal biomass, selective extraction and fractionation of valuable bio-molecules is crucial. Moreover, for sustaining biorefinery, green solvents extraction (Chemat et al., 2012) or multistage extraction (Zhu et al., 2018) were developed. They allowed reducing the amount of toxic solvents, increasing the extraction efficiency, and reducing the energy consumption. These approaches correspond to the “green extraction concept” (Chemat et al., 2017).

The objective of this chapter was to investigate the effect of HVED as pretreatment on the extraction and fractionation of bio-molecules from microalgae during a multi-step extraction process. The multistage extraction process included the application of cell disruption pretreatment in combination of aqueous and non-aqueous extractions. The efficiency of recovery of ionic components, proteins, carbohydrates, pigments and lipids at different stages of extraction procedures were estimated.

In this chapter, the first part (details are presented in article 5: *Multistage aqueous and non-aqueous extraction of bio-molecules from microalga Phaeodactylum tricornutum*) was published in the journal “Innovative Food Science and Emerging Technologies”. The second part (details are presented in article 6: *Two-step procedure for selective recovery of bio-molecules from microalga Nannochloropsis oculata assisted by high voltage electrical discharges*) was published in the journal “Bioresource Technology”. These works were



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carried out under the direction Dr. Nabil Grimi, Prof. Luc Marchal and in collaboration with Prof. Nikolai Lebovka and Prof. Eugène Vorobiev.

V.2 *Article 5*: Multistage aqueous and non-aqueous extraction of bio-molecules from microalga *Phaeodactylum tricornutum*

(The article is presented on the following pages)

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**Multistage aqueous and non-aqueous extraction of bio-molecules from  
microalga *Phaeodactylum tricornutum***

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## Abstract

A multi-step aqueous and non-aqueous extraction procedure was applied to recovery bio-molecules from *Phaeodactylum tricornutum*. The process include that physical pre-treatments (high voltage electrical discharges (HVED, 40 kV/cm, 1-8 ms, HVED samples) or high pressure homogenization (HPH, 1200 bar, 10 passes, P samples)) (1<sup>st</sup> step); aqueous extraction (2<sup>nd</sup> step); pigments extraction in ethanol (3<sup>rd</sup> step); and lipids extraction in CHCl<sub>3</sub>/MeOH (4<sup>th</sup> step). The extractability of ionics, carbohydrates, proteins, pigments and lipids for untreated, HVED and P samples were evaluated. The results evidenced that HVED allowed selective extraction of water soluble ionic products at 1<sup>st</sup> and 2<sup>nd</sup> steps. The maximum ionic concentrations were found for HVED samples. However, P samples resulted in higher contents of extracted components as compared to HVED samples ( $\approx$  1.5-fold of carbohydrates,  $\approx$  2.5-fold of proteins,  $\approx$  5-fold of carotenoids, and  $\approx$  3-fold of chlorophylls). Moreover, the non-aqueous extraction (3<sup>rd</sup> and 4<sup>th</sup> steps) allowed supplementary extraction of pigments and lipids.

**Keywords:** Microalgae; *Phaeodactylum tricornutum*; High voltage electrical discharges; High pressure homogenization; Selective extraction; Biorefinery

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## 1. Introduction

Microalgae have recently emerged as a potential biomass feedstock due to their capability of producing compounds of great economic value, including antioxidants, dyes, sterols, proteins, phycocolloids, amino acids, polyunsaturated fatty acids, and vitamins (Irshad, Myint, Hong, Kim, & Sim, 2019). For example, the microalgal biomass can contain high levels of lipids (up to 75 wt%) and it has been considered as a most promising feedstock to produce biodiesel (Hernández-Pérez, Sánchez-Tuirán, Ojeda, El-Halwagi, & Ponce-Ortega, 2019; Veillette, Giroir-Fendler, Faucheux, & Heitz, 2017). The microalgal proteins can be used instead of conventional food supplements due to their nutritional values and amino acid profiles (Becker, 2007), and polysaccharides can be hydrolyzed to reduced sugars which have potential application in the production of bioethanol (Fu, Hung, Chen, Su, & Wu, 2010).

The microalga *Phaeodactylum tricornutum* (*P. tricornutum*), a typical unicellular diatom, was found throughout marine and freshwater environments (Xu et al., 2010). *P. tricornutum* is also the only species of microalgae that can exist in three morphotypes (fusiform, triradiate, and oval) (Flori, Jouneau, Finazzi, Maréchal, & Falconet, 2016). It contains 36.4% of crude protein, 26.1% of available carbohydrates, 18% of lipids on a dry weight basis (Reboloso-Fuentes, Navarro-Pérez, Ramos-Miras, & Guil-Guerrero, 2001).

For the recovery of microalgal bio-molecules, the wet extraction (with no preliminary drying) is the most adopted and low-energy demand strategy, and it starts with the carbohydrates and proteins release in the aqueous phase (Orr, Plechkova, Seddon, & Rehmman, 2015; Zinkoné, Gifuni, Lavenant, Pruvost, & Marchal, 2018). In this line, the selective extraction of different bio-molecules is highly influenced by the method used for their release from the cells (Angles, Jaouen, Pruvost, & Marchal, 2017). Therefore, cell disruption is a crucial pretreatment step in the biorefinery process.

Recently, some attention has been focused on applications of physical pretreatment methods (such as high pressure homogenization (HPH), ultrasound, microwave, pulsed electric fields (PEF), and high voltage electrical discharges (HVED)) for the selective extraction of bio-molecules from different microalgal species ('t Lam et al., 2017; Parniakov, Barba, et al., 2015; Zhang, Parniakov, et al., 2019). Particularly, the HPH treatment has great potential for a recovery of pigments and high molecular weight proteins (Zhang, Grimi, Marchal, & Vorobiev, 2018). The HPH treatment followed with with chloroform: methanol (2 :

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1, v/v) extraction was applied to release a good quality lipids of microalgae *Scenedesmus* sp. useful for biodiesel production (Cho et al., 2012). In addition, using green solvents (Chemat, Vian, & Cravotto, 2012) or multistage extraction (Zhu et al., 2018) allowed reducing the amount of toxic solvents, increasing the extraction efficiency, and reducing the energy consumption. These approaches correspond to the “green extraction concept” (Chemat et al., 2017). The several research groups (Gnapowski, Akiyama, Sakugawa, & Akiyama, 2013; Grimi et al., 2014) have also reported that application of HVED treatment was suitable for selective extraction in a biorefinery process. It allows effective recovery of low molecular weight components from microalgae such as water soluble intracellular ions, vitamins, carbohydrate, bio-active acids (folic, pantothenic, nicotinic), microelements (Ca, K, Na, Mg, Zn, Fe, etc.), and other micro- and macronutrients (Zhang, Parniakov, et al., 2019). Commonly, the HPH treatment results in non-selective release of the intracellular molecules, it requires high energy consumption, and can cause degradation of bio-molecules and production of high amount of cell debris (Zhang, Parniakov, et al., 2019). The HVED treatment is more “gentle”, and it can assist partial release of weakly bounded biomolecules, but it may be less effective for extraction of some intracellular components. Therefore, in order to become economically feasible the HPH and HVED assisted extraction techniques should be designed and rethought in an integrative way together with simplified downstream processes.

The valorisation of microalgal biomass can be improved by using the physical pretreatment in a multistage extraction processes. The multistage extraction processes can include the application of physical treatment in combination of aqueous and non-aqueous extractions. The previously discussed two-step procedures included PEF pre-treatment step before pH-assisted aqueous extraction of intracellular molecules from *Nannochloropsis* (Parniakov et al., 2015) and application of high pressure disruption in a two-step treatment for selective extraction of intracellular components from the microalga *Porphyridium cruentum* (Jubeau et al., 2013). However, at present time the available information about application of selective multistage extraction of bio-molecules from microalgae is still scarce. The objective of the present study was to investigate the efficiency of HVED-pretreatment on the selective recovery of bio-molecules from *P. tricornutum* during a multi-step extraction process. The efficiency of recovery of ionic components, proteins, carbohydrates, pigments and lipids at

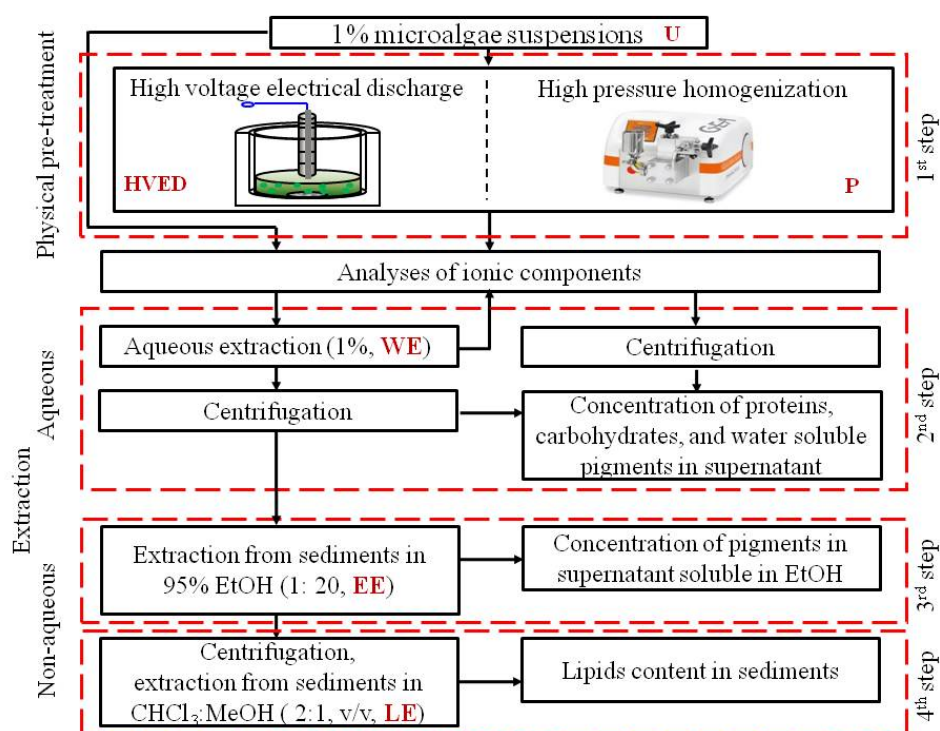
different stages of extraction procedures were estimated. The results were compared to the pretreatment with HPH.

## 2. Materials and methods

### 2.1 Microalgae

The microalga *P. tricornutum* used throughout this study were provided by Algosource Saint-Nazaire, France. The cells have approximately fusiform (a spindle-like) shape. The samples were obtained as frozen algae pastes ( $\approx 68.6 \pm 0.7\%$  moisture content) and stored at  $-20\text{ }^{\circ}\text{C}$  until use. The pastes were first thawed at ambient temperature and then diluted with deionized water in order to prepare 1% dry matter (hereinafter %) suspensions.

### 2.2 Four steps extraction procedures



**Fig. 1.** Schematic presentation of four step extraction procedures.

**Fig. 1** presents schematic of multistage extraction procedures for bio-molecules recovery from *P. tricornutum*. The multistage extraction procedure included four steps. The 1<sup>st</sup> step included HVED treatment (HVED samples, 40 kV, 1-8 ms) or HPH treatment (P samples, 1200 bar, 10 passes). Untreated suspension (U samples) was used in control experiments. The 2<sup>nd</sup> step included aqueous extraction from HVED, P and U samples for 1 h. After this step, the

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characterization analysis of ionic components, proteins, carbohydrates and water-soluble pigments were done.

The non-aqueous extraction included 3<sup>rd</sup> and 4<sup>th</sup> step extraction from sediments obtained after 2<sup>nd</sup> step. The 3<sup>rd</sup> step was done using ethanol (EtOH, 95%, v/v) (extraction of pigments) and the 4<sup>th</sup> step was done using chloroform/methanol (CHCl<sub>3</sub>: MeOH) mixture (2/1: v/v) (extraction of lipids). After non-aqueous extraction, the contents of pigments (3<sup>rd</sup> step) and total lipids (4<sup>th</sup> step) were determined. The optimal parameters of treatment and extraction procedures have been selected using the previously published data on different microalgal species (for a review, see (Vorobiev & Lebovka, 2020; Zhang, Parniakov, et al., 2019)) and according to the preliminary performed estimations and tests.

### 2.2.1 Physical pre-treatment (1<sup>st</sup> step)

HVED treatment involved a treatment of 500 g of suspension (1% dry matter). A high voltage pulsed power 40 kV-10 kA generator (Basis, Saint-Quentin, France) was used. The treatment was performed in a one-liter cylindrical batch treatment chamber with an electrode of needle-plate geometry. The voltage peak amplitude was fixed at 40 kV. The distance between the stainless steel needle and the grounded plate was fixed to 1 cm. HVED treatment comprised the application of  $n$  successive pulses ( $n = 1-800$ ), and a pulse repetition rate of 1 Hz, and a 1-3 min pause was applied after each 200 pulses to cool the sample in ice bath in order to avoid significant elevation of temperature. The total time of electrical treatment ( $t_{HVED}$ ) varied from 0.01 to 8 ms. The initial temperature of suspensions before HVED procedure was 22 °C and the temperature elevation after HVED treatment never exceeded 40 °C.

For HPH treatment (P procedure), a high pressure homogenizer GEA Niro Soavi PandaPlus 2000 (GEA Niro Soavi SpA, Parma, Italy) was used. In this work, 500 g of untreated suspensions (1% dry matter) were passed through the homogenizer. In all experiments, the HPH treatment at 1200 bar, 10 passes allowed obtaining the maximum disruption. The treatment at larger pressure can result in significant temperature induced degradation of the product during the one pass. In order to prevent excessive heating, the suspensions were maintained at 22 °C by a cooling system for the next pass through the homogenizer and following characterization.



