Pulsed electric field (PEF)-assisted protein recovery from microalgae biomass for food and feed applications

Zur Erlangung des akademisches Grades

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DISSERTATION

von

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Zudem erkläre ich, dass ich mich beim Anfertigen dieser Arbeit an die Regeln zur Sicherung guter wissenschaftlicher Praxis des KIT gehalten habe, einschließlich der Abgabe und Archivierung der Primärdaten, und dass die digitale Version mit der gedruckten übereinstimmt.

Karlsruhe, im September 2019

Daniel Scherer

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Zusammenfassung

Mikroalgen (einzellige Algen) werden aktuell sowohl als "Energiepflanze der nächsten Generation" als auch als Nahrungsquelle für Mensch und Tier diskutiert, da diese je nach Stamm große Mengen an Öl und/oder Proteine akkumulieren können, die für die menschliche/tierische Ernährung vorteilhaft sein könnten. Andere Mikroalgen dienen als Quelle für hochwertige Pigmente/Farbstoffe für die Nahrungsmittel-, Pharma- und Kosmetikindustrie. Essentiell für die Gewinnung dieser Komponenten ist der Zellaufschluss, der durch viele verschiedene Methoden erreicht werden kann. Es wurden bereits eine Bandbreite an mechanischen Verfahren erforscht, unter Anderem Kugelmühle, Ultraschall und Hochdruckhomogenisation. Eine Konsequenz dieser Methoden ist, dass die ohnehin schon mikroskopisch kleinen Zellen durch Scherkräfte in noch kleinere Teile zerkleinert werden sodass sich die Abtrennung des Zellschrotts schwierig gestaltet. Die Abtrennung ist im Labor in den kleinen Zentrifugen die aber hohe G-Kräfte fahren können verhältnismäßig einfach doch im industriellen Maßstab nicht, dort müssen große Mengen in kurzer Zeit umgesetzt werden müssen.

Diese Arbeit soll die Elektroimpulsbehandlung als Alternative zu den mechanischen Zellaufschlussmethoden diskutieren. Eine Konsequenz der Elektroimpulsbehandlung ist, dass die Zellmembran zunächst polarisiert wird und sich in Folge dessen durch elektrostatische Abstoßung Poren in der Membran bilden. Dieses Phänomen ist auch als Elektroporation bekannt und wird in der Biologie klassisch zum Einschleusen von DNA Material benutzt. Algen verfügen wie Pflanzenzellen auch über eine Zellwand, die i.d.R. hohen Drücken standhält und die Zelle in Form hält. Die Elektroporation ist also eine Möglichkeit, die Zellen aufzuschließen ohne sie in kleinere Teile zu zerschlagen. Dies wäre prinzipiell auch durch chemische Zusätze wie Detergentien möglich, doch diese würden das Endprodukt kontaminieren und müssten wieder auf irgendeinem Weg entfernt werden.

Der Ansatz Proteine mittels Elektroporation aus Algen zu extrahieren ist noch relativ jung und wurde bisher mit nur mäßigem Erfolg vollbracht: in den bisherigen Studien waren die Proteinextraktionseffizienzen sehr niedrig, daher wurde die Elektroporation als ineffizient abgeschrieben, allerdings ohne Bemühungen anzustellen die optimalen Voraussetzungen für die Proteinextraktion zu identifizieren und Ursachen für das nur geringe Austreten der Proteine zu ermitteln.

In dieser Arbeit wird anhand der Mikroalge *Chlorella vulgaris* gezeigt, dass es möglich ist, bis zur Hälfte der in der Zelle vorhandenen Proteine aus der Alge mit Hilfe der Elektroporation und einem Inkubationsschritt zu extrahieren. Die Freisetzung der Proteine erfolgt zeitabhängig und ist durch äußere Parameter wie Biomassekonzentration, Temperatur und pH Wert beeinflussbar. Es konnte in dieser Arbeit ein optimales Fenster für diese Parameter eingegrenzt werden, jenseits dessen die Extraktion schlechter abläuft. Weiterhin konnte mit Protease-Inhibitoren eine

vergleichbare Verschlechterung der Extraktionseffizienz hervorgerufen werden auch wenn die anderen Parameter optimiert waren. Dies führt zur Schlussfolgerung dass Enzyme bei der Freisetzung der Proteine beteiligt sind.

Der Umstand, dass nach der Elektroporation auch eine Fragmentierung der DNA einsetzt lässt die Schlussfolgerung zu, dass die Freisetzung der Proteine eine Konsequenz des einsetzenden programmierten Zelltodes ist, nach welchem sich die Zelle selbst schrittweise selbst abbaut. Und eben dieser Prozess ist empfindlich gegen Schwankungen der oben genannten Parameter. Es konnte auch gezeigt werden, dass die Zelle in ihrer Gesamtheit betroffen ist: über Western Blots wurden Proteine aus allen untersuchten Organellen detektiert, was bedeutet, dass das elektrische Feld nicht nur einen Effekt auf die äußere Membran, sondern auch einen Effekt auf die Organellen hat. Wiederholt man das gleiche Experiment unter dem Einfluss von Proteaseinhibitoren kann man beobachten, dass das Signal zurück geht und manche Proteine nicht mehr auf dem Blot detektierbar sind, was die Hypothese über die Notwendigkeit der Protease Aktivität erhärtet. Aus der Summe der Ergebnisse kann man hypothetisieren, dass die Freisetzung der Proteine auf dem Zelltod beruht, der nach der Elektroimpulsbehandlung eintritt. Das bedeutet, dass man die Elektroporation nur so einstellen muss, dass sie den Zelltod in Gang setzt.

Aus qualitativen Assays wie der SDS-PAGE geht hervor, dass sich die Proteinfraktionen, die durch mechanische Verfahren und durch Elektroporation gewonnen wurden, in ihrer Zusammensetzung unterscheiden: die Elektroporation extrahiert so gut wie keine membranständigen Proteine. Membranassoziierte Proteine können sich unter Umständen im Überstand wieder finden. Dies könnte Auswirkungen auf das Aminosäureprofil der Proteinfraktionen haben, welches zum "Nährwert" der Proteine beiträgt. Weiterhin ist eine offene Frage, welche Unterschiede es in der Qualität der beiden Proteinfraktionen im Hinblick auf technofunktionale Eigenschaften hat. Die Proteine sind dann vielleicht besser oder schlechter wasserlöslich, oder haben bessere/schlechtere Eigenschaften wie Schäumen, Emulgieren, Gelieren, Kleben o.Ä. Dies muss noch durch technofunktionale Analysen geklärt werden. Der Umstand, dass bevorzugt wasserlösliche, "frei schwimmende" Proteine extrahiert werden könnte in anderen Anwendungen (z.B. rekombinanten Proteinen) von Vorteil sein.

Ein letzter wichtiger Punkt ist, dass die Elektroporation eine sequentielle Extraktion der Algenbiomasse erlaubt, beispielsweise können zunächst die löslichen (sprich: nicht membranständige) Proteine und danach die Öle extrahiert werden ohne die Biomasse vorher zu trocknen oder vollständig entwässern zu müssen, was eine große Energieeinsparung in einem industriellen Prozess bringt und die Algenbiomasse effizient auf ihre Wertstoffe ausgeschlachtet werden kann.

Abstract

Microalgae are currently being discussed as a next-generation energy plant and as a source for food and feed. Some of these microalgae are proven to contain valuable compounds that are beneficial to human and animal health such as poly-unsaturated fatty acids, antioxidant properties and a favorable amino acid composition. Depending on the cultivation parameters algae can be engineered to produce high amounts of proteins or lipids. Other algae contain high-value compounds like pigments that are interesting for food and pharmaceutical industry, and pigments like astaxanthin and phycocyanine also have strong antioxidant properties.

In order to extract these valuable compounds, cell disruption is necessary. There is a broad variety of cell disruption methods that are being studied as of now, and in most cases it boils down to mechanical methods like high pressure homogenization (HPH), ultrasonication or bead milling. The consensus of these methods is that the already microscopically small cells are being shredded into even smaller pieces by shearing forces. While separating the cell debris is no problem in lab-scale experiments and centrifuges, this poses a problem for industrial scale applications because industrial scale centrifuges can process large amounts of liquid in a short time but at much less g-force, making separation of the cell debris is very challenging in large dimensions.

As an alternative to mechanical cell disruption methods, this work aims to explore the potential of pulsed electric field (PEF) treatment as an alternative. When a cell is exposed to a strong enough electric field, the cell membrane is polarized and then in consequence forms pores due to electrostatic repulsion of lipid molecules. This phenomenon is also known as electroporation. Therefore, PEF treatment can be exploited to permeabilize the cell membrane without smashing the cells into smaller bits and without chemical additives that might interfere and contaminate the desired final product and thus render it useless for food or pharmaceutical applications.

Using the microalgae *Chlorella vulgaris* in this work, it can be shown that up to half of the proteins present in the cell can be extracted via PEF treatment and a subsequent incubation step. The release of proteins is time-dependent and can be influenced by parameters such as biomass concentration, temperature and pH value. Optima for each one of these parameters could be determined, outside of which the extraction efficiency generally decreases. The same reduction of the yield can be mimicked with protease inhibitors under otherwise suitable conditions. This leads to the conclusion that protein release is catalyzed by proteases.

A process that also can be witnessed upon PEF is the fragmentation of the DNA, also called DNA laddering, which is seen as a hallmark process for programmed cell death (PCD). The conclusion of this work is that protein release after PEF treatment is a consequence of cell death and the subsequent autolysis ("self-digestion") of the cell, and that this process is prone to unfavorable conditions. Using western blots, it can be

shown that the cell is affected in its entirety by PEF treatment: proteins from every organelle of interest could be detected. When this same experiment is repeated under the influence of protease inhibitor, the signals on the western blots are diminished, with one signal for a specific protein disappearing completely. This further strengthens the hypothesis that protein release upon PEF requires a protease activity. Telling from the results it can be hypothesized that the release of proteins is a consequence of cell death. This means the energy input of the PEF has only to be sufficient enough to trigger cell death.

Qualitative assays reveal clear differences in the protein fractions obtained by PEF treatment and HPH: PEF treatment doesn't seem to extract membrane proteins. Membrane-associated proteins can be detected under the identified optimal conditions but not under the influence of protease inhibitors. These circumstances have an impact on the composition of the protein fractions in terms of their amino acid profile, which indirectly also translates to "nutritional value" as well as their technofunctional properties that still have to be elucidated. It has to be determined which protein fraction has got more favorable characteristics in terms of solubility, foaming, gelling and/or emulsifying properties.