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### 2.2.2 Aqueous extraction (2<sup>nd</sup> step)

After pre-treatment processing (1<sup>st</sup> step), U, HVED and P samples were immediately used for aqueous extraction (WE, 2<sup>nd</sup> step) for 1 h under stirring at 150 rpm ( $\approx 22\text{ }^{\circ}\text{C}$ ) to allow the water-soluble components to diffuse out of cells. The diffusion experiments were done in 1% aqueous suspensions. After diffusion, the suspensions were centrifuged using a Sigma 3-16 instrument (Fisher Scientific, Illkirch, France) at  $3,600\times g$  for separation of supernatants and sediments.

### 2.2.3 Non-aqueous extraction (3<sup>rd</sup> and 4<sup>th</sup> steps)

For EtOH extraction (EE, 3<sup>rd</sup> step) procedure, the collected microalgal sediments was mixed with 95% EtOH for 500 s under stirring at 150 rpm, and solid liquid ratio was 1:20. The concentrated EtOH was accepted as the best solvent to maximize pigments extraction (Kim et al., 2012). After the EE procedure, microalgal sediments were re-collected by centrifugation and dried until reach a constant solid mass for further lipids analysis. Drying experiment was carried out in a vacuum chamber (Cole-Parmer, USA) connected with a vacuum pump (Rietschle, Germany). The pressure of chamber was maintained at 30 kPa and the drying temperature was fixed at  $50\text{ }^{\circ}\text{C}$ .

For lipids extraction (LE, 4<sup>th</sup> step) procedure, the lipids content of dried sediments was determined by the method of “whole cell analysis” (WCA) as described by Van Vooren et al. (Van Vooren et al., 2012). Briefly, in order to prevent oxidation of lipids, the dried sediments ( $\approx 0.5\text{ g}$ ) were first mixed with 20  $\mu\text{L}$  of distilled water and 10  $\mu\text{L}$  of butylated hydroxytoluene (20  $\mu\text{g}/\mu\text{L}$ ) in clean vials. The 6 mL of a  $\text{CHCl}_3$ : MeOH mixture (2:1, v/v) was added in the sample by three times. Vials were maintained 6 h in the dark and under slow agitation (Van Vooren et al., 2012).

## *2.3 Characterization*

Optical microscopy images of microalgae cells after different treatments were obtained at 40 folds magnification using a VisiScope light microscope (VWR, Italy) (The optical microscopy images presented in Fig. S1 in Supplementary materials). All spectra were measured by a UV/vis spectrophotometer (Thermo Electron Corporation, MA).

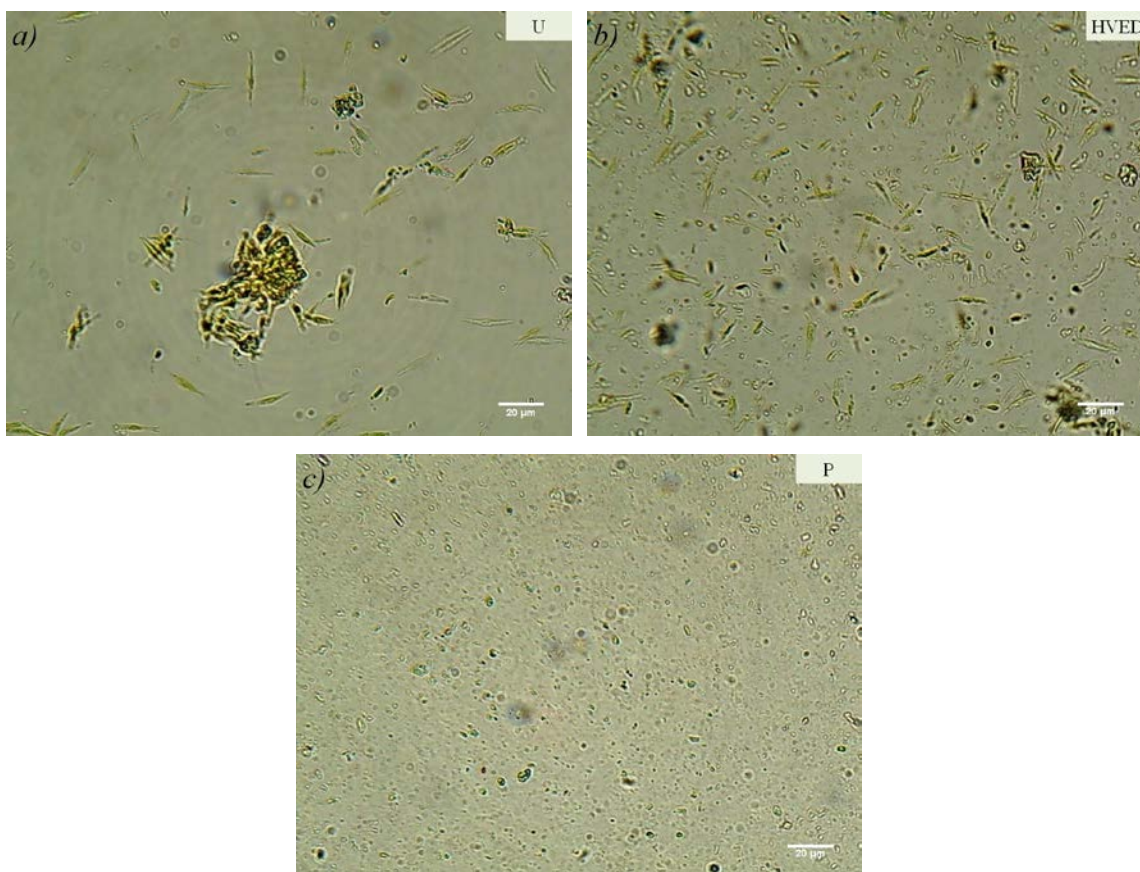


Fig. S1. Examples of optical microscopy images of the microalgal cells (*Phaeodactylum tricornerutum*) obtained using U (a), HVED (40 kV/cm, 8 ms) (b), and P (1200 bar, 10 passes) (c) procedures.

### 2.3.1 Ionic components

The release extent of ionic components was estimated by the measurements of the electrical conductivity,  $\sigma$ , of the suspensions after and before the treatment. The electrical conductivity was always measured by a conductivity meter InoLab pH/cond Level 1 (WTW, Weilheim, Germany) at 22°C.

### 2.3.2 Analyses of supernatant

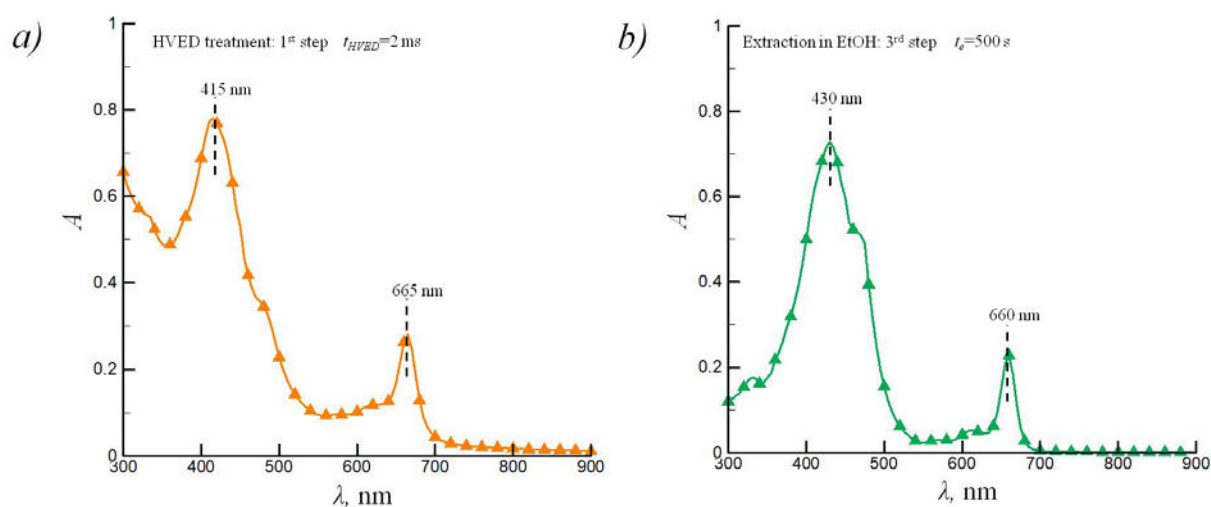
The 2 mL of suspensions were centrifuged using a mySPIN6 Mini Centrifuge (Thermo Fisher Scientific, China) at 2,000×g for 10 min and the supernatants were collected (**Fig. 1**).

The content of carbohydrates,  $C_c$ , was tested using a phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). In brief, the color reaction was initiated by mixing 1 mL of appropriately diluted supernatants with 100 µL of 5% phenol solution and 5 mL of concentrated sulfuric acid (Sigma-Aldrich, France). The incubation was kept at 20 °C

for 20 min. Absorbance was measured at 490 nm. D-glucose (Sigma-Aldrich, France) was used as a standard. The results were expressed as mg of glucose equivalent per gram of dry microalgae (mg glucose/g DM).

The content of proteins,  $C_p$ , was determined by means of the method of Bradford (Bradford, 1976). Briefly, 100  $\mu$ L supernatants (diluted if required) were mixed with 1 mL of Bradford Dye Reagent (Thermo Fisher, Kandel, Germany) on the Vortex for 10 s. After incubation at ambient temperature for 5 min, absorbance was measured at 595 nm. Bovine serum albumin (BSA) (Sigma-Aldrich, France) was used for the calibration curve. The results were expressed as mg of BSA equivalent per gram of dry microalgae (mg BSA/g DM).

Absorption spectra of supernatants (diluted if required) obtained from different procedures was measured in the wavelength range of 300-900 nm against the blank (with the precision of  $\pm 1$  nm) (The UV absorption spectra of pigments obtained after aqueous (1<sup>st</sup> step) and non-aqueous extraction (3<sup>rd</sup> step) presented in Fig. S2 in Supplementary materials).



*Fig. S2. Examples of the UV absorption spectra of supernatants, obtained after treatment using HVED ( $t_{HVED} = 2$  ms) procedures (1<sup>st</sup> step) (a), and obtained after treatment using EtOH extraction (EE) procedure ( $t_e = 500$ s) (3<sup>rd</sup> step) (b).*

### 2.3.3 Lipid analyses

After 6h of lipids extraction, organic and aqueous phases were separated and the solvent of the extracts was evaporated under  $N_2$  flux. 1 mL of  $CHCl_3$ : MeOH (2:1, v/v) mixture was then added and stored at  $-20$  °C until analysis. All lipids extracted from sediments were analyzed using gas chromatography-flame ionization detector (GC-FID)

(Agilent Technologies Inc., Santa Clara, CA) after a transesterification step to obtain fatty acid methyl ester. More details can be found in (Van Vooren et al., 2012). The content of lipids,  $C_l$ , was expressed as mg of lipids per gram of dry microalgae (mg/g DM).

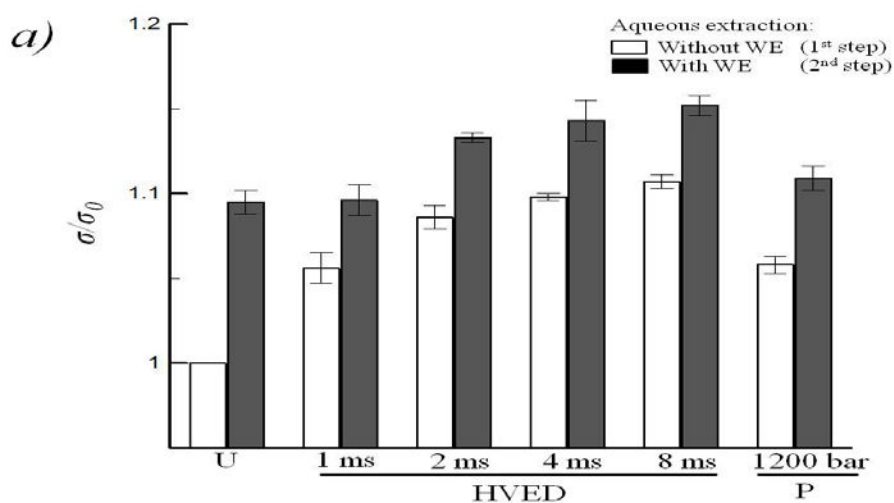
## 2.4 Statistical analysis

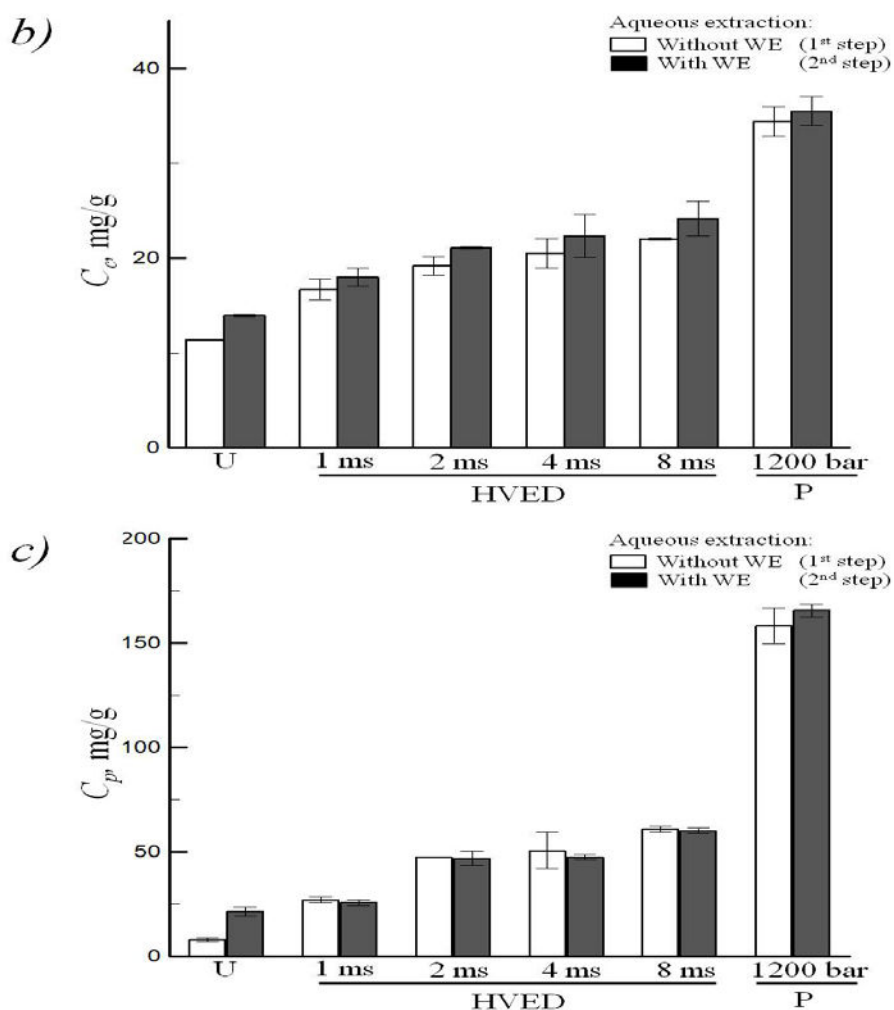
Each experiment was repeated three times. Data are expressed as mean  $\pm$  standard deviations. The error bars, presented on the figures, correspond to the standard deviations. One-way analysis of variance was used for statistical analysis of the data with the help of OriginPro 8.0. A probability value ( $p$  value) of  $< 0.05$  was considered statistically significant.

## 3. Results and discussion

**Fig. 2** presents ratio of electrical conductivities of suspensions after and before treatments,  $\sigma/\sigma_0$ , (a), the contents of carbohydrates,  $C_c$ , (b) and proteins,  $C_p$ , (c) of supernatants. The data are presented for U, HVED ( $t_{HVED} = 1-8$  ms) and P samples without WE (1<sup>st</sup> step) and with WE (2<sup>nd</sup> step).

The obtained data shows that for U samples only a small number of extracellular components presented on the cell walls ( $\sigma_0 = 2.69 \pm 0.01$  mS/cm,  $C_c = 11.39 \pm 0.02$  mg/g and  $C_p = 7.9 \pm 0.87$  mg/g) can be released by spontaneous cell lysis (Carullo et al., 2018). The application of physical pre-treatments (HVED ( $t_{HVED} = 1-8$  ms) and HPH) can significantly increase of the value of  $\sigma/\sigma_0$ ,  $C_c$  and  $C_p$  compared to U samples ( $p < 0.05$ ). For the HVED pre-treatment, the value of  $\sigma/\sigma_0$ ,  $C_c$  and  $C_p$  increased with the increase of  $t_{HVED}$ , and the maximum values were obtained by using the longest applied treatment time,  $t_{HVED} = 8$  ms.



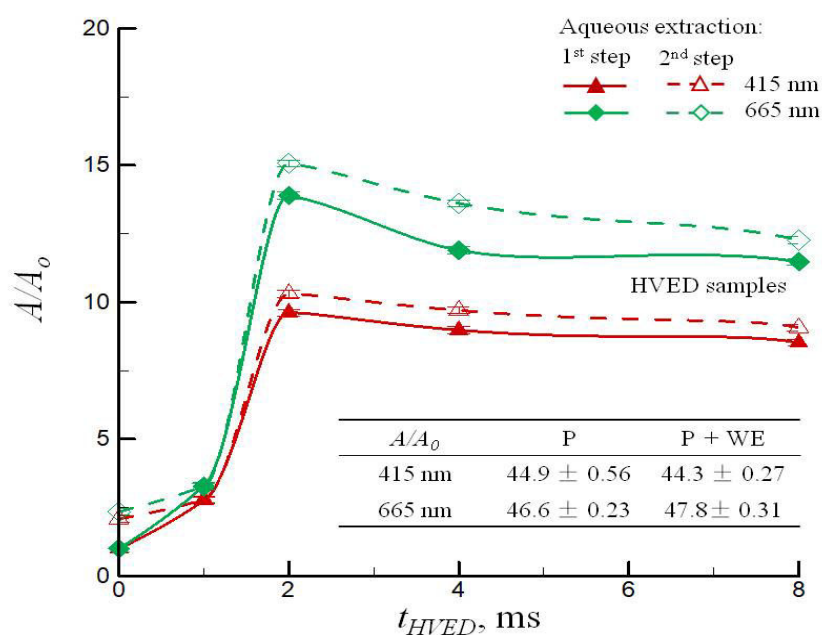


**Fig. 2.** Ratio of electrical conductivity of suspensions after and before treatment,  $\sigma/\sigma_0$ , (a), the contents of carbohydrates,  $C_c$ , (b) and proteins,  $C_p$ , (c) of supernatants. The data are presented for U, HVED ( $t_{HVED} = 1-8$  ms) and P samples obtained without WE (1<sup>st</sup> step) and with WE (2<sup>nd</sup> step).

For P samples, the value of  $\sigma/\sigma_0$  was lower than that obtained for HVED samples at  $t_{HVED} \geq 2$  ms (**Fig. 2a**). This observation is in the line to those reported by Zhang et al. (Zhang et al., 2018), for the release of ionic components from microalga *Parachlorella kessleri*. However, the better efficiency in recovery of carbohydrates and proteins was observed for P samples as compared for HVED samples. For example, for P samples, the  $\approx 1.5$ -fold increase in carbohydrates content (**Fig. 2b**) and  $\approx 2.5$ -fold increase in proteins content (**Fig. 2c**) were achieved for both the 1<sup>st</sup> and 2<sup>nd</sup> steps. Moreover, the extraction of ionic components and carbohydrates was more efficient in the 1<sup>st</sup> step as compared with the 2<sup>nd</sup> step (**Fig. 2a and b**). However, the extraction of proteins was not very efficient for the HVED samples. It can

reflect the relatively mild nature of disruptions of microalgal cells caused by HVED as compared with HPH. HVED treatment can partially release of the proteins present in cytoplasm or inside weak organelles of microalgae. The complete extraction proteins require more intensive cell disintegration methods like HPH or bead milling (Pataro et al., 2017).

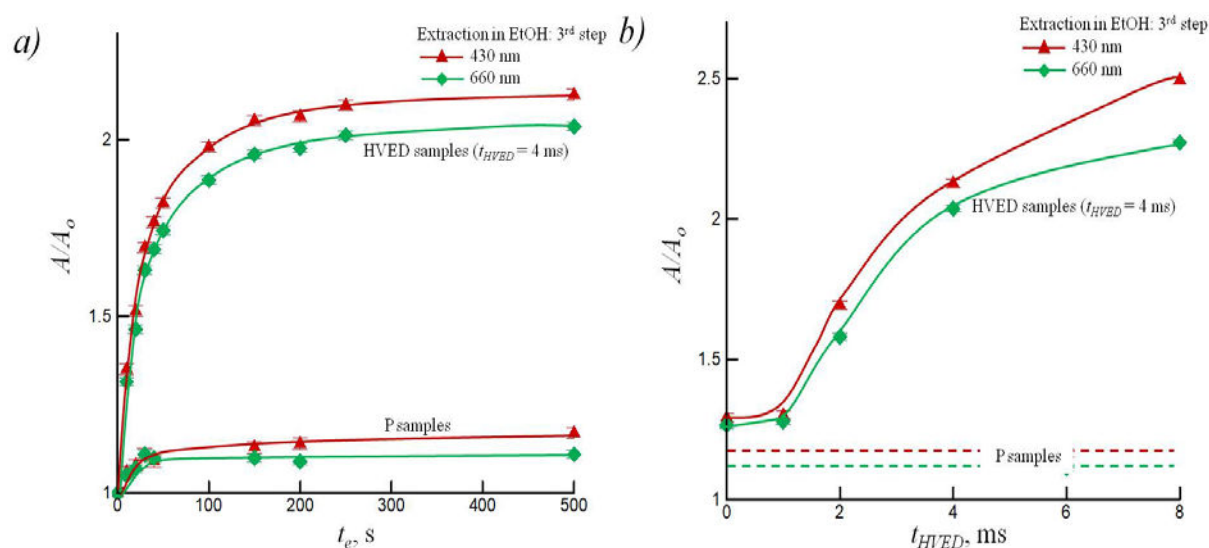
**Fig. 3** depicts ratio of absorbance,  $A/A_0$ , of supernatants versus the HVED treatment time,  $t_{HVED}$ , measured at two different wavelengths  $\lambda_v = 415$  nm (violet) and  $\lambda_r = 665$  nm (red) for HVED samples. Here,  $A_0$  is the absorbance measured in absence of HVED pre-treatment (at  $t_{HVED} = 0$  ms), and the data are presented for samples without WE (1<sup>st</sup> step) and with WE (2<sup>nd</sup> step). The observed changes of  $A/A_0$  at  $\lambda_v = 415$  nm (carotenoids) and  $\lambda_r = 665$  nm (chlorophylls) were rather similar, but they were more pronounced at  $\lambda_r = 665$  nm. Moreover, the maximum value of  $A/A_0$  was observed at  $t_{HVED} = 2$  ms. This effect can be attributed to the temperature increase during the long HVED treatment time (up to 40 °C at  $t_{HVED} = 8$  ms) and possible degradation of some pigments (Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015; Parniakov, Apicella, et al., 2015).



**Fig. 3.** Ratio of absorbance,  $A/A_0$ , of supernatants versus the HVED treatment time,  $t_{HVED}$ , measured at two different wavelengths  $\lambda_v = 415$  nm (violet) and  $\lambda_r = 665$  nm (red). Here,  $A_0$  is the absorbance measured at  $t_{HVED} = 0$  ms. The data are presented for suspensions without WE (1<sup>st</sup> step) and with WE (2<sup>nd</sup> step).



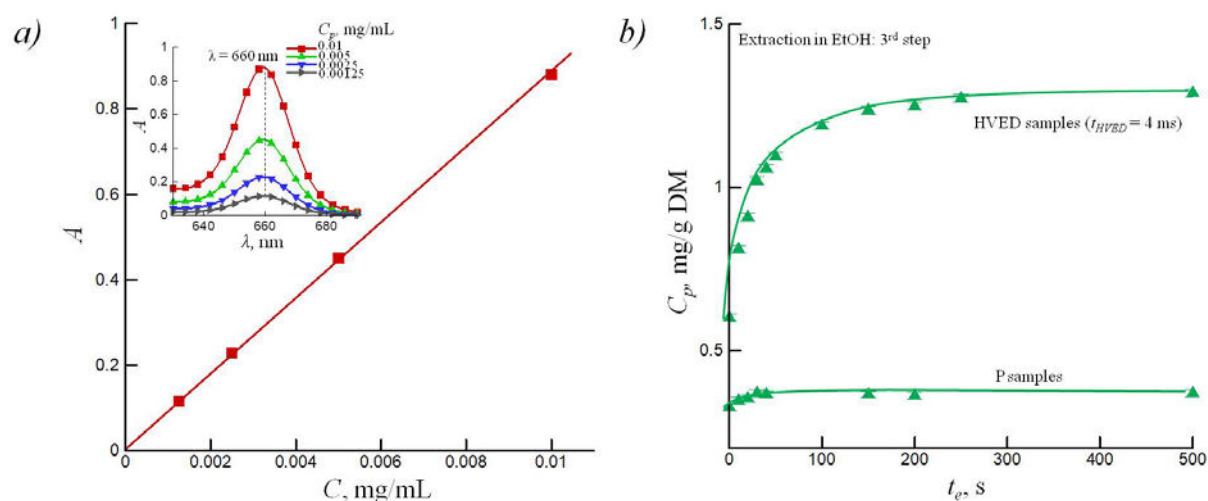
Even the more effective extraction of pigments in aqueous phase was observed for P samples. For example, for P samples, the values  $A/A_0 = 44.9 \pm 0.56$  (415 nm) and  $A/A_0 = 46.6 \pm 0.23$  (655 nm) (1<sup>st</sup> step), and  $A/A_0 = 44.3 \pm 0.27$  (415 nm) and  $A/A_0 = 47.8 \pm 0.31$  (655 nm) (2<sup>nd</sup> step) was obtained. That's correspond to the  $\approx 5$ -fold increase of carotenoids content and  $\approx 3$ -fold increase of chlorophylls content compared to the HVED samples ( $t_{HVED} = 2$  ms). This results are in correspondence with previously reported data on the release of pigments using the HPH (Grimi et al., 2014) and bead milling (Postma et al., 2015). However, for HPH assisted extraction of pigments was more energy-effective and as compared with HVED. For example, application of HPH at  $\approx 64$  kJ/g dry matter allowed releasing more pigments in the aqueous phase as compared with application of HVED at energy of  $\approx 70$  kJ/g dry matter (Zhang et al., 2018). Therefore, the applied treatment for assistance of extraction should be optimized accounting for the required selectivity of the target molecules and energy consumptions. Additionally, WE procedure applied in the 2<sup>nd</sup> step allowed supplementary release of some quantity of pigments (**Fig. 3**).



**Fig. 4.** Ratio of absorbance,  $A/A_0$ , of supernatants versus the EtOH extraction time,  $t_e$ , (a) and ratio of absorbance,  $A/A_0$ , obtained at  $t_e = 500$  s versus the HVED treatment time,  $t_{HVED}$ , (b).

Here,  $A_0$  is the absorbance measured at  $t_e = 0$  s, the values of  $A/A_0$  were measured at two different wavelengths  $\lambda_v = 430$  nm (violet) and  $\lambda_r = 660$  nm (red). The data are presented for HVED ( $t_{HVED} = 4$  ms) and P samples (1<sup>st</sup> step), in both the cases the WE ( $t_e = 1$  h, 2<sup>nd</sup> step) procedure was applied.