These results however suggest that PEF extraction is selective for soluble, "free-floating" enzymes that are not tethered to membranes, which could make PEF treatment interesting for other applications (e.g. isolation of recombinant proteins from cell cultures).

But the most important point is that PEF would allow for a sequential extraction cascade in which first soluble proteins and then lipids could be extracted by solvent extraction afterwards, and all this in a "wet" process without the necessity to dry the biomass in between. This would contribute to significant energy savings in industrial scale processes and also fully valorizes the algae biomass.

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List of abbreviations

ALA	alpha linolenic acid
AS	Amino acid
BSA	Bovine serum albumine
bp	Base pair
°C	Degrees celsius (unit)
Ca^{2+}	Calcium
CO2	Carbon dioxide
COXII	Cytochrome c oxidase II
Cv	Chlorella vulgaris
DBM	dry bio mass
DNA	Desoxyribonucleic acid
E	Energy
EDTA	Ethylene diamine tetraacetate
g	Gram (unit)
x g	Gravitational acceleration (centrifugal force)
CG-MS	gas chromatography with coupled mass spectrometry
GLV	Green leaf volatiles
GRAS	generally regarded as safe
h	Hour (unit)
HCl	Hydrochloric acid
HPH	High pressure homogenization
J	Joule (unit)
JA	Jasmonic acid
l	Liter (unit)
LOX	lipooxygenase
min	Minute (unit)
NaOH	Sodium hydroxide
OD	Optical density
PBR	Photo bioreactor
PCD	Programmed cell death
PEF	Pulsed electric field
RuBisCo	Rubulose-1,6-bisphosphate carboxylase
ROS	Reactive oxygen species
s	Second (unit)
S	Siemens (unit)
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gelelectrophoresis
TAE	TRIS acetate EDTA
TAP	TRIS, acetate and phosphate
TBST	TRIS-buffered saline with TWEEN-20
TP	TRIS and phosphate
TRIS	Tris(hydroxymethyl)-aminomethan
V	Volt (unit)

1 Introduction

1.1 Why study algae?

A popular question posed in biology lectures is often "Why study plants?" (Reinsvold, 2009). And while it is trivial that plants are the very reason why animal life is possible in the first place, it is worth to look at algae from which these plants have evolved during the course of evolution (Heckman *et al.*, 2001). As the world population constantly grows (Raftery *et al.*, 2012), so does the demand for food, feed and energy resources. Some plants and crops such as *Miscanthus* are currently being discussed as a source for biomass for fuel production (De Vrije *et al.*, 2002; Sørensen *et al.*, 2008) as it grows very fast and produces a fair amount of biomass in a short time (Lewandowski *et al.*, 2000), but this leads to a competition for arable land which is in turn will be needed to grow crops that secure our nutrition. So, shall we use our arable land to cultivate plants that we convert to energy or plants that we can actually feed people and animals with? This circumstance evolved into a debate that got more serious during the food and fuel crisis in 2008 (Ruel *et al.*, 2010; Vilar-Compte *et al.*, 2014). In Germany, this debate is also known as the "Tank vs. Teller" debate, which translates into "fuel tank vs. plate" and basically refers to the final destination of the plants. Cultivating plants for both the fuel tank and the plate will eventually have spatial constraints and we cannot expect them to satisfy the demands for both food and fuel simultaneously. So how can we deal with this problem? Enter microalgae. Microalgae are loosely defined as microscopic organisms capable of photosynthesis. This would include cyanobacteria, but for the sake of consistency, this work will refer to microalgae as microscopic eukaryotic organisms capable of photosynthesis. Most algae are quite robust and can be cultivated in many different ways like in open pond systems (Brennan and Owende, 2010) or in closed reactors that come in many shapes and designs (Kojima and Zhang, 1999; Degen *et al.*, 2001; Zhang *et al.*, 2001; Qiang and Richmond, 2004; Chisti, 2007). Therefore they don't require arable land and can be cultivated virtually anywhere if a suitable reactor type is available, and on top of that out-class energy crops such as *Miscanthus* in biomass productivity by a factor of five (Lewandowski and Heinz, 2003; Eing *et al.*, 2013). And for this reason microalgae are being investigated as a novel source of fuel, food, feed and high-value compounds such as pigments, protein, lipids and pharmaceuticals (Rosello Sastre and Posten, 2010). When browsing through the previously cited literature, it is apparent that most microalgae have been studied in the context of biofuel production. Some microalgae can accumulate a fair amount of lipids and oil under nitrogen starvation *(*Illman *et al., 2000; Mata et al.*, 2010; Stephenson *et al.*, 2010). One of the most prominent algae for fuel production is *Botyrococcus braunii,* an algae that can naturally produce very high amounts of hydrocarbons, taking up to 61 % of the cell dry weight (Metzger and Largeau, 2005).

Since biofuel production from microalgae alone is not yet feasible or competitive, the focus of research is slowly shifting back towards a food and feed context. Microalgae like *Chlorella vulgaris* and the cyanobacterium *Spirulina platensis* are already certified for food and feed applications as they are generally regarded as safe (GRAS) (Halperin *et al.*, 2003) by the food and drug administration (FDA) in the US and are being marketed as "super food" as well (Wells *et al.*, 2017). The use of algae in human diet dates back to a couple of thousands of years ago, but large scale industrial cultivation of microalgae started in the 70's (Borowitzka, 1999; Spolaore *et al.*, 2006), and dietary supplementation with *C. vulgaris* and *Spirulina* are considered to have positive benefits for human health (Soheili and Khosravi-Darani, 2012; Panahi *et al.*, 2015). For *Chlorella* specifically, there is a broad variety of health benefits reported. In animal models, it has been observed that *Chlorella* has got anti-cancer properties (Konishi *et al.*, 1985; Tanaka *et al.*, 1990). It has also been shown that polysaccharides from *Chlorella* have anti-inflammatory and immunomodulatory effects on mice (Guzman *et al.*, 2003), and anti-microbial properties have also been reported (Hasegawa *et al.*, 1989). It has also been shown that supplementation with *C. vulgaris* can help lowering serum cholesterol (Ryu *et al.*, 2014) and thus is considered to have preventive effects in cardiovascular diseases. And consumption of *Chlorella* is also supposed to have a preventive effect for diabetes as it lowers blood sugar level and mitigates insulin-resistance (Cherng and Shih, 2005; Lee and Kim, 2009). So, telling from these benefits, *C. vulgaris* should be marketable. However, the social acceptance of *C. vulgaris* as a food source is still not fully evaluated for the European and local markets, but this would be essential if an algae-derived product is supposed to sell well: (Morimura and Tamiya, 1953) already pointed out that the taste of algae might be pleasant for East Asian people but was not to westerners. The topic of microalgae is thus often accompanied by interdisciplinary studies that evaluate whether algae would be accepted as food or food additive, which criteria an algae-derived product should fulfill and also work out marketing strategies that contribute to a change of the zeitgeist in that regard.

1.2 The model organism *C. vulgaris*

Like most microalgae from the genus *Chlorella, C. vulgaris* is a unicellular, spherical, non-motile algae with a cell diameter of 2 - 10 µm with a single chloroplast (Bock *et al.*, 2011) that reproduces by auto sporulation (Yamamoto *et al.*, 2004). This means they reproduce asexually via mitosis, commonly forming 4 daughter cells that burst from the shell that was the former mother cell's wall (Figure 1).

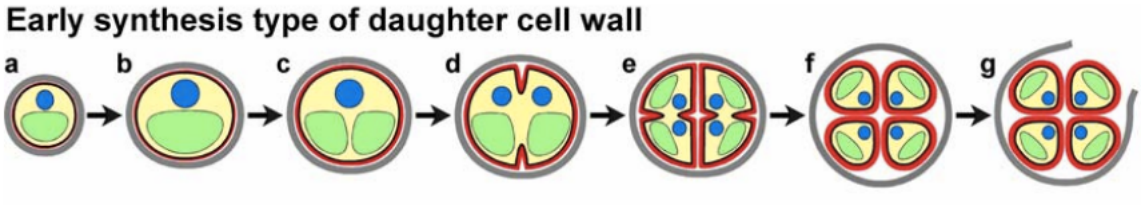


Figure 1: Stages of cell-wall formation in daughter cells of *C. vulgaris*: (a) early cell-growth phase; (b) late cell-growth phase; (c) chloroplast dividing phase; (d) early protoplast dividing phase; (e) late protoplast dividing phase; (f) daughter cells maturation phase and (g) hatching phase; adapted from (Yamamoto *et al.,* 2005; Safi *et al.*, 2014)

C. vulgaris was first isolated more than 100 years ago by Martinus Willem Beijerinck (Beijerinck, 1890) and was among the first model organisms to serve for studies on photosynthesis (Warburg, 1919). Calvin & Benson also used *Chlorella* to identify the Calvin cycle (Calvin and Benson, 1948) for which Melvin Calvin earned the Noble Prize in 1961 (Calvin, 1961).

As it can be seen in the previously cited literature, microalgae have long been discussed as a source for biofuel, dating back to 1942 (Harder and von Witsch, 1942). This topic has a revival nowadays due to the dwindling fossil fuel resources and the necessity to create CO_2-neutral energy cycles. Between all these decades, the motivation to make people more independent from fossil fuels seems to be unchanged, still. However, depending on the cultivation parameters *C. vulgaris* is rich in protein. The idea to use *Chlorella* algae as a food additive also isn't exactly new, as (Morimura and Tamiya, 1953) have already experimented with adding the algae to various foods and formulations like noodles, cookies and other things to boost the protein content and to provide more essential amino acids through them. They described the taste of most *Chlorella* algae to be similar to green tea (which I can confirm after this dissertation). Protein contents between 42 - 58% of dry biomass ($\%_{DBM}$) are often reported in literature (Morris *et al.*, 2008; Seyfabadi *et al.*, 2011; Servaites *et al.*, 2012; Safi *et al.*, 2013) and the amino acid profile is shown to be similar to the one of eggs (Safi *et al.*, 2014). It is important to know that a product derived from these GRAS algae has to be tested again to ensure that the product is not harmful. Some oils and proteins extracted from *Chlorella* are already certified for food applications (García *et al.*, 2017). This is in so far important because this makes it easier to establish a new product on the market that is derived from these algae. According to (Brennan and Owende, 2010), the biggest producers of *C. vulgaris* are Germany, Japan and Taiwan,

which produced 2000 t of the algae combined in 2009, which indicates that there is in fact a market for it and thus also potential for algae-derived products.