In fact, the extracted pigments (carotenoids and chlorophylls) are poorly soluble in water, but they can present in water in the form of pigment-macromolecular water-soluble complexes (Zhang et al., 2019a). For further recovery of pigments in cells, the green solvent EtOH was used. **Fig. 4a** presents example of ratio of absorbance,  $A/A_0$ , of supernatants versus the extraction time,  $t_e$ , during the 3<sup>rd</sup> step of extraction. Here,  $A_0$  is the absorbance measured at  $t_e = 0$  s. The kinetic curves of carotenoids (430 nm) and chlorophylls (660 nm) were rather similar, the values of  $A/A_0$  increased with the increase of  $t_e$ , and reached equilibrium at  $t_e \approx 500$  s. For HVED ( $t_{HVED} = 4$  ms) sample, the EE extraction allowed up to the  $\approx 2$ -fold increase of pigments content (**Fig. 4a**). Note that the extraction was more efficient for carotenoids. The differences between extraction of carotenoids and chlorophylls in EtOH (3<sup>rd</sup> step) can reflect the different release of these pigments at the previous aqueous extraction step (2<sup>nd</sup> step). Moreover, the EtOH extraction efficiency of pigments was significantly higher for HVED ( $t_{HVED} = 4$  ms) samples than for P samples. For example, for chlorophylls (660 nm) after  $t_e = 500$  s of extraction the values of  $A/A_0 \approx 2.04$  and  $A/A_0 \approx 1.17$  were obtained for HVED ( $t_{HVED} = 4$  ms) and P samples. The corresponding content of chlorophyll *a* ( $\lambda_r = 660$  nm),  $C_{chl a}$ , were calculated using calibration curve for chlorophyll *a* (#C5753, Sigma-Aldrich, France) (See, Supplementary materials Fig. S3).



*Fig. S3. UV absorbance,  $A$ , at  $\lambda_r = 660$  nm versus the concentration of chlorophyll *a* in 95% EtOH,  $C$ , (calibration curve) (a); examples of the content of chlorophyll *a*,  $C_p$ , versus the extraction time,  $t_e$ , in EE procedure (3<sup>rd</sup> step) (b). Insert of Fig. S3a shows examples of UV absorption spectra at different concentration of chlorophyll *a*.*



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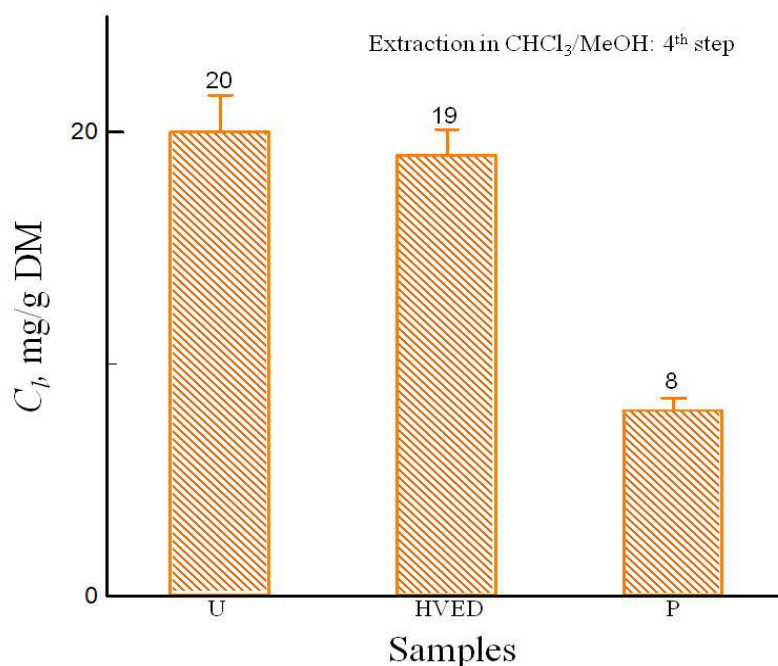
**Fig. 4b** presents ratio of absorbance,  $A/A_0$ , obtained at  $t_e = 500$  s versus the HVED treatment time,  $t_{HVED}$ , (3<sup>rd</sup> step). Here,  $A$  is absorbance of samples obtained after  $t_e = 500$  s. The value of  $A/A_0$  obtained for P samples are also shown (dashed lines). The highest values of  $A/A_0$  were obtained for HVED ( $t_{HVED} = 8$  ms) samples. The values of  $A/A_0$  obtained for U ( $t_{HVED} = 0$  ms) and HVED ( $t_{HVED} = 1$  ms) samples were comparable, but the value of  $A/A_0$  continuously increased at  $t_{HVED} \geq 2$  ms. For both the carotenoids (430 nm) and chlorophylls (660 nm), the values of  $A/A_0$  were significantly higher for HVED samples as compare with P samples. For example, for HVED ( $t_{HVED} = 8$  ms) samples, the values of  $A/A_0 \approx 2.49$  (430 nm) and  $A/A_0 \approx 2.27$  (660 nm) were almost  $\approx 2$ -fold higher than those for P samples. The small efficiency of P procedure at 3<sup>rd</sup> step can be explained by the following reasons. The application of HPH pretreatment leads to the intensive generation of cell debris and allowed good recovery of proteins into the aqueous phase. It can be speculated that losses of pigments before EtOH extraction (3<sup>rd</sup> step) is related with good adsorption affinity of pigment to the cell debris and formation of pigment-proteins complexes during the aqueous extraction step (Zhang et al., 2019a). These parts of pigment content can be removed from supernatant as a result of centrifugation after the 2<sup>nd</sup> step.

At the 4<sup>th</sup> step, the supplementary extraction of lipids from U, HVED ( $t_{HVED} = 8$  ms) and P samples was studied using the  $\text{CHCl}_3/\text{MeOH}$  mixture of solvents. For determination of total (initial) content of lipids in biomass, the washed biomass was used. The washing procedure: the biomass was diluted to 1%, agitated at 150 rpm for 10 min, and centrifuged for 10 min at 4600 g. Then supernatant was removed and the washing procedure was repeated 3 times. Finally, the sediment was separated and freeze-drying lyophilisation was applied for 64 h at  $-20$  °C using a MUT 002A pilot freeze-drier (Cryotec, France). In this condition, the total content of lipids in biomass was  $39 \pm 1.2$  mg/g DM.

**Fig. 5** compares the lipids content,  $C_l$ , in sediment obtained at 4<sup>th</sup> step of extraction for U, HVED ( $t_{HVED} = 8$  ms), and P samples. The values of  $C_l$  decreased at 4<sup>th</sup> step for all the samples and they were significantly smaller as compared with total content of lipids in the biomass. Obtained data allowed concluding that the prior applications of 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> steps may results in loss of some quantity of lipids from the biomass.

The recovery of lipids was the smallest for the P samples where the lipids content decreased up to  $C_l \approx 8 \pm 0.5$  mg/g DM. It demonstrated the presence of significant losses of

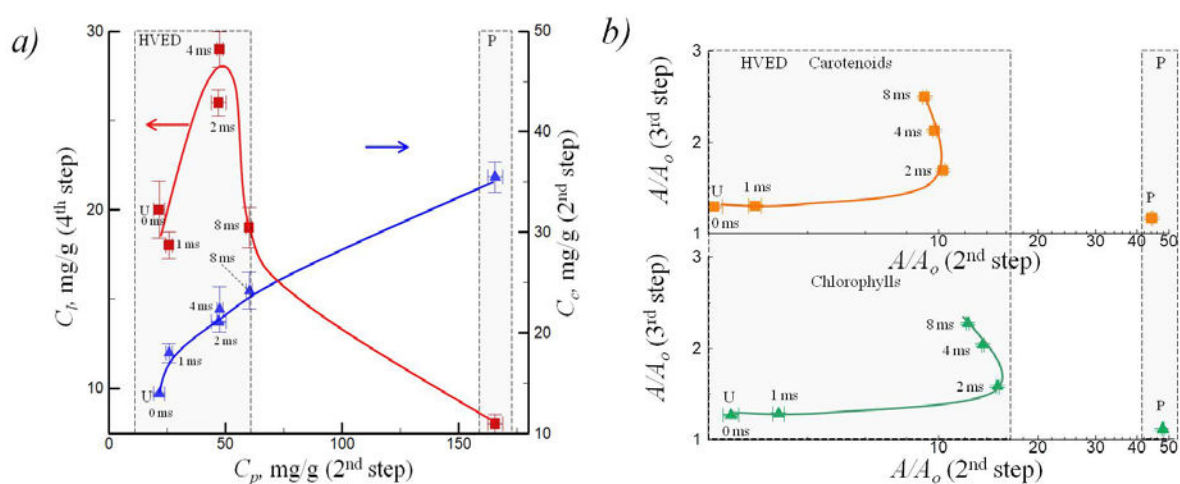
lipids for P samples prior the extraction in the  $\text{CHCl}_3/\text{MeOH}$  mixture of solvents. Moreover, for the P samples, the extraction of lipids during the 3<sup>rd</sup> step (EE procedure) may be also important. For example, the results of the study of the effects of different solvent on extraction of lipids from microalga *Choricystis minor var. minor*. evidenced about the similar extraction yields in EtOH (30.5%) and  $\text{CHCl}_3/\text{MeOH}$  mixture (30.9%) (da Cruz Lima et al., 2018).



**Fig. 5.** The lipids content,  $C_l$ , extracted at the 4<sup>th</sup> step for U, HVED ( $t_{HVED} = 8$  ms) and P samples. The total lipids content in biomass was  $C_l = 39 \pm 1.2$  mg/g DM.

**Fig. 6** presents correlations between extraction efficiencies of different components obtained during different extraction steps for U, HVED and P samples. For the 2<sup>nd</sup> step, the direct proportionality between content of carbohydrates,  $C_c$ , and proteins,  $C_p$ , was observed (**Fig. 6a**). This is in line with the existing data on efficiency of HPH and HVED assisted extraction of bio-molecules from microalgae (for a review, see (Vorobiev & Lebovka, 2020; Zhang, Parniakov, et al., 2019). The amount of lipids,  $C_l$ , extracted at the 4<sup>th</sup> step gone through the maximum with increasing of content of proteins,  $C_p$ , extracted at the 2<sup>nd</sup> step. However, the maximum lipids contents at 4<sup>th</sup> step can be obtained for relatively moderate HVED treatment at  $t_{HVED} = 2$  and 4 ms (**Fig. 6a**). Moreover, for P samples, the minimum extraction of lipid at the 4<sup>th</sup> step and maximum extraction of proteins at the 2<sup>nd</sup> step were observed. Hence, the intensive HVED treatment is desirable for effective extraction of

proteins but it can be undesirable for extraction of lipids. The correlations for extractions of pigments (both the carotenoids and chlorophylls) obtained at the 2<sup>nd</sup> and 3<sup>rd</sup> steps are presented in **Fig. 6b**. For P samples, the maximum extraction of pigments at the 2<sup>nd</sup> step corresponds to the minimum extraction of pigments at the 3<sup>rd</sup> step. It simply reflects to mass balance of intracellular pigments for extraction assisted severe disruption technique, HPH treatment. However, for HVED samples, the effects of pigment extraction were dependent upon HVED protocol. The maximum extraction at 2<sup>nd</sup> step was observed at the moderate HVED treatment ( $t_{HVED} = 2$  ms) and at the maximum extraction at 3<sup>rd</sup> step require the more intensive HVED treatment ( $t_{HVED} = 8$  ms).



**Fig. 6.** Correlation between lipids content,  $C_l$ , (4<sup>th</sup> step), carbohydrates content,  $C_c$ , (2<sup>nd</sup> step) and proteins content,  $C_p$ , (2<sup>nd</sup> step) (a); correlations of absorbance ratios,  $A/A_o$ , for 2<sup>nd</sup> and 3<sup>rd</sup> steps of extraction (b) obtained for different physical pre-treatment procedures (U, HVED and P samples).

#### 4. Conclusions

The efficiency of multi-step extraction procedure for the valorisation of *P. tricornutum* biomass has been investigated. The extraction procedures included physical pre-treatments (HVED or HPH, 1<sup>st</sup> step), aqueous extraction for 1 h (2<sup>nd</sup> step), EtOH extraction (3<sup>rd</sup> step), and lipids extraction in the  $\text{CHCl}_3/\text{MeOH}$  (4<sup>th</sup> step). At the 1<sup>st</sup> step, the HPH treatment was more effective for microalgal cell disruption and extraction of water-soluble compounds (carbohydrates and proteins). However, HVED has more selective for extraction ionic components. The 2<sup>nd</sup> step allowed further supplementary release water-soluble compounds. For non-aqueous extractions (3<sup>rd</sup> and 4<sup>th</sup> steps), the extraction of pigments and lipids with

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assistance of HVED was more efficient in comparison with HPH. The combined multistage extraction procedures assisted by HVED or HPH allow selective and cleaner extraction of individual bio-molecules soluble in water or organic solvent. However, further LCA (life cycle assessment) studies are necessary (Bussa, Eisen, Zollfrank, & Röder, 2019) to optimize the industrial implementation of the proposed multistage extraction techniques from microalgal biomass. This study provided an integrated ideas and methods for improvements of HPH and HVED assisted techniques in valorisation of microalgal biomass. For industry application, the feasibility study can be done in the future studies.

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### **Conflict of interest**

The authors declare that they have no conflict of interest.