For all these reasons, *C. vulgaris* is a good model organism to use in bioprocess engineering contexts. It is also fairly well-characterized how *C. vulgaris* has to be cultivated to boost production of lipids *(*Illman *et al., 2000; Mata et al.*, 2010; Stephenson *et al.*, 2010), carbohydrates (Ho *et al.*, 2013) or of proteins (Lai *et al.*, 2019). It all boils down to the algae's "diet composition" in form of light, CO_2 and nitrogen source. When it comes to cultivation of *C. vulgaris*, there are usually three different modes: autotrophic growth, which basically means the algae are supplied with light, CO_2 and a couple of nutrients (trace elements and nitrogen source for example) and grow based on that, and heterotrophic growth in which the algae are supplied with an organic carbon source that makes them independent from light. Organic carbon sources that can be used for *C. vulgaris* include glucose, acetate, glutamate and also glycerol, with glucose showing the highest productivity. Lastly, there is mixotrophic growth, which is a combination of both auto- and heterotrophic cultivation, with the purpose to bridge the "dark" phase of cultivation with an organic carbon source. The supplementation with an organic carbon source has in general shown to increase biomass and lipid productivity (Ogawa and Aiba, 1981; Martinez *et al.*, 1991; Liang *et al.*, 2009; Yeh and Chang, 2012) but it usually increases the cost of the cultivation and in the case of heterotrophic growth the question arises which advantages *C. vulgaris* has over yeast for example.

Even though *C. vulgaris* has been isolated more than 100 years ago, it is still a fairly young model organism from a molecular-biological point of view; the genome of one strain has just been sequenced very recently (Guarnieri *et al.*, 2018). This might open up further biological works to better understand the genetics, metabolism and cell biology of *C. vulgaris* in the future. This would be especially important because most of the research on microalgae in bioprocess engineering contexts doesn't take much of the biology of microalgae into account and they are treated more or less as unspecific biomass. As it has been reported by (Krienitz *et al.*, 2015) some algae that were considered to belong to the genus *Chlorella* were slowly transferred to other genera with growing understanding about them. For microalgae, there is AlgaeBase (Guiry & Guiry, 2019), an online database for all kinds of microalgae, that currently has 177 (as of 21.08.2019) hits for "*Chlorella",* and this number is expected to constantly change in the future.

1.3 Cell disruption methods that are currently being discussed in research

1.3.1 Mechanical cell disruption methods

The focus in this study lies on protein extraction since these proteins from *C. vulgaris* could be used for food and feed applications. Various extraction methods for proteins are being investigated during the last years, most of them being mechanical methods like ultra sonication (Gerde *et al.*, 2012; Greenly and Tester, 2015), high-pressure homogenization (HPH) (Samarasinghe *et al.*, 2012; Ursu *et al.*, 2014) or bead-milling (Postma *et al.*, 2017) for instance. In short, mechanical methods rely on shearing forces that tear the cells apart. While these methods are reported to be quite efficient in terms of protein extraction efficiency, they have a few downsides that are relevant for industrial scale applications. Some of these disruption methods cannot be scaled up properly to process large amounts of algae, they create cell debris that is hard to separate in industrial settings and they can also create a fair amount of heat that might be detrimental to the compound of interest (Carullo *et al.*, 2018; Kapoore *et al.*, 2018).

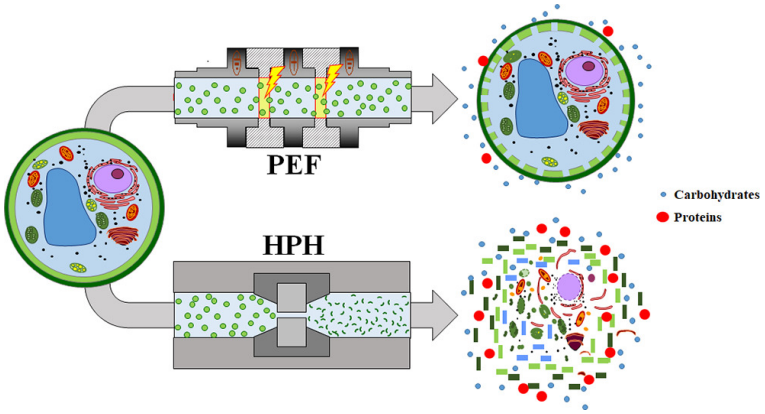


Figure 2: An illustration of the processing of a microalga cell via PEF in comparison to HPH. PEF permeabilizes the cell without destroying it whereas HPH tears the cell apart mechanically, taken from (Carullo *et al.*, 2018).

1.3.2 PEF treatment as cell disruption method

As an alternative cell disruption method, PEF are also being investigated and have already been applied to microalgae (Eing *et al.*, 2013; Goettel *et al.*, 2013; Coustets *et al.*, 2014; 't Lam *et al.*, 2017). The consequence of PEF treatment is membrane electroporation (Saulis, 2010). In a more detailed way, this electroporation can be described in 4 steps. Step 1 is the interfacial polarization of the membrane. Step 2 is the formation of hydrophilic pores in the membrane (Figure 3). Step 3 is the evolving of the pores and in the final step, once these pores are established, intracellular components can leak out of the cell or other components can enter. These pores can either re-seal (reversible electroporation) or they don't (irreversible electroporation), which is dependent on the treatment energy.

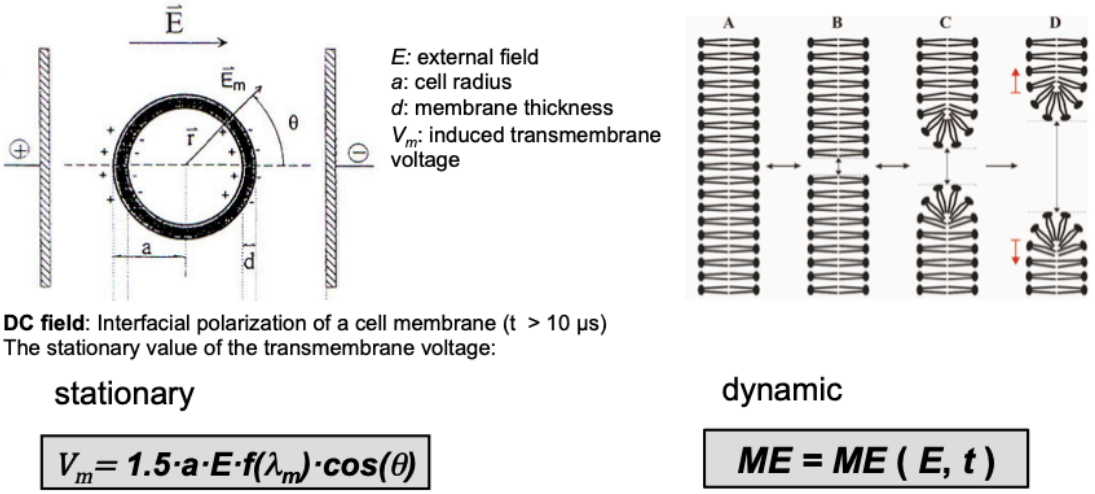


Figure 3: Stages of membrane electroporation. When a cell is introduced to a strong enough field, the first step is polarization of the membrane which can be described as a stationary phase (Neumann *et al.*, 1999). The consequence is pore formation, which can be described as a dynamic process as in (Neu and Krassowska, 1999). ME = membrane electroporation, t = time.

Since PEFs can reversibly or irreversibly permeabilize membranes, this phenomenon is used in a broad array of applications such as gene electro transfer (Miklavcic *et al.*, 2016), pasteurization of beers, milk and juices (Qin *et al.*, 1996; Aguilar-Rosas *et al.*, 2007; Mosqueda-Melgar *et al.*, 2008; Barbosa-Cánovas and Bermúdez-Aguirre, 2010; Milani *et al.*, 2015), decontamination of hospital wastewaters (Gusbeth *et al.*, 2009) and also in cancer therapy (Nuccitelli, 2018). Companies like Südzucker also utilized PEF to improve extractability of sugar beets (El Belghiti and Vorobiev, 2004), and PEF is also explored in the making of vine as it improves/accelerates extractability of pigments in the case of red vine and also can substitute thermal or enzymatic disruption of grapes as the heat and/or enzymes can alter the quality of wine (Sack *et al.*, 2009, 2010; Ozturk and Anli, 2017).

Another application of PEF treatments in basic research is to apply nsPEF to the cells which creates a unique stress for the cells by either reversible electroporation or charging the membrane for a brief moment, which would disrupt membrane potentials and electric signals. This form of PEF treatment can have many sub-lethal effects on biological cells. It has been used to enhance growth of *Arabidopsis thaliana* seedlings for example (Eing *et al.*, 2009). The original idea here was to make the roots of the seedling more permeable for nutrients for a short time. But it also has been shown that physiological responses such as re-arrangement and also disintegration of the cytoskeleton can be triggered by nsPEF in BY-2 tobacco cells (Berghöfer *et al.*, 2009). Using *Chlamydomonas reinhardtii* it has also been demonstrated that nsPEF stimulation can trigger developmental responses such as early and late ROS bursts that seem to induce palmella-formation (Bai *et al.*, 2017). In this way, PEF treatment can be used as a form of electro-stimulation/electro-manipulation of biological cells to trigger stress responses.

For extraction of valuables from algae, PEF treatment has already been shown to increase extraction yields for lipid extraction (Silve *et al.*, 2018a), but the utilization of PEF for protein extraction has not been fully investigated so far. There are reports confirming that protein extraction is conceptually possible (Coustets *et al.*, 2014), although in some studies only moderate success has been reported as of now (Postma *et al.*, 2016; 't Lam *et al.*, 2017; Safi *et al.*, 2017). However, investigating PEF-assisted extraction is appealing because it is potentially possible to get a higher degree of extraction selectivity towards certain valuable fractions such as proteins and lipids, whereas mechanical methods such as HPH scramble these fractions together, emulsifying lipids and proteins as reported in (Ursu *et al.*, 2014) or illustrated by (Carullo *et al.*, 2018) for instance. This means that PEF allows for fractionation of the biomass into different phases (e.g. lipids, proteins). There are attempts to combine PEF as a pre-treatment to mechanical methods such as HPH, however no combinatory effect can be seen with PEF and HPH (Carullo *et al.*, 2018).