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V.3 *Article 6*: Two-step procedure for selective recovery of bio-molecules from microalga *Nannochloropsis oculata* assisted by high voltage electrical discharges

(The article is presented on the following pages)

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**Two-step procedure for selective recovery of bio-molecules  
from microalga *Nannochloropsis oculata* assisted by high voltage electrical  
discharges**

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## Abstract

Two-step procedure with the initial aqueous extraction from raw microalga *Nannochloropsis oculata* and secondary organic solvent extraction from vacuum dried (VD) microalgae were applied for selective recovery of bio-molecules. The effects of preliminary aqueous washing and high voltage electrical discharges (HVED, 40 kV/cm, 4 ms pulses) were tested. The positive effects of HVED treatment and washing on selectivity of aqueous extraction of ionics and other water-soluble compounds (carbohydrates, proteins and pigments) were observed. Moreover, the HVED treatment allowed improving the kinetic of vacuum drying, and significant effects of HVED treatment on organic solvent extraction of chlorophylls, carotenoids and lipids were determined. The proposed two-step procedure combining the preliminary washing, HVED treatment and aqueous/organic solvents extraction steps are useful for selective extraction of different bio-molecules from microalgae biomass.

**Keywords:** Microalgae; High voltage electrical discharges; Vacuum drying; Pigments; Lipids

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## 1. Introduction

Nowadays, the recovery of bio-molecules from microalgae has attracted wide attention of academic and industrial researchers (Mittal and Raghavarao, 2018). Microalgae have high growth rate, photosynthetic efficiency, worldwide distribution, and valuable bio-contents (Daneshvar et al., 2018). Due to high proportion of proteins and micronutrients in extracts (Buchmann et al., 2019), they present promising source for food and feed production. Moreover, the extracted pigments and polyphenols from microalgae can be used in cosmetics and pharmaceutical industries (Rivera et al., 2018), and their lipid extracts can serve as raw material for biofuels (Talebi et al., 2013).

*Nannochloropsis* sp. are a marine green microalgae belonging to the Eustigmataceae family (Parniakov et al., 2015). The major photosynthetic pigments are violaxanthin, vaucheraxanthin, and chlorophylls (Reboloso-Fuentes et al., 2001). In favorable growing conditions (with adjustable temperature, salinity and additives), they can also accumulate considerable amounts of lipids ranging from 12 to 60% w/w (Chiu et al., 2009; Doan and Obbard, 2015; Mitra et al., 2015). However, the extraction of bio-molecules from *Nannochloropsis* sp. is not easy task. Their cells are near spherical with relatively small size ( $\approx 2.5 \mu\text{m}$ ) and they are covered by rather thick rigid walls ( $\approx 60\text{-}110 \text{ nm}$ ) (Gerken et al., 2013). To facilitate extraction of intracellular compounds, different chemical, enzymatic (Zhu et al., 2018a) and physical methods (Zhu et al., 2018b) have been tested (for a recent review see (Zhang et al., 2018b)).

The applications of pulsed electric energy (pulsed electric fields (PEF) (Parniakov et al., 2015a, 2015b) and high voltage electrical discharges (HVED)) (Grimi et al., 2014) for recovery intracellular compounds from *Nannochloropsis* sp. have been reported. The PEF provoke electroporation of cell membranes, while the HVED can provoke the damage of cell walls due to electrical breakdown and different secondary phenomena (liquid turbulence, intense mixing, shock waves, and bubble cavitation, etc) (Barba et al., 2015). They can be easily done for algal slurries with the high moisture content of ( $\approx 80\% \text{ wt}$ ). However, the effective extraction of hydrophobic bio-molecules, such as pigments, lipids and phenolic compounds requires applications of more complex techniques, including organic solvents extraction (Barba et al., 2015), high pressure homogenization (Zhang et al., 2018a) and ultrasonication (Zhang et al., 2019), etc.

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Moreover, the extraction of hydrophobic bio-molecules requires drying of algal slurries with significant reducing of a moisture content up to  $\approx 10\%$  that is time and energy consuming process (Ansari et al., 2018; Bagchi et al., 2015; Hosseinizand et al., 2018). Vacuum drying (VD) has been recently applied for processing of algal cells (Makkar et al., 2016). Effects of VD on seaweed *Pyropia orbicularis* at the pressure of 15 kPa and drying temperatures at 40-80 °C were investigated (Uribe et al., 2018). The high recovery yields of total phenolic, carotenoids, phycoerythrin and phycocyanin were demonstrated. Furthermore, the pre-treatment by pulsed electric energy can also affect the efficiency of VD (Liu et al., 2018).

The main aim of this work was efficiency testing of the two-step procedure for selective recovery of bio-molecules from microalga *Nannochloropsis oculata* (*N. oculata*) assisted by HVED. The procedure combined washing with HVED treatment at the initial aqueous extraction step, and VD before at the final non-aqueous extraction step. The effects of HVED pre-treatment on the extraction efficiencies of hydrophilic components (ionics, carbohydrates, proteins and water-soluble pigments) (first step), and hydrophobic components (pigments and lipids) (second step) were investigated. Moreover, the impact of HVED treatment on VD kinetics was evaluated for the first time.

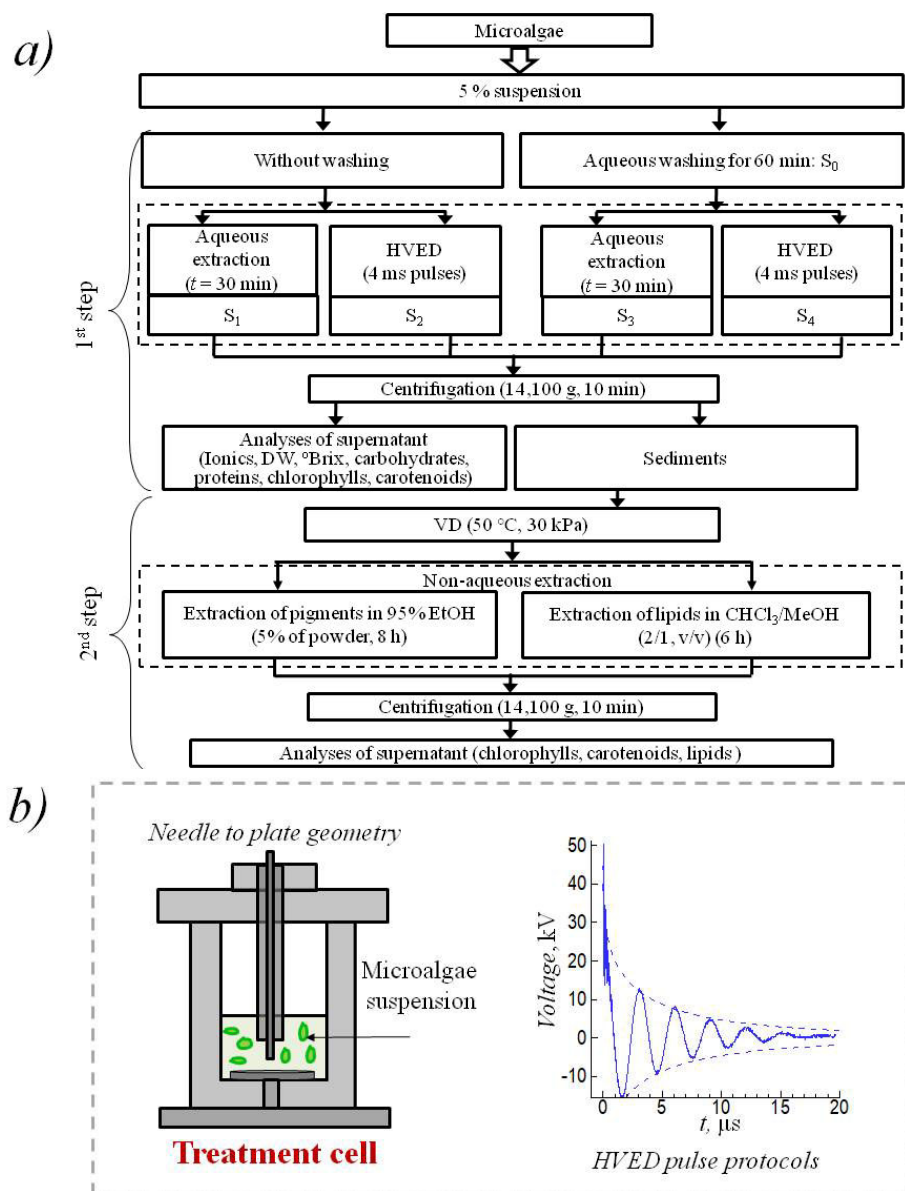
## 2. Materials and methods

### 2.1 Microalgae

Microalga *Nannochloropsis oculata* (*N. oculata*) (provided by AlgoSolis, Saint-Nazaire, France) was obtained as a frozen paste. The moisture content of biomass was measured at 105 °C for 24 h. Accounting for this content, the biomass, at the initial step, was diluted with deionized water to obtain 5% dry matter (DM) concentration.

### 2.2 Design of experiments

**Fig. 1a** presents the schema of experiments applied in the present study. It includes the preparation of samples (without or with preliminary washing), HVED treatment and aqueous extraction (first step for extraction of water-soluble components), and VD of sediments and organic solvent extraction (second step for extraction of pigments and lipids).



**Fig. 1.** The schema of the applied extraction procedures (a), and high voltage electrical discharges (HVED) treatment cell applied in the present experiment (b).

### 2.2.1. Preparation of the samples

In the supplied biomass paste, the water-soluble components were present in the extracellular aqueous solution. In order to evaluate effects of these components on extraction efficiency for different applied procedures, the samples with and without washing were prepared.

The initial samples with preliminary washing for 60 min were designed as S<sub>0</sub>. The aqueous extraction for 30 min was performed for biomass (both unwashed and washed) (**Fig.**



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**1a).** Samples processed without preliminary washing were designed as S<sub>1</sub> (untreated) and S<sub>2</sub> (HVED treated). Samples processed with preliminary washing were designed as S<sub>3</sub> (untreated) and S<sub>4</sub> (HVED treated). In the first washing step, the suspension was diluted to 1% DM, agitated at 150 rpm for 10 min, and centrifuged for 10 min at 4600 g. Then supernatant was removed and sediment was diluted to 1% DM and the next washing step was applied. The duration of one washing step was 20 min. The analysis of supernatant for presence ionic components, proteins, and carbohydrates was done (see Section 2.3 for the details).

### 2.2.2. High voltage electrical discharges (HVED) treatment

HVED treatment was applied using a high voltage pulsed power 40 kV-10 kA generator (Basis, Saint-Quentin, France). HVED treatment was done in a 1-L cylindrical batch treatment cell with an electrode of needle-plate geometry (**Fig. 1b**). The distance between stainless steel needle and grounded plate was fixed to 1 cm, which corresponding to  $E = 40$  kV/cm of electric field strength. HVED treatment comprised of the application of  $n$  successive pulses ( $n = 1-400$ ) and a pulse repetition rate of 0.5 Hz. The total time of electrical treatment was 0.01-4 ms. This discharge protocol with  $E = 40$  kV/cm and  $n = 400$  was shown to be effective for extraction of water-soluble proteins (Grimi et al., 2014). The damped oscillations with effective decay time  $t_p \approx 10 \pm 0.1 \mu\text{s}$  were observed in HVED mode (**Fig. 1b**). The 2 min of pause was done after each 100 pulses to maintain temperature elevation after HVED treatment never exceeded 30 °C. The total operation time for HVED experiment is 30 min. The 200 g of suspension of microalgae (5% DM) was used in this study (samples S<sub>2</sub> and S<sub>4</sub>). The samples without HVED treatment keep for 30 min was used as control (samples S<sub>1</sub> and S<sub>3</sub>). Then these samples were centrifuged at 14,100 g for 10 min using a MiniSpin Plus Rotor F-45-12-11 (Eppendorf, France). The supernatants were used for analyzing of extracts and the sediments were dehydrated by VD.

### 2.2.3. Vacuum drying (VD) of sediments

The sediments were mixed with absolute EtOH (2:1, w/w). The 10 g of mixture were spread uniformly with 7 mm of initial thickness in Petri dishes (6 cm inner diameter and 2.5 cm inner depth) and kept for VD. The absolute EtOH was added to centrifuged samples to facilitate detachment of cells from the centrifugation glass tubes. Then the EtOH was evaporated quickly from the samples. VD experiment was carried out in a vacuum chamber (Cole-Parmer, USA) connected with a vacuum pump (Rietschle, Germany). The pressure of

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drying chamber was maintained at 30 kPa and the drying temperature was fixed at 50 °C. The initial temperature of samples ( $\approx 25^\circ\text{C}$ ) was measured before VD experiment. During the drying, the temperature inside centre of the sample,  $T$ , was recorded in the online mode using a thermocouple (K-type, NiCr–Ni). The weight of the sample,  $m$ , was measured using a balance (GF-600, A & D, Japan).

The moisture ratio,  $MR$ , of the sample during the drying was calculated as follows:

$$MR = (m - m_f) / (m_i - m_f) \quad (1)$$

where  $m$  is the running weight of the sample, and the subscripts  $i$  and  $f$  refer to the initial and final (completely dried) values, respectively. In experiments, the final (completely dried) value was determined by oven drying samples at 105 °C for 24 h.

After VD processing, the dried biomass was respectively used for analysis of pigments and lipids extraction.

### 2.3 Analysis of extracts

#### 2.3.1 Aqueous extracts

The following aqueous suspensions were centrifuged at 14,100 g for 10 min. The supernatants were used for analyzing microalgae extracts. All characterization measurements were done at ambient temperature.

The extent of the releasing of ionic components was characterized by measurements of the electrical conductivity by the instrument InoLab pH/cond Level 1 (WTW, Weilheim, Germany). The soluble matter content (°Brix) was measured by a digital Atago refractometer (PR-101, Atago, 50 Tokyo, Japan). For determination of dry weight content (DW), 150 mL of supernatant was placed in glass beaker and dried in an oven at 105 °C for 24 h. The value of DW was gravimetrically determined by weighting the samples before and after drying. The results were expressed as mg of dry matter/g of supernatant. The contents of carbohydrates,  $C_c$ , and proteins,  $C_p$ , were determined using a phenol-sulfuric acid (Dubois et al., 1956) and Bradford's method (Bradford, 1976), respectively. The pigments were determined using spectroscopic-based techniques by UV/vis spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA).

Briefly, for determination of the content of carbohydrates, D-glucose standard provided by Sigma-Aldrich (Saint-Quentin Fallavier, France) was used for the calibration curve. The 1 mL of supernatants (diluted if required), 0.1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid were mixed in glass tubes. The mixture was incubated at 20 °C for 20 min. Absorbance was measured using at the wavelength of 490 nm.

For determination of the content of proteins, the diluted supernatant (0.1 mL) was mixed 1 mL of Bradford Dye Reagent (Thermo Fisher, Kandel, Germany) and kept for 5 min. The absorbance was measured at the wavelength of 595 nm. Bovine serum albumin (BSA) provided by Sigma-Aldrich (Saint-Quentin Fallavier, France) was used for the calibration curve.

For determination of the content of pigments, the supernatant was mixed with EtOH (95%, v/v) (50 µL sample + 950 µL 95% EtOH). The absorbances of chlorophyll *a*, chlorophyll *b*, and total carotenoids were measured at the wavelengths of 664, 649 and 470 nm using 95% EtOH as blank. The concentrations of chlorophyll *a*,  $C_{ch}^a$ , chlorophyll *b*,  $C_{ch}^b$ , total chlorophylls,  $C_{ch}$ , and total carotenoids,  $C_{cr}$ , (µg pigment/mL supernatant) were calculated using the following equations (Gerde et al., 2012):

$$C_{ch}^a = 13.36 \times A_1 - 5.19 \times A_2, \quad (2a)$$

$$C_{ch}^b = 27.43 \times A_2 - 8.12 \times A_1, \quad (2b)$$

$$C_{ch} = C_{ch}^a + C_{ch}^b, \quad (2c)$$

$$C_{cr} = (1000 \times A_3 - 2.13 \times C_{ch}^a - 97.64 \times C_{ch}^b) / 209, \quad (2d)$$

where  $A_1$ ,  $A_2$ ,  $A_3$  are the absorbances measured at the wavelengths of 664, 649, and 470 nm, respectively.

The content of components released from microalgae was expressed as mg/g dry microalgae.

### 2.3.2 Organic solvent extracts

For analysis of extraction of pigments, the biomass obtained by VD with a final *MR* of 0.01 and 0.2 was diluted with 95% EtOH to solid-liquid ratio of 1: 20. The extraction was

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studied for 8 h under the stirring at 150 rpm. To avoid any evaporation, the extraction cells were covered with aluminum foil during the extraction process.

For analysis of extraction of lipids, the standard lipid extraction procedure was used according to the “whole cell analysis” (WCA) method (Van Vooren et al., 2012). Briefly, in order to prevent oxidation of lipids, the dried samples ( $MR_f = 0.01$ ) were first mixed with 20  $\mu\text{L}$  distilled water and 10  $\mu\text{L}$  butylated hydroxytoluene (BHT, 20  $\mu\text{g}/\mu\text{L}$ ) in clean vials. Then the mixture was suspended in 6 mL of a chloroform/methanol ( $\text{CHCl}_3$ : MeOH, 2:1, v/v) mixture. Vials were maintained for 6 h in the dark under slow agitation. After extraction, the organic and aqueous phases were separated and the solvent of the extracts was evaporated under  $\text{N}_2$  flux. 1 mL of  $\text{CHCl}_3/\text{MeOH}$  (2/1, v/v) mixture was then added and stored at  $-20\text{ }^\circ\text{C}$  until analysis. Total fatty acids (TFA) contents in the lipid extracts were quantified by Gas Chromatography-Flame Ionization Detector (GC-FID) (Agilent Technologies Inc., Santa Clara, CA) analysis. Fatty acid methyl ester (FAME) contents in the lipid extracts were quantified after a transesterification step. More details can be found in (Van Vooren et al., 2012). The values of the total lipids content,  $C_l$ , and relative content of fatty acids (saturated fatty acids (SFA, all single bonds between carbon atoms), monounsaturated fatty acids (MUFA, one double bond) and polyunsaturated fatty acids (PUFA, at least two double bonds) were evaluated.

### 2.3.3 Content of bio-molecules in totally disintegrated cells

For total disintegration of cells, the biomass was grinded using a bead-beating method at 30 Hz (MM400 mixer mill, Retsch GmbH & co. KG, Haan, Germany). In this method, the cells are mechanically disrupted by ceramic beads in the reaction vials. For determination of maximum content of proteins, carbohydrates, chlorophylls, and carotenoids in microalgae the washing procedure was initially applied. The biomass was diluted to 1% DM, agitated at 150 rpm for 10 min, and centrifuged for 10 min at 4,600 g. Then supernatant was removed and the washing procedure was repeated 3 times. Finally, the sediment was separated and lyophilization was applied for 64 h at  $-20\text{ }^\circ\text{C}$  using a MUT 002A pilot freeze-drier (Cryotec, France). The final  $MR$  of the sample was 0.08. Then the dried biomass was grinded in a wet mode. The dried biomass was initially diluted with distilled water for analysis of proteins and carbohydrates and 95% EtOH for analysis of chlorophylls and carotenoids. The solid-liquid ratio was 1: 20 and the grinding was done for 15 min. To avoid overheating during the grindings the 15 s pauses after each minute were applied. The maximum contents were

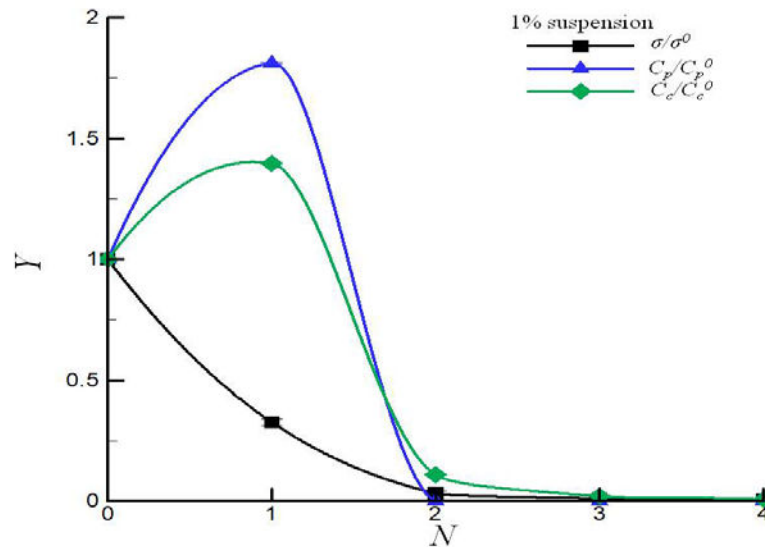
obtained:  $C_p = 47.67 \pm 0.83$  mg/g DM (proteins),  $C_c = 67.36 \pm 0.46$  mg/g DM (carbohydrates),  $C_{ch} = 1.17 \pm 0.01$  mg/g DM (total chlorophylls),  $C_{cr} = 0.256 \pm 0.001$  mg/g DM (carotenoids).

#### 2.4 Statistical analysis

Each experiment was replicated three to five times. The error bars, presented on the figures, correspond to the standard deviations. One-way analysis of variance (ANOVA) was used to determine significant differences ( $p < 0.05$ ) among the samples with the help of OriginPro 8.5 (OriginLab Corporation, USA). Differences between means were detected using Tukey's test.

### 3. Results and Discussion

#### 3.1. Preliminary washing



**Fig. 2.** Relative quantities,  $Y$ , ( $Y = \sigma/\sigma^0$  for electrical conductivity,  $Y = C_p/C_p^0$  for concentration of proteins, and  $Y = C_c/C_c^0$  for concentration of carbohydrates) versus the number of washing steps,  $N$ . The  $\sigma^0$ ,  $C_p^0$ , and  $C_c^0$  are the initial values before washing. The measurements were done for 1% dry matter (DM) suspensions.

**Fig. 2** presents relative electrical conductivity,  $Y = \sigma/\sigma^0$ , concentration of proteins,  $Y = C_p/C_p^0$ , and concentration of carbohydrates,  $Y = C_c/C_c^0$ , versus the number of washing steps,  $N$ . Here, the  $\sigma^0$ ,  $C_p^0$ , and  $C_c^0$  correspond to the values for the initial suspension before washing. The relative electrical conductivity,  $Y = \sigma/\sigma^0$ , continuously decreased with increase of  $N$  that corresponds to the dilution of ionic solution in the extracellular aqueous solution. In contrast,

the values of  $Y = C_p/C_p^o$  (proteins) and  $Y = C_c/C_c^o$  (carbohydrates) remarkably increased after the first washing step and then decreased. The observed phenomena can be explained by release after first washing a certain amount of water-soluble proteins and carbohydrates initially captured on the surface of algal cells. Obtained data evidenced that application of washing for three times allows significant purification of extracellular solution from water-soluble components. Therefore, in our experiments three washing step of 60 min were applied.

### 3.2. Aqueous extraction

*Table S1 Comparisons of contents of different bio-molecules for untreated suspensions in the samples S<sub>1</sub> and S<sub>3</sub>: Electrical conductivity,  $\sigma$ , soluble matter, °Brix, dry weight, DW, contents of carbohydrates,  $C_c$ , proteins,  $C_p$ , chlorophylls,  $C_{ch}$ , and carotenoids,  $C_{cr}$ , for the untreated samples (S<sub>1</sub>, without washing) and (S<sub>3</sub>, with washing).*

	Conductivity ( $\sigma$ , mS/cm)	°Brix (%)	Dry weight (DW, mg/g supernatant)	Carbohydrates ( $C_c$ , mg/g)
S <sub>1</sub>	9.37 ± 0.042 <sup>a</sup>	2.0 ± 0.001 <sup>a</sup>	11.88 ± 0.53 <sup>a</sup>	26.76 ± 0.81 <sup>a</sup>
S <sub>3</sub>	1.057 ± 0.008 <sup>b</sup>	0.9 ± 0.001 <sup>b</sup>	2.33 ± 0.11 <sup>b</sup>	14.32 ± 1.17 <sup>b</sup>

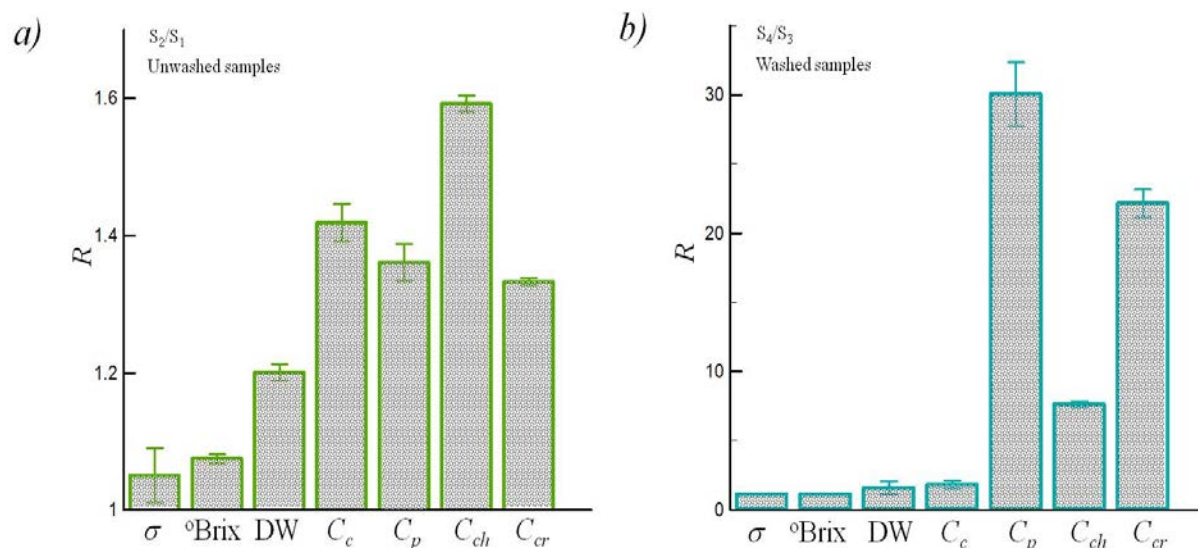
	Proteins ( $C_p$ , mg/g)	Chlorophylls ( $C_{ch}$ , mg/100g)	Carotenoids ( $C_{cr}$ , mg/100g)
S <sub>1</sub>	24.09 ± 0.93 <sup>a</sup>	1.74 ± 0.0012 <sup>a</sup>	0.03 ± 0.0001 <sup>a</sup>
S <sub>3</sub>	0.61 ± 0.06 <sup>b</sup>	0.29 ± 0.0005 <sup>b</sup>	0.0023 ± 0.0001 <sup>b</sup>

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test.

<sup>a</sup> or <sup>b</sup> letters within the same column indicate a significant difference (p < 0.05) according to Tukey's test.

In the first step, the aqueous extraction was applied (**Fig. 1a**). The contents of different bio-molecules (electrical conductivity,  $\sigma$ , soluble matter, °Brix, dry weight, DW, contents of carbohydrates,  $C_c$ , proteins,  $C_p$ , chlorophylls,  $C_{ch}$ , and carotenoids,  $C_{cr}$ ) for the untreated samples (S<sub>1</sub>, without preliminary washing) and (S<sub>3</sub>, with preliminary washing) were compared. All characteristics obtained for the samples S<sub>1</sub> and S<sub>3</sub> were significant different ( $p < 0.05$ ). For example, the content of carbohydrates obtained for the sample S<sub>1</sub> ( $C_c = 26.76 \pm 0.81$  mg/g) was  $\approx 2$  folds higher than those obtained for the sample S<sub>3</sub> ( $C_c = 14.32 \pm 1.17$  mg/g). The content of proteins obtained for the sample S<sub>1</sub> ( $C_p = 24.09 \pm 0.93$  mg/g) was  $\approx 39$  folds higher than those obtained for the sample S<sub>3</sub> ( $C_p = 0.61 \pm 0.06$  mg/g). Electrical conductivity, the

contents of chlorophylls and carotenoids obtained for the sample  $S_1$  were also significantly higher (approximately one order of magnitude) than those obtained for the sample  $S_3$  ( $p < 0.05$ ). This fact can be explained by the presence of some compounds on the surface of the microalgal cells that can be released by procedure of washing.