From physical point of view the extraction of protein is also interesting in that mathematical models that exist for electroporation and pore formation cannot fully explain the release of proteins or the passage of molecules. In general, the pores created upon electroporation are considered to be barely big enough for some protein molecules to penetrate. The pore sizes reported range mostly around 1 - 15 nm (Krassowska and Filev, 2007; Smith *et al.*, 2014), although it has to be mentioned that the energy input/pulse duration can influence the distribution of size of the pores (Saulis and Saule, 2012; Smith *et al.*, 2014). How the pore size increases after the pores have been formed is still cryptic from the modeling point of view. Using BY-2 protoplasts (tobacco cells whose cell wall has been removed enzymatically) and patch clamp, (Wegner *et al.*, 2011) observed that permeabilized cells show different permeabilities for different ions. This means there must be further processes following electroporation that lead to enlargement of the pores and facilitate the release of proteins from permeabilized cells, and that enable extraction or introduction of other compounds of interest in general.

Besides PEF as a non-mechanical method is it noteworthy that microwave-assisted extraction also exists and is currently explored further, mostly for lipid extraction (de Moura *et al.*, 2018; Kapoore *et al.*, 2018). Microwave-assisted extraction however also heats up the sample significantly and this heat development can be problematic as many compounds of interest might degrade under high heat.

1.3.3 Chemical and enzymatic methods

For the sake of completeness, it also has to be briefly mentioned that among the myriad of extraction methods (Günerken *et al.*, 2015; Bleakley and Hayes, 2017) are also chemical and enzymatic extraction approaches. In comparison with these, PEF would have the advantage that no chemical additives would be necessary besides the adjustment of conductivity, which can be accomplished with salts, providing a non-toxic extraction method that could provide food-grade proteins. Enzymes are usually quite expensive and the amount of enzyme needs to be scaled with the substrate concentration, this can ramp up the costs tremendously (Klein-Marcuschamer *et al.*, 2012). Enzymatic cell disruption of algal or plant cells typically involve cellulases, but this clade of enzymes work slow and are considered to be inefficient at what they are supposed to do (Saddler, 1986; Coughlan, 1992). Nevertheless there are studies that combine chemical and enzymatic approaches with mechanical methods with the aim for maximizing the cell disruption efficiency (Wang *et al.*, 2015; Phong *et al.*, 2018; Zhang *et al.,* 2018). However, it has to be considered that some chemical extraction procedures will stand in conflict with GRAS status requirements and might render extraction products useless for food and feed applications if the contaminants introduced cannot be removed sufficiently.

1.3.4 Protein purification methods

When it comes to protein extraction, it also has to be discussed about protein purification methods. As mentioned before, the extraction method per se can render the proteins useless for food and feed applications depending on which chemical additives are used. Detergents of lysis buffers such as SDS, Triton-X100 or sodium-desoxycholate are efficient in disrupting cells and solubilizing proteins but are definitely not safe to ingest. Cell lysis via alkaline hydrolysis - essentially boiling the cells in NaOH like done in (Pruvost *et al.*, 2011) is efficient but needs neutralization (with HCl for example) afterwards, in which case a fair amount of salt is created. Once proteins are extracted from the source material, they have to be somehow concentrated/purified, which also means to get rid of other substances that were present before and the ones that were introduced during extraction.

In the laboratory, there are many established protocols for this. One of the easiest and cheapest way is solvent precipitation. Conceptually, an excess of solvent that is more polar than the proteins "dehydrates" the proteins, taking away the hydrate shell so that the proteins have nothing else to stick to than themselves. This works with cold acetone in a ratio of 4:1 (Botelho *et al.*, 2010; Crowell *et al.*, 2013), 50 % ethanol/50 % acetone 8:1 precipitation (Grossmann *et al.*, 2018a; Grossmann *et al.*, 2018b) or even methanol 9:1 (Bychkov *et al.*, 2011). These solvents and solvent blends have proven to work well for protein precipitation in laboratory scale experiments but are per se toxic and hence it is unlikely that they are useful in a food and feed context, especially since the ratio of solvent to extract can scale up drastically in industrial scale processes. Needless to say that it would cost a lot of energy to recover these big amount of

solvents by distillation for example. Another way of precipitating protein that relies on a similar mechanism would be "salting out" of proteins. In this case, salt is used to achieve the same effect as described for solvents. The most popular salt is ammonium sulfate (Wingfield, 2010), although other salts/ions also work. The precipitation efficiency of different ions is described by the Hofmeister Series (Hofmeister, 1888; Zhang and Cremer, 2006). For the production of tofu for example, "nigari" is used (Yang and James, 2013), a sea water derived saline consisting mostly of magnesium chloride and other salt impurities. For this reason it is often substituted by magnesium chloride alone (Li *et al.*, 2015), but sometimes calcium sulfate (Kao *et al.*, 2003) is also used to make tofu. Of course, when proteins are precipitated with salts, it has to be considered whether the salts are toxic, which contents of salt are acceptable and in consequence whether it's necessary to somehow reduce or remove the salt. In laboratory scale, this would be possible by appropriate washing protocols or dialysis kits. Another way to precipitate proteins would be isoelectric precipitation, which can be accomplished with acids or bases. In this case, the pH of the environment is adjusted with acids or bases to a pH value that matches the isoelectric point of the protein. At this point, the net charge of the protein is neutral and thus it likely won't form a hydration shell anymore, or protein molecules rather tend to interact with each other and essentially end up aggregating. These forms of precipitation are often applied to "simple" protein mixtures like soymilk that mostly contains glycinin and ß-conglycinin (Krishnan *et al.*, 2009) or cow milk that mostly contains whey and casein (Rafiq *et al.*, 2016). Casein and whey that are isolated/concentrated by acid precipitation can be bought as "acid casein" or "acid whey" for supplement. However, acid precipitation is likely inefficient for complex protein mixtures as every protein has an own isoelectric point. It would only make sense for a step-wise precipitation of proteins, but not to precipitate the proteins in bulk. One special case of acid precipitation is the precipitation by trichloroacetic acid combined with sodium desoxycholate, also called NaDOC-TCA precipitation (Bensadoun and Weinstein, 1976; Arnold and Ulbrich-Hofmann, 1999). In this method the acid works like a hybrid between "stealing" the hydration shell and acidifying the proteins.

Heat can be another way to precipitate proteins if one thinks of boiling an egg, and in industrial applications there are also chromatography methods if a special, high-value protein has to be purified (e.g. vaccine antigens, antibodies, therapeutic enzymes etc.). An overview for these kind of industrial methods can be found in (Kumar and Sharma, 2015). These purification methods are usually very expensive and only worth doing when the product is of very high value, so this form of purification usually applies to the pharmaceutical industry but is not feasible for food production.

While protein purification and precipitation is not the main task of this work, it has to be taken into account when attempting to establish a novel processing cascade for algal protein extraction.

1.4 Establishing a bio refinery concept via PEF

When browsing through the literature about extraction procedures for microalgae in general it becomes clear that the extraction research is split up into roughly three camps: lipids for biodiesel, proteins for food and feed, and high-value compounds such as phycocyanin (Dianursanti *et al.*, 2018) and astaxanthin (Zgheib *et al.*, 2018) that are used as pigments for food coloring or as pharmaceuticals due to their strong antioxidant properties and benefits to human health (Fassett and Coombes, 2011; Ambati *et al.*, 2014; Memije-Lazaro *et al.*, 2018). However, focusing on one compound of interest is likely not feasible. Very recent calculations suggest that fuel production from algal biomass is only feasible in a very optimized system, a state that the current technology has not achieved yet (Chisti, 2013; Slade and Bauen, 2013; Dasan *et al.*, 2019). This is also the reason why fuel from microalgae will be more expensive than fuel generated from conventional plants like rapeseed or sugar cane and sugar beet (Klein-Marcuschamer *et al.*, 2013). In the work of (Klein-Marcuschamer *et al.*, 2013) it is also illustrated that the costly part of producing fuels from microalgae actually stems from the cultivation and harvest of the algae itself, so even when most extraction procedures are optimized, it is the cultivation itself that needs to be improved to lower the cost, so essentially the microalgae biomass itself needs to become even cheaper. Extraction procedures for certain compounds are usually optimized for pinpoint accuracy, but the production of fuel, food and valuables from microalgae can only be profitable if most of the valuables are extracted from the biomass. It is therefore important to focus on establishing and optimizing a bio refinery concept in which the valuables are extracted step-by-step. But so far no bio refinery concept has been realized for microalgae. By optimizing PEF extraction parameters for proteins, it could be possible to establish a sequential, "wet" processing cascade (Figure 4) in which water-soluble proteins are extracted first and then lipids e.g. as in (Silve *et al.,* 2018a), all this without the need to dry the biomass first. Investigating extraction parameters for various compounds might enable a bio refinery cascade for microalgae biomass as it is being applied to other biomasses already (Golberg *et al.*, 2016). The residual biomass that should at this point be devoid of proteins and lipids could then be used for feed or energetic processes like hydrothermal liquefaction (Galadima and Muraza, 2018; Xu *et al.*, 2018). This would realize full fractionation and valorization of the algae biomass and reduce waste products. There have already been attempts to use lipid-extracted algae as a source for proteins (Ansari *et al.*, 2015), which would be the other way around (lipids first, then protein) compared to what is suggested for PEF processing cascade, and ever since then the question came up which extraction sequence is/could be the most efficient one (Ansari *et al.*, 2017) with minimal overall losses of valuables.

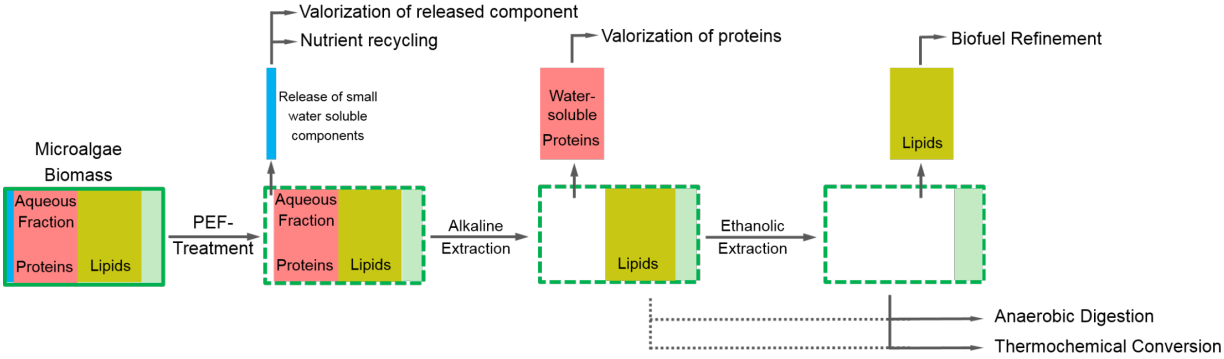


Figure 4: A visual concept of a microalgae bio refinery concept based on PEF technology. Algae are made permeable with PEF treatment, small water-soluble compounds and then proteins are recovered, and in the end lipids/oils can be extracted via solvent extraction. The residual biomass can then be used in energetic processes or further valorized (e.g. digestion of the cell wall to create platform chemicals). Illustration adapted from (Kotnik *et al.*, 2015).

1.5 A brief introduction to cell death

1.5.1 Programmed cell death / apoptosis

As mentioned before, biological cells can be killed via PEF treatment. Depending on the energy input, the cell is either killed by the irreparable damage to its membrane and/or by inducing cellular pathways that trigger programmed cell death (PCD, also known as apoptosis). This phenomenon has been well-described for animal and cancer cell lines and is thus exploited for cancer treatment (Cabuy, 2012). This circumstance is important to keep in mind because the physics and mathematical models behind electroporation alone cannot explain why extraction of proteins could ever work. However, cell death can play a crucial role in explaining protein release from the cells as the observations from the works on PEF-assisted protein extraction cannot be explained by diffusion processes alone.

PCD is considered to be a physiological process to get rid of unwanted or unnecessary cells (Ellis *et al.*, 1991) and in multicellular organisms is also part of normal development. PCD can be induced by a broad variety of signals. In plants, the most prominent ones are reactive oxygen species (ROS) bursts (Levine *et al.*, 1994; Jacobson, 1996; Huang *et al.*, 2016), calcium (Levine *et al.*, 1996; Zhivotovsky and Orrenius, 2011) and/or activation of caspases (Beebe *et al.*, 2013). Briefly, the signal for PCD starts with production of a ROS burst and Ca^{2+} influx, which activates pro-apoptotic transcription factors and also activate caspases and endonucleases. In plant and algae there are usually no caspases, this function is taken over by caspase-like proteins (Jiménez *et al.*, 2009; Xu and Zhang, 2009). When browsing through PCD-related literature for plants it is often stated that ROS bursts precede the Ca^{2+} signal, but in cancer therapy and/or in mammalian cell contexts the opposite is reported like in

(Nuccitelli *et al.*, 2013) for example, which put calcium signals upstream of ROS bursts. It is also for this reason that calcium electroporation is discussed as a cheap cancer therapy method (Hoejholt *et al.*, 2019). In short, the idea is to inject a calcium solution into the surrounding of the tumor and in consequence flood the cancer cells with Ca^{2+} ions upon electroporation to induce PCD. Overall, the consensus is that ROS and Ca^{2+} go hand-in-hand in the PCD context and can activate caspases/caspase-like proteins that will then start to degrade the cell (Van Durme and Nowack, 2016).

In plants, the plastids (mitochondria, chloroplast and peroxisomes) can also contribute to PCD via signaling (Figure 5) that is induced under stress via release of apoptosis-inducing proteins and/or also ROS bursts (Bras *et al.*, 2005; Scott and Logan, 2008) as they are basically the parts of the cell where most ROS are produced naturally as a by-product of photosynthesis or cell respiration (Choudhury *et al.*, 2013). It is reported that the release of cytochrome c from the mitochondria triggers PCD (Balk *et al.*, 1999; Balk and Leaver, 2001; Yao *et al.*, 2004; Vacca *et al.*, 2006). And a similar mechanism has also been described for cytochrome f from chloroplasts (Wang *et al.*, 2014). PCD signaling from the plastids usually occurs upon abiotic stress.

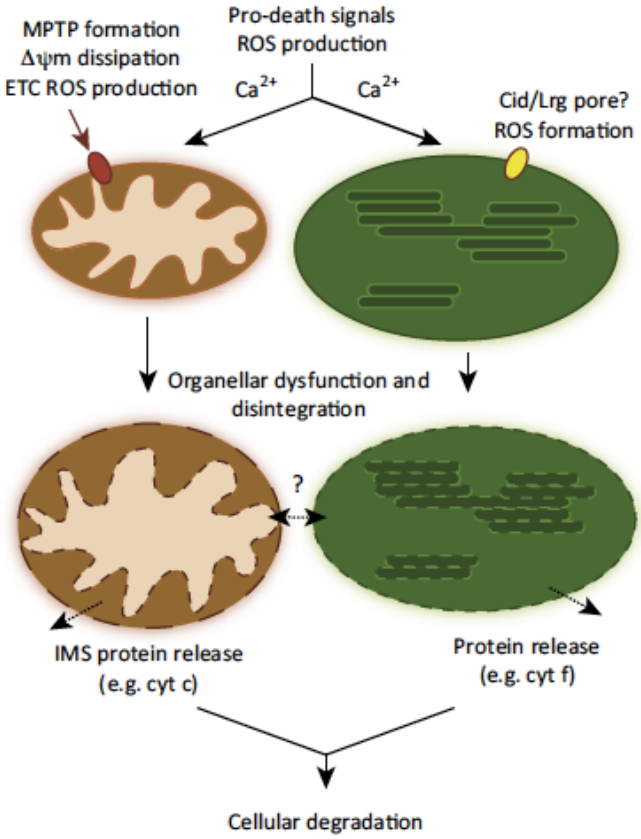


Figure 5: A simplified model of how plastids can be involved in PCD and which processes underlie for each organelle, adapted from (Van Aken and Van Breusegem, 2015). MPTP = mitochondrial permeability transition pore; ΔΨm = mitochondrial membrane potential; ETC = electron transport chain; ROS = reactive oxygen species, IMS = intermembrane space

Another signal for PCD can also be mediated via hormones. In plants, phytohormones such as ethylene (Trobacher, 2009) or jasmonic acid (Reinbothe *et al.*, 2009) can trigger PCD, as well as mobile signals such as green leaf volatiles (GLVs) that most green plants can emit when they are wounded or mechanically damaged. GLVs are also the compounds that smell like cut grass (mostly cis-3-hexenol and cis-3-hexenal), and these compounds can also trigger cell death as shown in the work of (Akaberi *et al.*, 2018). Phytohormones such as ethylene and jasmonic acid are common in land plants but have also been shown to exist in microalgae (Ueda *et al.*, 1991; Lu and Xu, 2015), although it's still somewhat cryptic which functions they fulfill in microalgae, as most phytohormones coordinate plant development, cell differentiation and systemic stress responses that are not existent in microalgae in the same way. It is also unclear which phytohormones *C. vulgaris* in particular can synthesize or sense.

But in summary, most of the time these signals happen in concert and ultimately drive a cell to commit suicide. For multicellular organisms, PCD is part of normal development and also a protection mechanism against cancer: most eukaryotic cells have safety mechanisms during their cell division that arrest the cell cycle and trigger PCD when something goes wrong during cell division (Ca *et al.*, 1998; Pietenpol and Stewart, 2002). In higher plants, PCD also serves as a protection mechanism to prevent the spread of diseases and pathogens in that infected cells quickly commit suicide, which in this context is also called "hypersensitive response" (Levine *et al.*, 1994; Levine *et al.*, 1996; Heath, 2000; Lam *et al.*, 2001). The ability to commit PCD is also found in unicellular organisms and algae (Jiménez *et al.*, 2009; Bidle, 2016), and the role of PCD in unicellular organisms is a hot topic to date (Franklin *et al.*, 2006), because why should a single-cell organism be able commit suicide? It obviously doesn't make much sense if it comes to survival and passing on genetic information. But PCD-like phenomena have also been described for bacteria and are reported to help in biofilm production (Oleskin *et al.*, 2000; Webb *et al.*, 2003). This example already shows that PCD in unicellular organisms is essentially a very altruistic mechanism: the suicide, the sacrifice of a few cells is meant to secure survival of the overall population. In some way the mechanism of skin formation is similar to this phenomenon: keratinocytes that emerge from epithelial cells commit PCD upon differentiation to form "dead" skin that shields our body and cells from most environmental stresses (Maruoka *et al.*, 1997). For microalgae, the ability to commit suicide doesn't make much sense from the perspective of individual cells but in the context of the whole cell population and conservation of species, it is an essential tool. These mechanisms have been described for bacteria but are still not as well researched for algae yet, but certainly have to be taken into account as there are also attempts to cultivate microalgae such as *Chlorella* in biofilm reactors (Rincon *et al.*, 2017).

Ways to assay PCD involve detection of signs of DNA degradation. The *in vitro* approach would be to screen for DNA laddering: DNA is degraded upon cell death into fragments of 180 bp, resulting in a ladder pattern on an agarose gel. This is considered

to be a hallmark of PCD (Hale *et al.*, 1996). As an alternative, degradation of DNA can also be detected *in vivo* with the help of TUNEL staining (Gavrieli *et al.*, 1992). TUNEL stands for "TdT-mediated dUTP-biotin nick end labeling", where "TdT" stands for "terminal deoxynucleotidyl transferase". Basically, the enzyme TdT attaches a fluorescently labeled nucleotide onto the ends of each fragment, and the more the DNA is fragmentized the more it will be labeled. Alternatively, the activity of caspases (or in the case of plants and algae caspase-like proteins) itself can be measured using substrates that are cleaved by caspases to form fluorescent by-products (Tawa *et al.*, 2001). Of course the expression or upregulation of caspase/caspase-like proteins can also be used as a read-out for PCD. During developmental processes that involve PCD, many caspases/caspase-like proteins are induced to contribute to proper development of an organism. In (Van Durme and Nowack, 2016) there is a table that summarizes which caspase-like proteins are induced during developmental PCD in different tissues of various model plants. Morphological changes that can occur during PCD involve cell shrinkage, nuclear condensation and/or fragmentation, externalization of phosphatidylserine in the membrane and membrane blebbing, which is the formation of apoptotic bodies (Kerr *et al.*, 1972; Franklin *et al.*, 2006; Reape and McCabe, 2008). In the mammalian context, apoptotic bodies make it easier for immune cells like macrophages to clean up dead cells.