**Fig. 3.** The ratios of different measured values,  $R = \text{high voltage electrical discharges (HVED) treated/untreated}$ , (electrical conductivity,  $\sigma$ ; °Brix, dry weight, DW; contents of carbohydrates,  $C_c$ , proteins,  $C_p$ , chlorophylls,  $C_{ch}$ , and carotenoids,  $C_{cr}$ ) obtained for unwashed samples ( $R = S_2/S_1$ ) (a) and for washed samples ( $R = S_4/S_3$ ) (b).

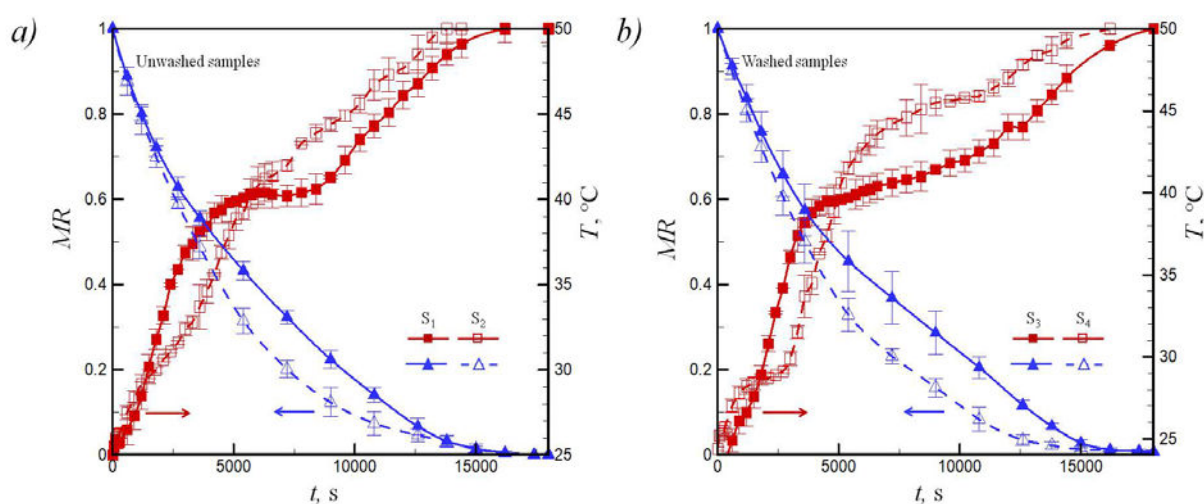
Moreover, the effect of HVED treatment on extraction of different components can be characterized by ratio of different measured values,  $R = \text{HVED treated/untreated}$ . **Fig. 3** presents the values of  $R$ , (ratios of electrical conductivity,  $\sigma$ ; soluble matter, °Brix; dry weight, DW; contents of carbohydrates,  $C_c$ , proteins,  $C_p$ , chlorophylls,  $C_{ch}$ , and carotenoids,  $C_{cr}$ ) obtained for unwashed samples ( $R = S_2/S_1$ ) (a) and for washed samples ( $R = S_4/S_3$ ) (b). In all cases, the values of  $R$  were higher than 1. It reflects the positive effect of HVED treatment on extraction of intracellular components. For unwashed samples, the maximum ratios were observed for total chlorophylls ( $R \approx 1.59 \pm 0.01$ ), carbohydrates ( $R \approx 1.42 \pm 0.03$ ), proteins ( $R \approx 1.36 \pm 0.03$ ) and carotenoids ( $R \approx 1.33 \pm 0.01$ ). For washed samples, the maximum ratios were observed for proteins ( $R \approx 30.1 \pm 2.3$ ), carotenoids ( $R \approx 22 \pm 1.0$ ), and total chlorophylls ( $R \approx 7.6 \pm 0.2$ ). The obtained data evidenced the high efficiency of HVED application for recovery of different bio-molecules. It can be explained by the feasibility of HVED to electroporate the cell membrane and induce damage the microalgal cell walls (Grimi et al.,



2014). All these findings also confirmed the possibility to attain the selective extraction of different bio-molecules by using the different modes of washing combined with HVED treatment.

### 3.3. Organic solvent extraction

In the second step, the organic solvent extraction was proceeded. To remove water from sediment the vacuum drying was initially performed. **Fig. 4** presents the moisture ratio,  $MR$ , and temperature inside centre of the sample,  $T$ , versus the drying time,  $t$ , for untreated (solid lines, filled symbols) and HVED treated (dashed lines, open symbols) suspensions of unwashed samples ( $S_1$  and  $S_2$ ) (a) and washed samples ( $S_3$  and  $S_4$ ) (b). During the VD, the values of  $MR$  continuously decreased for all samples ( $S_1$ - $S_4$ ), and HVED treatment noticeably accelerated the drying processes. No significant differences in  $MR(t)$  were observed for samples without and with washing. Three different stages of the variation of temperatures  $T(t)$  during the VD were observed. During the first heating stage, the initial increase of temperature with water evaporation from the surface of slurry was observed. During the drying stage, the intensive evaporation of moisture with the stabilization of the temperature at near constant temperatures level of  $T \approx 40$  °C was observed. Finally, at long drying time and relatively low  $MR$  below  $\approx 0.2$ , the reduced drying rate stage with further increase in temperature up to the temperature of VD chamber,  $T = 50$  °C, was observed.



**Fig. 4.** Moisture ratio,  $MR$ , and temperature inside centre of the sample,  $T$ , versus the vacuum drying (VD) time,  $t$ , for unwashed samples ( $S_1$  and  $S_2$ ) (a) and washed samples ( $S_3$  and  $S_4$ ) (b) obtained for untreated (solid lines, filled symbols) and high voltage electrical discharges (HVED) treated (dashed lines, open symbols) suspensions.



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The evolutions of temperature  $T(t)$  were rather different for untreated (samples  $S_1$  and  $S_3$ ) and HVED treated (samples  $S_2$  and  $S_4$ ) suspensions. Particularly, periods of the near constant temperature ( $T \approx 40$  °C) were more clearly displayed for untreated suspensions (they lasted for  $\approx 4000$ - $7500$  s, samples  $S_1$  and  $S_3$ ), and the reduced drying rate stages were started earlier for HVED treated suspensions (they lasted for  $\approx 6000$  s for sample  $S_2$ , and for  $\approx 5000$  s for sample  $S_4$ ). The HVED treatment accelerated the drying, and the final moisture content of  $MR = 0.01$  required VD time of  $\approx 16200$  s and  $\approx 15000$  s for untreated (samples  $S_1$  and  $S_3$ ) and HVED treated ( $S_2$  and  $S_4$  samples) suspensions, respectively. It evidently reflected positive effects of HVED treatment of acceleration of VD process.

### 3.3.1 Extraction of pigments

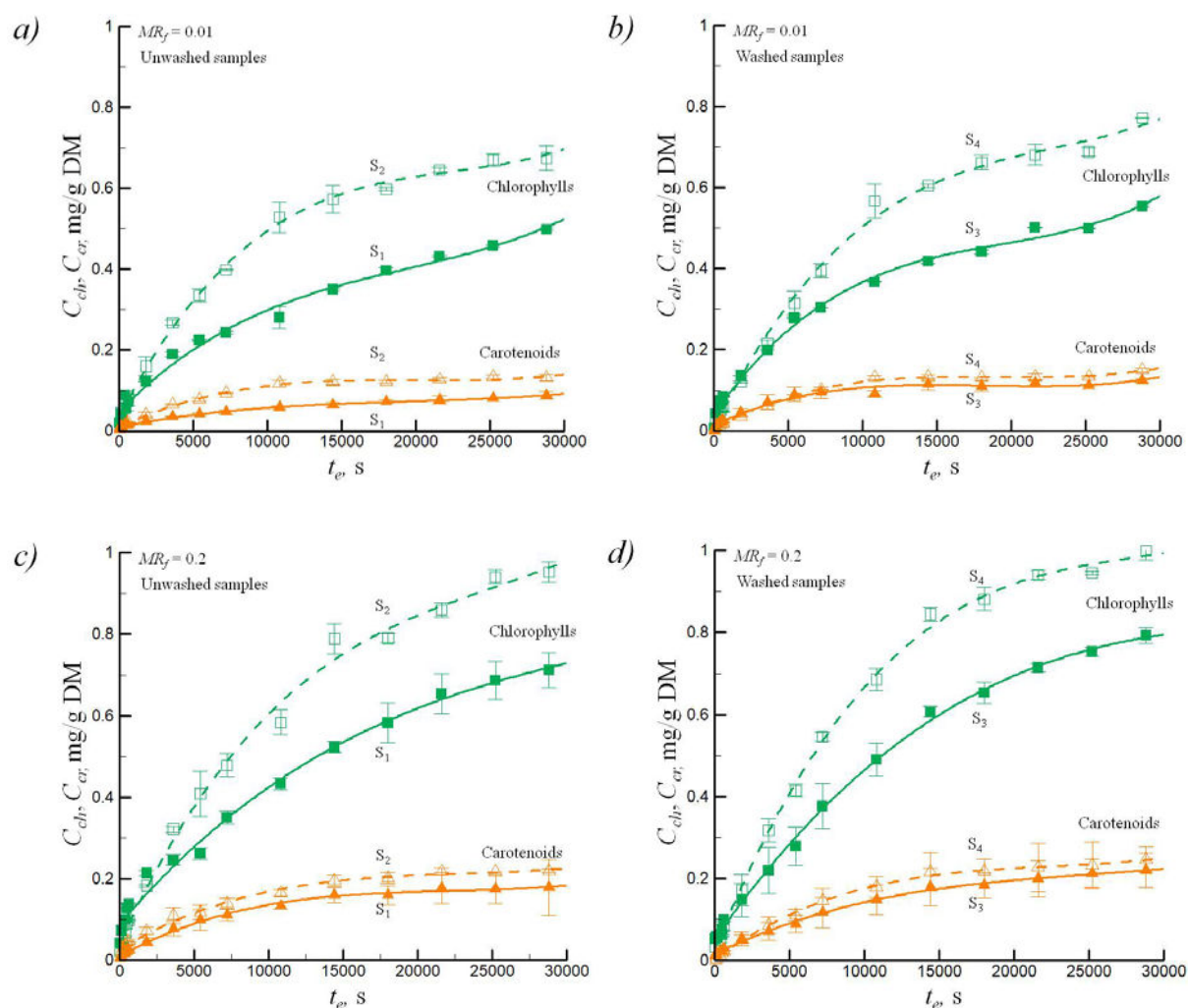
**Fig. 5** presents the examples of extraction kinetics of pigments for unwashed ( $S_1$  and  $S_2$ ) and washed ( $S_3$  and  $S_4$ ) microalgae with different final values of  $MR_f$  after VD ( $MR_f = 0.01$  and  $0.2$ ). The content of total chlorophylls,  $C_{ch}$ , continuously increased with increasing of extraction time,  $t_e$ , and no saturation was observed even at relatively long extraction time of  $t_e = 28800$  s (8 h). For carotenoids, the near-saturation behaviour was observed for extraction time above  $t_e \approx 10000$ - $15000$  s.

For HVED pretreated samples, the larger contents of extracted chlorophylls and carotenoids were obtained. For example, for samples with  $t_e = 28800$  s and  $MR_f = 0.2$ , the following values were obtained:

$C_{ch} \approx 0.95$  mg/g DM (unwashed),  $C_{ch} \approx 1$  mg/g DM (washed) for HVED treated suspensions;

$C_{ch} \approx 0.71$  mg/g DM (unwashed),  $C_{ch} \approx 0.80$  mg/g DM (washed) for untreated suspensions.

The washing favored the extraction efficiency, but the differences for unwashed (**Fig. 5a, c**) and washed (**Fig. 5b, d**) were less significant. However, the extraction efficiency of both chlorophylls and carotenoids for less dried samples with  $MR_f = 0.2$  was significantly higher than for the samples with  $MR_f = 0.01$ . It evidently reflects the retardation of extraction from overdried samples with less developed porous structure.

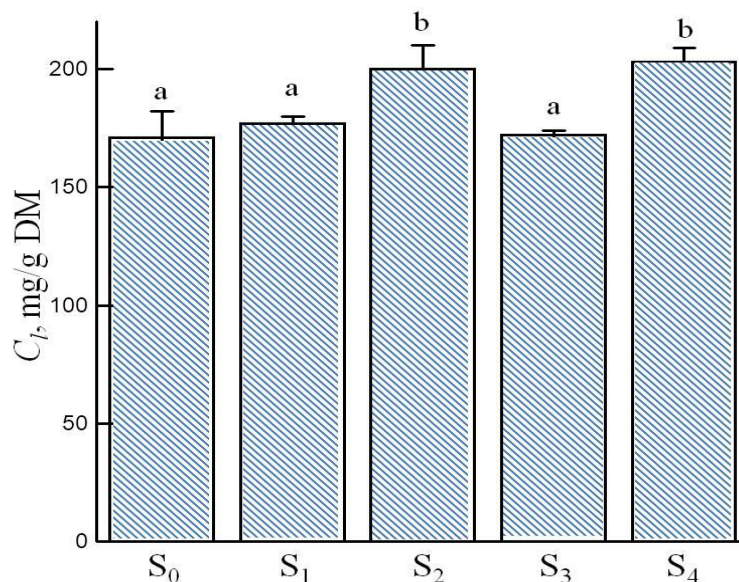


**Fig. 5.** The content of total chlorophylls,  $C_{ch}$ , and carotenoids,  $C_{cr}$ , versus the time of EtOH (95%, v/v) extraction,  $t_e$ , for unwashed ( $S_1$  and  $S_2$ ) and washed ( $S_3$  and  $S_4$ ) samples with different final moisture ratio,  $MR_f$ :  $S_1$  and  $S_2$ ,  $MR_f = 0.01$  (a),  $S_3$  and  $S_4$ ,  $MR_f = 0.01$  (b),  $S_1$  and  $S_2$ ,  $MR_f = 0.2$  (c) and  $S_3$  and  $S_4$ ,  $MR_f = 0.2$  (d) obtained for untreated (solid lines, filled symbols) and high voltage electrical discharges (HVED) treated (dashed lines, open symbols) suspensions.

### 3.3.2. Extraction of lipids

The presence of moisture can hamper the lipids extraction from the microalgae and moisture removal is an important factor to obtain high lipids extraction yield (Bagchi et al., 2015). In our experiments, the extraction of lipids was studied for unwashed (samples  $S_1$  and  $S_2$ ) and washed (samples  $S_3$  and  $S_4$ ) microalgae biomass with the final value of  $MR_f = 0.01$

after VD. For comparison, the extraction for the initial sample  $S_0$  (with washing for 60 min without any treatment) was also studied.



**Fig. 6.** Total lipids content,  $C_l$ , extracted in chloroform/methanol ( $\text{CHCl}_3/\text{MeOH}$ , 2/1, v/v) for 6 h for different samples  $S_0$ - $S_4$  obtained for different procedures performed in this study. The microalgae sediment with the final value of moisture ratio,  $\text{MR}_f = 0.01$  after vacuum drying (VD) was used. Di

0.05) according to Tukey's test.

**Fig. 6** compares the effects of different procedures performed in this study (Fig. 1) on total lipids content (TLC),  $C_l$ , extracted from the microalgae sediment using non-aqueous solvent  $\text{CHCl}_3/\text{MeOH}$  (2:1, v/v). The values of  $C_l$  for the samples  $S_0$ ,  $S_1$  and  $S_3$  (without HVED treatment) were approximately the same ( $C_l \approx 170 \pm 3.2$  mg/g DM). However, for the samples with HVED treatment ( $S_2$  and  $S_4$ ), the lipid content increased up to  $C_l \approx 200 \pm 2.1$  mg/g DM. The significant effect of HVED treatment ( $p < 0.05$ ) on extraction of lipids can be explained by the breakdown of the microalgal cells. Commonly, the HVED treatment is accompanied with different processes including the electrical breakdown, propagation of streamer, bubble formation and cavitations, light emission, appearing of localized regions with high pressure, and formation of shock and acoustic waves (Boussetta and Vorobiev, 2014). The previous experiments evidenced the presence of strong fragmentation of suspended biocells by HVED treatment (Grimi et al., 2014; Shynkaryk et al., 2009). However,

the differences between the unwashed ( $S_1$  or  $S_2$ ) and washed ( $S_3$  or  $S_4$ ) samples were insignificant because the small extraction efficiency of lipids in water.

*Table S2 Relative content of fatty acids (saturated fatty acids (SFA, all single bonds between carbon atoms), monounsaturated fatty acids (MUFA, one double bond) and polyunsaturated fatty acids (PUFA, at least two double bonds) after extraction in  $CHCl_3/MeOH$  (2/1, v/v) for 6 h for different samples  $S_0$ - $S_4$ . The microalgae sediment with the final value of moisture ratio,  $MR_f = 0.01$  after vacuum drying was used. Relative content of fatty acids,  $R$ ,  $R_{SFA} + R_{MUFA} + R_{PUFA} = 100\%$ .*

Relative content of fatty acids ( $R$ , %)			
Samples	$R_{SFA}$	$R_{MUFA}$	$R_{PUFA}$
$S_0$	$32.1 \pm 1.1$	$33.5 \pm 1.0$	$34.3 \pm 1.1$
$S_1$	$31.4 \pm 0.3$	$32.7 \pm 0.2$	$35.9 \pm 0.2$
$S_2$	$29.6 \pm 0.6$	$37.2 \pm 0.8$	$33.1 \pm 0.5$
$S_3$	$27.0 \pm 0.5$	$38.9 \pm 0.3$	$34.1 \pm 0.2$
$S_4$	$30.9 \pm 0.9$	$37.9 \pm 1.0$	$31.2 \pm 1.0$

The TLC includes different fatty acids such as SFA, MUFA and PUFA. The composition of these lipids directly influences the efficiency of biofuel conversion and its quality, being rich in SFA and MUFA (such as palmitoleic acid and oleic acid) are most favourable for biodiesel production (Nascimento et al., 2013). The relative content of the fatty acids (SFA, MUFA, and PUFA) in extracts were found to be rather similar for different samples  $S_0$ - $S_4$  ( $R_{SFA} \approx 27$ - $32\%$ ,  $R_{MUFA} \approx 33$ - $39\%$ , and  $R_{PUFA} \approx 31$ - $36\%$ ). It reflects that washing mode and HVED treatment did not affect significantly the composition of fatty acids in extracted lipids.

*Table S3 The relative content of different fatty acid methyl ester, FAME, obtained from the samples  $S_0$ - $S_4$ .*

FAME	Sample, %				
	$S_0$	$S_1$	$S_2$	$S_3$	$S_4$
$R_{C16:0}$	26.2	25.0	27.5	25.8	28.4
$R_{C16:1n-7}$	28.5	27.8	30.5	28.8	32.2
$R_{C18:1n-9}$	4.1	4.4	6.0	4.3	4.8

$R_{C18:2n-6}$	2.3	2.4	2.4	2.3	2.5
$R_{C18:3n-6}$	1.3	1.4	1.2	1.4	1.4
$R_{C20:4n-6}$	5.5	0.8	5.2	5.5	0.9
$R_{C20:5n-3}$	25.2	30.7	23.7	24.3	25.9
$R_{others}$	6.9	7.5	3.5	7.6	3.9

The relative content of different fatty acids methyl ester (FAME) was also determined. A transesterification step was used to obtain FAME content. The palmitic C16:0 (25-29%), palmitoleic C16:1n-7 (28-32%) and eicosapentaenoic C20: 5n-3 (24-31%) acids were predominant in FAME profiles. For palmitic acid C16:0 and palmitoleic acid C16:1n-7, the highest extractions were observed for the samples with HVED treatment (samples S2 and S4). It correlates with data obtained for the TLC (**Fig. 6**). However, for eicosapentaenoic acid C20: 5n-3, the biggest value of the relative content was observed for the sample S1. It reflects that preliminary washing and HVED treatment can selectively affect the content of some FAME in non-aqueous extracts.

#### 4. Conclusions

Two-step procedure with the initial aqueous extraction from raw microalgae and secondary organic solvent extraction from vacuum dried microalgae were applied for selective recovery of bio-molecules from *N. oculata*. The effects of preliminary washing and HVED pre-treatment were tested. The application of combined washing and HVED treatment significantly enhanced efficiency of aqueous extraction of ionics, carbohydrates, proteins and pigments. Moreover, HVED treatment noticeably accelerated the VD process, and increased extraction yield of chlorophylls, carotenoids and lipids in organic solvent. Partial drying (to 2% of residual moisture content) favored extraction of chlorophylls and carotenoids.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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#### V.4 Chapter conclusion

This chapter focus on the maximum valorisation of microalgal biomass by a multi-step process, with respond to concept of “microalgae biorefineries”. The feasibility of a multi-step process on the extraction and fractionation of bio-molecules from microalgae was investigated by two sections. The proposed multistage extraction process based on a cell disruption pretreatment with HVED in order to extract the water-soluble molecules, then centrifugation, followed by an organic extraction in order to extract the liposoluble molecules. A drying step was sometimes carried out before the organic solvent extraction. This extraction protocol allows selective recovery of bio-molecules with a high yield and purity, and considerably simplify the post-treatment stages.

In this chapter, the obtained results for extraction of water-soluble compounds are consistent with the results obtained from Chapter III and IV. Moreover, the results evidenced that HVED pretreatment significantly enhanced efficiency of solvent extraction of pigments and lipids in the multi-step extraction process, compared to the untreated samples. However, the application of more intensive cell disruption pretreatment (such as HHP technology) can be cause significant losses of pigments and lipids during the previous water extraction step. All these findings evidence that HVED (as a mild cell disruption technology) protocols allowed obtaining an optimal integrated biorefinery with defined selectivity and maximum valorisation of microalgal biomass.

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## General Conclusion and Prospects

Besides as bioenergy feedstocks, the valorisation of the microalgal bio-molecules became of major importance, and it cannot be neglected due to their highly added value. They can target multiple areas in the market (cosmetics, pharmaceuticals, nutrition, and aquaculture, animal feed, as well as waste-water treatment). Based on these reasons, algae scientists worldwide are largely agreeing the concept of biorefinery.

This thesis was focus on downstream process of microalgae biorefineries in order to maximize extract and fractionate the intracellular bio-molecules. Such processes must start from the understanding of cell structure as a basis to develop an optimal fractionation strategy, and must include selective and mild disintegration processes, in order to preserve the functionality of the target molecules. In order to solve these problems, three main objectives have been set:

- (1) Compare the impact of three physical treatments on cell disruption and release of intracellular bio-molecules from different microalgal species;
- (2) Verify the feasibility of combined treatment (physical treatments + HPH) for improve extraction efficiencies of bio-molecules and reduce processing energy consumption;
- (3) Optimize a multi-step extraction process for the maximum selective extraction and fractionation of intracellular bio-molecules from microalgae.

At first, the feasibility of physical treatments (PEF, HVED and US) for extraction of bio-molecules from different microalgae species was evidenced. The application of physical treatments ( $\approx 704$  kJ/kg suspension) can significantly increase extraction yields of carbohydrates, proteins and pigments compared to untreated samples. For all tested species, the extraction efficiencies of target molecules depends on the applied methods. At the equivalent applied energy, the HVED treatment was the most effective technique for extraction of carbohydrates, while the US treatment was the most adapted technique for extraction proteins. However, the extraction degree of three physical treatments were all  $\leq 40\%$  for carbohydrates and  $\leq 10\%$  for proteins. The smallest efficiency of carbohydrates and proteins was both observed for the PEF treatment. For each tested technology, they allowed selective extraction more carbohydrates than proteins. The relative mild PEE technologies have the higher extraction selectivity than US technology.

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Moreover, the extraction efficiencies of bio-molecules also depends on the extracted target molecules. Different molecules are located within different parts of the microalgal cells, thus resulting in different extraction behavior. For physical treatments assisted solvent extraction of chlorophyll *a*, the US treatment was more effective than the PEE treatments. However, the different extraction behaviors were observed between PEE and US treatments. The extraction of chlorophyll *a* using PEF or HVED occurs in one step of diffusion; while the extraction using US occurs in two stages: convection and diffusion. Moreover, the extraction behaviour of chlorophyll *a* also reflects the cell wall of *P. tricornutum* was more fragile than *Nannochloropsis* sp. or *P. kessleri*.

By contrast, for individual treatment, the most efficient method tested in our work was mechanical HPH treatment in terms of extraction efficiency. It can almost completely damage microalgal cells and simultaneous release all the bio-molecules. However, HPH causes the non-selective release of bio-molecules and produces large amounts of cell debris and high energy consumption. The application of HPH should be done in the latter step for the recovery of remaining cell compounds. In this line, the feasibility of combined treatment (physical treatments + HPH) for improve extraction efficiencies of bio-molecules and reduce processing energy consumption was investigated for the first time. The concentration of the treated suspensions is important for extraction efficiencies and total process energy consumption. The instrument's operating conditions restrict the diluted suspension ( $\leq 1\%$ ) was always used in the process of HPH treatment. In this line, the results evidenced that for preliminary physical treatments of diluted suspension ( $\leq 1\%$ ), combined procedures are less effective or negative for extraction of bio-molecules. However, the preliminary physical treatments of more concentrated suspensions ( $\geq 1\%$ ) followed by HPH of diluted suspension ( $\leq 1\%$ ) allowed increasing the extraction efficiency, and decreasing the total energy consumption.

In order to further maximize the valorisation of microalgal biomass, a multi-step process was investigated for the selective extraction and fractionation of various bio-molecules from microalgae. This multi-step process starts with the extraction of hydrophilic compounds (e.g. carbohydrates and proteins) by HVED treatment release in the aqueous phase, following by the extraction of hydrophobic compounds (e.g. pigments and lipids) in the organic solvent. The results evidenced that HVED pretreatment or/combined

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preliminary washing mode favored selective extraction of different bio-molecules in the aqueous phase.

Moreover, the impact of selected pretreatment method on the extraction hydrophobic compounds (e.g. pigments and lipids) was investigated. In general, the application of HVED pretreatment can increase the quantity of extractable chlorophylls, carotenoids and lipids in the solvent extraction process. By contrast, the application more intensive cell disruption (e.g. HPH) in pretreatment procedure can result in significant losses of extracted components (pigments and lipids) in previous extraction steps. Therefore, all these findings evidenced that HVED (as a mild cell disruption technology) protocols allowed obtaining an optimal integrated biorefinery with defined selectivity and maximum valorisation of microalgal biomass.

Additionally, the results obtained from this thesis raise some new questions and suggest some future prospects:

(1) Find the reason why the combined HVED and HPH treatment with the concentrated suspension resulted in a lower extraction efficiency of proteins comparing with individual HPH treatment;

(2) Study the morphological modifications of different microalgal cells during the process of cell disruption (tomography, SEM,...);

(3) Study the extraction of bio-molecules on a semi-industrial scale by continuous PEF or HVED treatment;

(4) Integrate pulsed electric energy assisted bio-molecules extraction and membrane filtration for continuous high added value bio-products production;

(5) A more precise characterization of bio-molecules (types, size, etc) present in the extracts would be desirable to seek an optimal purification;

(6) Study on the refining of extracts to produce powders of the bio-molecules of interest (polyphenols, proteins, pigments) would be necessary.

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