1.5.2 Necrosis

Contrary to PCD or apoptosis, which are terms for a controlled way of dying there is also necrosis, which would be "accidental cell death" due to injury or stress. Differences in morphological changes involve cell swelling and loss of membrane integrity. Both apoptosis and necrosis have been quite well characterized for mammalian cell models, and while differences between morphological changes can essentially happen for both modes of cell death, the consensus is that necrosis does not involve activation of genes such as the aforementioned caspases, and dying cells might release toxic substances that affect neighboring cells like ROS or inflammatory signals (Majno and Joris, 1995; Franklin *et al.*, 2006; Reape and McCabe, 2008), which is also harmful for the organism itself, and a good example for this would be the tumor lysis syndrome in cancer patients (Howard *et al.*, 2011). The boundaries between PCD and necrosis however are often blurry, there are a few debates about how to define necrosis on a mechanistic and/or molecular basis (Proskuryakov *et al.*, 2003; Golstein and Kroemer, 2007), and this debate is still not resolved to date.

2 Aim of the study

The aim to extract proteins from microalgae is a relatively young field of research. (Coustets *et al.*, 2014) have identified that an incubation step in a buffer is necessary after PEF treatment. Other works like the ones from (Postma *et al.*, 2016; ’t Lam *et al.*, 2017; Safi *et al.*, 2017) that compare PEF treatment to mechanical methods like HPH and bead milling only achieved low yields and consider PEF to be inefficient for protein extraction but without ever identifying ideal extraction parameters for PEF-assisted protein extraction. A series of open questions that came up during this work were:

2.1 What does PEF treatment do to the cell?

In works like (Goettel *et al.*, 2013; A. Silve *et al.*, 2018a) standard parameters (field strength of 40 $kV \cdot cm^{-1}$, duration of 1 µs, specific treatment energy of 150 $J \cdot g^{-1}$) have been established that positively impact lipid extraction from microalgae such as *Auxenochlorella protothecoides.* However, these parameters have to be transferred to *C. vulgaris* and characterized for their effect on it. How efficient is the process, how many cells are permeabilzed and is the permeabilization irreversible? The permerabilization efficiency can be evaluated via Evans blue staining and cell counting. Morphological changes can also be evaluated during this process as well.

2.2 What happens to the cell afterwards?

Which effects can be expected once the cell is successfully electroporated in an irreversible fashion? Irreversible damage to the cell membrane should allow for some proteins to leak out of the cell, but it also means that the cell won't be viable anymore. In the case of irreversible membrane permeabilization, cell death is expectable, but in which way? How does the cell die?

The protein content in the supernatant can be measured using a Lowry assay, and as a hallmark for programmed cell death (PCD), DNA laddering can be checked.

2.3 How can proteins be extracted and what are their properties?

Of course, the biggest question is whether the technique is able to successfully extract proteins from *C. vulgaris,* and there are many sub-questions to this. The consensus of some previous works on PEF-assisted protein extraction is that the protein extraction efficiency is usually too low for PEF treatment to be a viable option for industrial scale purposes (Postma *et al.*, 2016; ’t Lam *et al.*, 2017; Safi *et al.*, 2017), but they don't identify which parameters impact protein release from the cells. (Coustets *et al.*, 2014)

reported that an incubation step in a salty buffer is necessary after PEF treatment of the algae. In this work, the influence of incubation time, biomass concentration, temperature, pH value were elucidated, and also inhibitor experiments were done to investigate biological phenomena that underlie the process of protein release.

Once proteins are successfully extracted from the cells via PEF, the question arises whether there are differences between the protein fractions obtained via PEF treatment and mechanical methods such as HPH.

Mathematical models of PEF and electroporation suggest that the outer membrane of a cell is usually the most affected one, and inner membranes like the ones of organelles should not be affected unless more energy is applied in form of multiple pulses. It is for this reason that (Coustets *et al.*, 2014) claim that PEF treatment exclusively extracts cytosolic proteins from *C. vulgaris*, and without disrupting the vacuole which would leak lytic enzymes, but it is unclear how they came to this conclusion other than these mathematical models. It is therefore useful to investigate whether organelles of the cells are affected by PEF treatment as well. The proteins extracted via PEF and HPH treatment were thus visualized by SDS-PAGE and subsequent Coomassie staining to get a first glance on whether there are apparent qualitative differences in the protein fractions that allow to make qualitative predictions about the selectivity of PEF: does it yield only soluble proteins or are also some membrane proteins included? If PEF has got selectivity towards soluble proteins that are not membrane-bound or membrane associated, this should also have an effect on the amino acid composition for example. In the next step, hallmark proteins of specific organelles were probed on western blots (Histone H3 for Nucleus, RuBisCo for chloroplast, Actin for cytosol and COXII for mitochondria) to have a better resolution on which parts of the cell are affected and hence where the extracted proteins originate from. While it is hard to find a marker protein for vacuoles, an alternative approach is to stain the vacuole with Neutral Red and see whether the staining disappears after PEF treatment and/or whether the staining still works after PEF treatment. Lastly, the extracted proteins are precipitated by solvent precipitation and submitted for analysis of protein content and amino acid composition to obtain more resolution on the properties of the protein fractions.

3 Material & Methods

3.1 The model organism: *C. vulgaris*

3.1.1 *C. vulgaris* cultivation in flasks

C. vulgaris (strain 211-12 purchased from the SAG University of Göttingen) cells were usually cultivated in 1000 ml flasks containing 400 ml 1x TAP medium (0.02 M TRIS, 0.001 potassium phosphate buffer, 1x TAP salts (Gorman and Levine, 1965), 1x Hutner's trace elements (Hutner *et al.*, 1950), 0.001 % acetic acid, pH 7.0) in 1000 ml. They were inoculated at an OD of 0.1 and left to grow on a shaker under constant agitation, 23 °C and 60 $\mu E \cdot s^{-1} \cdot m^{-2}$ of light illumination with fluorescent lamps. They were grown for 7 d until they reach an OD around 2 and then concentrated by centrifugation (10000 x g, 2 min). At this point, the cells are in stationary phase of their growth. The biomass concentration and dry weight was determined gravimetrically using a precision scale after drying the suspension in aluminum cups in an oven at 80 °C. The biomass concentration was calculated as follows:

$$DBM\ [\frac{g}{kg}] = \frac{m\ (algae, dry)}{m\ (algae, wet)} * 1000 - \frac{m\ (medium, dry)}{m\ (medium, wet)} * 1000$$

The result is the biomass concentration in $g \cdot kg_{suspension}^{-1}$. However, taking into account that the density of water is 1 $kg \cdot l^{-1}$, the dry biomass can be expressed in $g \cdot l^{-1}$. The concentration was then adjusted to a desired concentration, typically 5 or 10 $g \cdot l^{-1}$, using leftover medium from the centrifugation steps. Under these conditions, the algae have an average protein content of 45 % related to dry biomass ($\%_{DBM}$).

3.1.2 *C. vulgaris* cultivation in indoor 25 l photo bioreactor

The cultivation can be scaled up to 25 l in a photo bioreactor using 1.5x TP medium (TAP medium without acetate) and 100 $\mu E \cdot s^{-1} \cdot m^{-2}$ of light illumination. The algae were inoculated at an OD of 0.1 and are stirred by gas bubbling (970 $cm^3 \cdot min^{-1}$ of air and 30 $cm^3 \cdot min^{-1}$ of CO_2) whereas the CO_2 serves as a carbon source (in the flasks, it's the acetate). After 5 days of cultivation, the CO_2 supply is shut off in order for the algae to enter a stationary phase prior to harvesting after 7 days of cultivation. The algae were harvested using a disk separator (GEA Westfalia Separatoren, STC 3-06-170) and the paste was re-suspended to a desired working concentration using spent medium. For the upscaling the algae paste was resuspended with medium to a final volume of 2 l with an average concentration of 18 $g \cdot l^{-1}$. One liter was subjected to PEF treatment and the other liter was treated with HPH.

3.1.3 Establishing a growth curve and determining doubling time

Growth was monitored via measuring the OD_{750} over the course of 7 days. In order to bridge the gap of the weekend, the cultivation was done twice in duplicates with a shift of 2 days and the generation time was determined using the OD_{750} measurements.

3.2 Cell disruption methods

3.2.1 PEF treatment

For the PEF treatment, a self-built cable impulse generator was used. In principle, the cable impulse generator utilizes a long cable that is arranged like a co-axial cylindrical capacitor. The length of the cable determines the pulse duration. For 1 µs, the cable is 100 m long. The cable is charged by a power supply unit. The spark gap is the switch that determines when the charge is unloaded onto the treatment chamber or cuvette. The spark gap works with gas, in this case sulfurhexafluoride SF_6. The gas acts as a resistance, and the gas pressure modulates it. The higher the gas is pressurized, the higher its resistance is, so more voltage builds up and the later the discharge occurs. This means that the field strength is determined by the pressure of SF_6: the higher the pressure, the higher the field strength.

Depending on the sample volume and sample size the algae suspension was pulsed either in a continuous flow chamber using silicon tubing and a peristaltic pump (MS-4/12-100 ISMATEC, Cole-Parmer GmbH, Wertheim, Germany) or in 500 µl electroporation cuvettes with a gap distance of 2 mm (Electroporation Cuvettes Plus, BTX Harvard Apparatus, Holliston MA, USA). The continuous flow chamber had a volume of approximately 500 µl enclosed by two planar electrodes with 2 mm gap distance as described in (Goettel *et al.*, 2013).

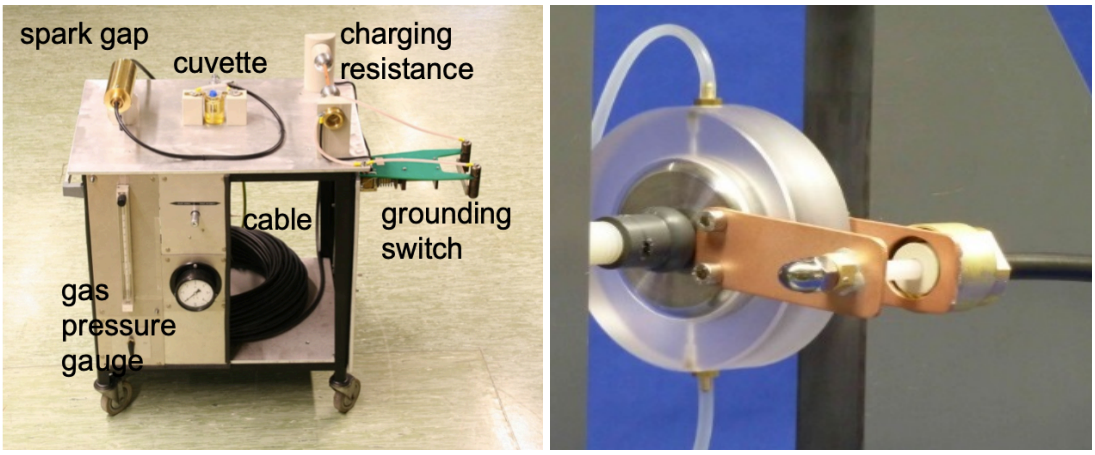


Figure 6: The cable impulse generator used in this study with its components indicated (left). The cuvette can be replaced by a continuous flow chamber (right).

The flow rate for continuous flow mode was set to 3 ml·min^{-1} and the pulse frequency was set to 4.5 Hz. For both methods, the rectangular pulses had a field strength of 40 kV·cm^{-1} and duration of 1 µs. The resulting specific treatment energy was 150 J·g^{-1}. In the cuvettes, the samples received 47 pulses with a pulse frequency of 1 Hz to mitigate heating. One pulse equals approximately 3.16 J·g^{-1}. The algae suspensions usually had an initial temperature of 21 °C and never exceeded 38 °C during the pulse treatment in either configuration. The field strength of 40 kV·cm^{-1} and the specific energy of 150 J·g^{-1} was used because based on empirical data, these parameters ensure that basically all cells are irreversibly electroporated (Eing *et al.*, 2013; Frey *et al.*, 2013; Goettel *et al.*, 2013) and thus were also applied for other works from this group (Silve et al. 2018a; Silve et al. 2018b). An increase of the treatment energy does not have a beneficial effect and essentially just converts into further heating of the sample, so 150 J·g^{-1} is basically the maximum meaningful energy.

The experiments were usually carried out at 5 g·l^{-1} if not specified otherwise.

3.2.2 High pressure homogenization (HPH)

For the HPH method, the cell suspension was passed through a high pressure homogenizer (Avestin EmulsiFlex-C3, 2 kbar, 5 passes) cooled with ice. A HPH works by pressurizing the liquid and essentially pressing it through a narrow gap, which creates high shearing forces that shred cells into smaller bits. This method proved to extract all proteins in our case. A reference for this method can be found in (Ursu *et al.*, 2014). The cell debris was separated by centrifugation (10000 x g, 10 min, 4 °C) afterwards. The cooling with ice is recommended as the process heats up the sample significantly. Without cooling, the suspension can reach up to 70 °C approximately. The suspension is always captured in a glass flask that is swimming in a water-ice bath to cool the suspension down.

3.3 Post-PEF incubation

Protein extraction after PEF treatment requires an incubation step (Coustets *et al.*, 2014). After the pulsing, the algae suspension was diluted with spent medium to 5 g·l^{-1} and incubated for a certain time and at different temperatures to test the influence of diffusion. For the biomass-concentration dependence experiment, the algae suspension was adjusted to a conductivity of 2 mS·cm^{-1} using NaCl and pulsed in cuvettes with 2 mm gap distances at 25, 20, 15, 10 and 5 g·l^{-1}. Afterwards these suspensions were diluted with an equal amount of water (in this case 500 µl deionized water) and incubated for 24 h at room temperature. After incubation the cells were spun down (10000 x g, 10 min) and the protein content/released protein in the supernatant was quantified with the BioRad DC-assay Kit.

This incubation step was modulated by incubating the samples at alkaline conditions with 0.1 M NaOH and/or protease inhibitor (c0mplete Plus by Roche) to test whether

pH has an influence on the protein extraction efficiency and whether enzymes such as proteases are involved in the process.

For the pH dependence experiment, water and/or leftover medium was adjusted using HCl or NaOH respectively to pH values ranging from 6 to 11. The algae were pelleted by centrifugation (10000 x g, 2 min) immediately after the pulse treatment, then the medium was taken off and the cells resuspended in the adjusted water/medium and incubated for 24 h until protein quantification.

3.4 Protein quantification methods

Protein content was determined using the Merck DirectDetect infrared spectrometer as well as the BioRad DC-assay, which is essentially a detergent-compatible Lowry assay (Lowry *et al.*, 1951). In order to determine the protein content of intact cells, we used infrared spectrometry. In this case the cells were diluted to a concentration within a range of 2.5–5 mg·ml^{-1} and 2 µl of the suspension was then pipetted in triplicate onto the PTFE membrane together with a blank and measured with the device:

$$Protein\ content\ in\ \%_{DBM} = \frac{c\ (protein)}{c\ (algae)} * 100$$

The supernatants and extracts obtained via HPH and PEF were quantified using the BioRad DC-assay Kit against a BSA calibration ranging from 0.2–2 mg·ml^{-1} according to the manufacturer's protocol. Afterwards, the protein extraction efficiency can be determined using the same principle:

$$Protein\ extraction\ efficiency\ in\ \%_{DBM} = \frac{c\ (protein\ in\ supernatant)}{c\ (algae)} * 100$$

3.5 Protein precipitation

Extracted proteins can be precipitated by acetone in a ratio of 4:1 (Botelho *et al.*, 2010; Crowell *et al.*, 2013). The samples were stored at -20 °C over 3 days if applicable and then the proteins were pelleted by centrifugation (10000 x g, 10 min, 4 °C). This procedure can be used to concentrate the proteins obtained by an extraction method. For large quantities, the samples were stored in the solvent cabinet and stored until processed. In this case, the precipitated protein was then separated using a funnel and coffee filters (JA!, Konos GmbH, Fabrikstr. 8, D-01683 Nossen). The residue was rinsed with acetone and then distributed in a glass petri dish and left to dry under a fume hood. The dry and brittle residue was further ground to a fine powder in a ball mill (Retsch MM400) using a steel vessel and 10 x 12 mm steel balls (30 Hz, 1 min).

3.6 Characterization of the protein precipitate

The characterization of the protein powder obtained by precipitation was out-sourced to the Landwirtschaftliche Untersuchungs- und Forschungsanstalt "LUFA" in Speyer, Rhineland-Palatinate. Briefly, the protein content of the powder was estimated by Dumas method (total nitrogen * 6.25) (Dumas, 1831) and the amino acid profile was determined by HPLC after acid hydrolysis and conjugation with Ninhydrin. Tryptophane was determined separately in the same manner, but after basic hydrolysis as it disintegrates during acid hydrolysis. The 18 amino acids covered are aspartic acid, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, arginine, tryptophane, lysine, methionine, cysteine and threonine (glutamine and asparagine are deaminated during acid hydrolysis, so they are "hidden" within the values for glutamic acid and aspartic acid respectively).

3.7 Evans blue staining of PEF-treated cells

Evans blue staining was used to check for membrane integrity after PEF treatment. Briefly, 500 µl of a cell suspension was pulsed in an electroporation cuvette (2 mm gap) and then an equal volume of a 5 % Evans blue solution (MW: 961 Da) was added at certain time points (immediately, 1, 6 and 24 h post-PEF). The sample was mixed with the pipette and then incubated for 10 min, then transferred to a 2 ml microfuge tube, spun down briefly (10000 x g, 2 min) and washed 3 times with 2 ml deionized water. Non-pulsed cells served as a control and were treated the same way. The samples were analyzed by microscopy (Zeiss Axioplan 2) at 63x magnification. Cells were counted manually using ImageJ (cell counter plugin) and the permeabilization efficiency in % was calculated by dividing the number of stained cells by the number of total cells multiplied by 100.

3.8 DNA extraction for investigation of DNA laddering

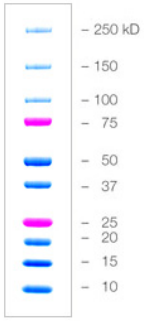
For the DNA extraction, the PEF treated algae were spun down (10000 x g, 10 min) in 2 ml microfuge tubes and separated into pellet and supernatant. Afterwards, these samples were frozen in liquid nitrogen and freeze-dried (CHRIST, Alpha 1-4 LDplus). The dried algae pellets were ground by inserting a 4 mm steel bead into the reaction tubes, cooling the tubes with the beads in liquid nitrogen and then agitating the tube at 30 Hz for 30 seconds (Retsch TissueLyser). The DNA was afterwards extracted from the ground material using the Invisorb Spin Plant Mini Kit according to the manufacturer's protocol. Briefly, 400 µl of lysis buffer P and 20 µl proteinase S were added to the samples and mixed by vortexing. The samples were then incubated at 65 °C for 30 min under constant agitation. Cell debris was removed by a pre-filter (2 min, 11100 x g). Then 40 µl of RNase A (Qiagen, 10 $mg \cdot ml^{-1}$) and then binding buffer A were added to remove RNA. The solution was vortexed thoroughly and then transferred to the spin column where it was incubated for 1 min prior to centrifugation

(2 min, 11100 x g). The filter was washed with 550 µl washing buffer II (1 min, 11100 x g) and then dried by another centrifugation step (4 min, 11100 x g). The DNA was then eluded using 45 µl of pre-warmed (65 °C) elution buffer, incubating the filter for 3 min before centrifugation (1 min, 11100 x g). The DNA can be stored at -20 °C afterwards. 25 µl of DNA were mixed with 6x DNA loading dye (Thermo Scientific) and visualized on a 1,5 % agarose gel in 0.5 % TAE (20 mM TRIS, 10 mM acetic acid, 0.5 mM EDTA) stained with 5 µl SYBR Safe DNA gel stain per 100 ml of agarose.

3.9 SDS-PAGE and western blot detection of organelle-specific proteins

The extracts obtained by PEF treatment were mixed with 4x Lämmli buffer (200 mM TRIS-HCl, 8 % (w/v) SDS, 40 % (v/v) glycerol, 4 % (v/v) ß-mercaptoethanol, 0.8 % (w/v) bromphenol blue) in a ratio of 4:1 (e.g. 300 µl extract + 100 µl 4x Lämmli) and then heated to 95 °C for 15 minutes (Lämmli, 1970). The proteins were separated on a 12 % polyacrylamide gel (selfmade) via SDS-PAGE, usually at 100 V (the equipment is all from BioRad). The gels can then be stained by Coomassie staining, in this case colloidal Coomassie as in (Candiano *et al.*, 2004) or subsequently blotted onto a nitrocellulose membrane (Roti-NC by Carl Roth). Transfer of proteins was confirmed via Ponceau S staining of the membrane (2 % (w/v) Ponceau S, 30 % (w/v) trichloroaceticacid, 30 % (w/v) salicylic acid). After blocking with cream liquor (Baileys Original Irish Cream), the membrane was washed with TBS-T (TRIS buffered saline; 50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 0.1 % (v/v) TWEEN-20) and antibodies were applied for organelle-specific proteins. The primary antibodies used in this work are directed against RuBisCo for chloroplast (Abcam, ab226002), Histone H3 for nucleus (Abcam, ab1791), Actin for cytosol (Agrisera, AS132640) and Cytochrome c oxidase subunit II (COXII) for mitochondria (Agrisera, AS04053A). These are all rabbit polyclonal antibodies, a goat anti-rabbit secondary antibody coupled to horseradish peroxidase (HRP, Abcam ab6721) served as secondary antibody for detection. The antibodies were all prepared at a concentration of 1:5000 in TBS-T with 5 % BSA and preserved with 0.01 % Thimerosal. The signals were developed colorimetrically using 1-Step TMB-Blotting Substrate Solution (Thermo Fischer Scientific).

The marker used in this work is the Dual Color Precision Plus Protein Standard by Bio-Rad:



Detailed protocols for the SDS-PAGE and Western Blot analysis can be found in the appendix.

3.10 Neutral red staining

C. vulgaris cells were stained in 0.5 µM Neutral Red (NR, Roth) for 1 h and the percentage of stained cells was determined by microscopy and manual cell counting. The percentage was determined by dividing the number of stained cells by the number of total cells. Then the cells were PEF treated and the percentage of stained cells was determined again after 5, 15, 30 and 60 min.

3.11 Statistical analysis & replication

Each experiment was repeated at least 3 times in triplicates each time. The graphs show average values of 3 independent experiments with their standard deviation. Statistical significance was determined by paired student's t-test when applicable. Gels, microscopy, western blots and DNA extraction were also done at least three times, the most representative were chosen.

4 Results

4.1 Evaluation of cell death after PEF treatment

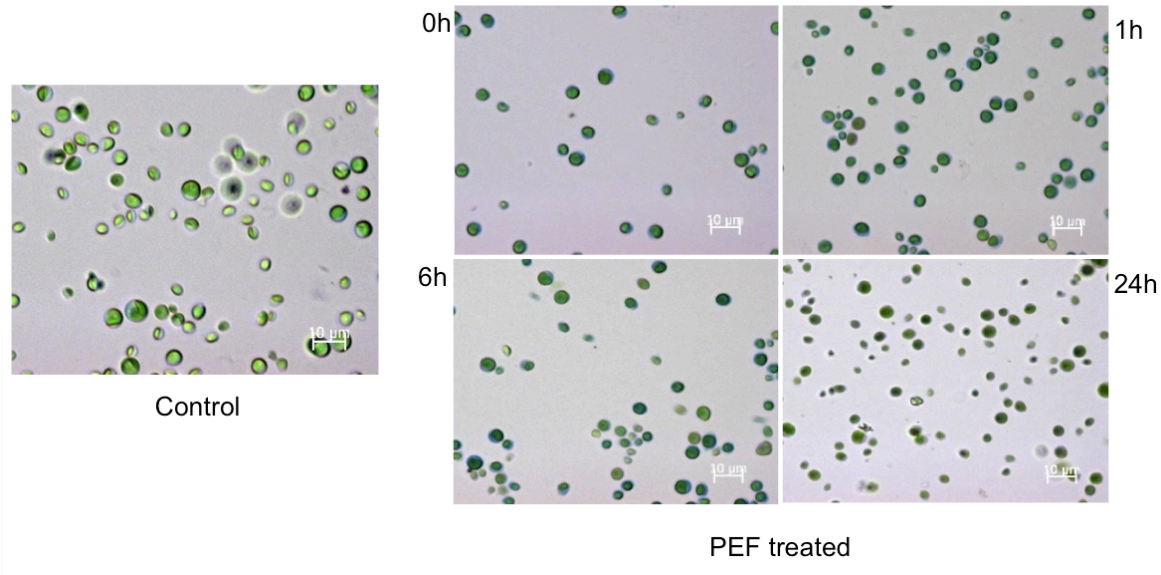


Figure 7: Evans blue staining of *C. vulgaris* after 0, 1, 6 and 24 h after PEF treatment, published in (Scherer *et al.*, 2019)

Evans blue is a dye that does not penetrate intact cells (Figure 2, Control) but can accumulate in permeabilized cells (Figure 7, PEF treated), so it is used to confirm that membrane integrity of *C. vulgaris* cells is in fact affected by the PEF treatment. Evans blue is able to penetrate cells at any point after the PEF treatment (i.e. 1, 6, 24 h). This reveals that the cells are immediately and irreversibly permeabilized by the PEF treatment under the parameters (40 $kV \cdot cm^{-1}$, 1µs, 150 $J \cdot kg^{-1}_{suspension}$). The staining efficiency was determined by cell counting and is approximately 99.92 %.

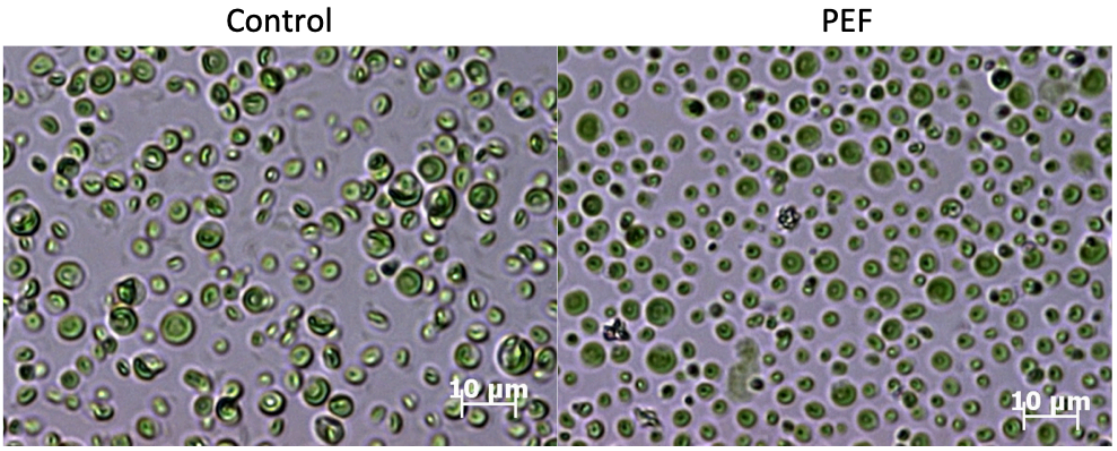


Figure 8: A picture of *C. vulgaris* cells before and after PEF treatment, without Evans blue.

Morphological changes are not evident, except for the observation that the inner compartments seem to expand. The shade of green slightly changes and the mostly sickle-or cup-shaped chloroplasts (depending on the orientation in the focus plane) seem to bloat (Figure 8).

Evans blue is often used as an indicator of cell death (Jacyn Baker *et al.*, 1994) although it is more an indicator of membrane integrity: cells can in principle recover and repair their membrane after uptake of the dye. Therefore the DNA of pulsed *C. vulgaris* was isolated and investigated for DNA laddering to back up the assumption that PEF treatment under these parameters is lethal to *C. vulgaris*. The genomic DNA appears as one clear band in the control (Figure 9, C). DNA extracts from the pellets of PEF-treated samples exhibit signs of DNA laddering that progress over time (Figure 9). DNA fragments can eventually be detected in the supernatants, indicating that the cells are also leaking their DNA material into their surrounding post-PEF. The samples processed by the HPH method only show a slight smear of DNA.

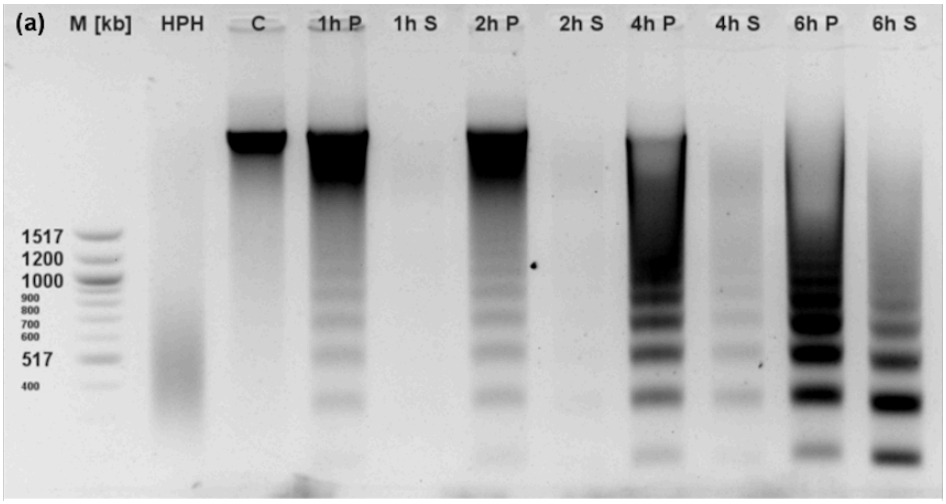


Figure 9: Visualization of DNA-laddering triggered by PEF treatment. Genomic DNA of PEF treated *C. vulgaris* was isolated from freeze-dried pellets (P) and supernatants (S) and visualized on a 1,5 % TAE agarose gel with SYBR Green. M [kb] = Marker in kilobases, C = control. Published in (Scherer *et al.*, 2019)

4.2 Effect of biomass concentration during incubation on protein extraction yield

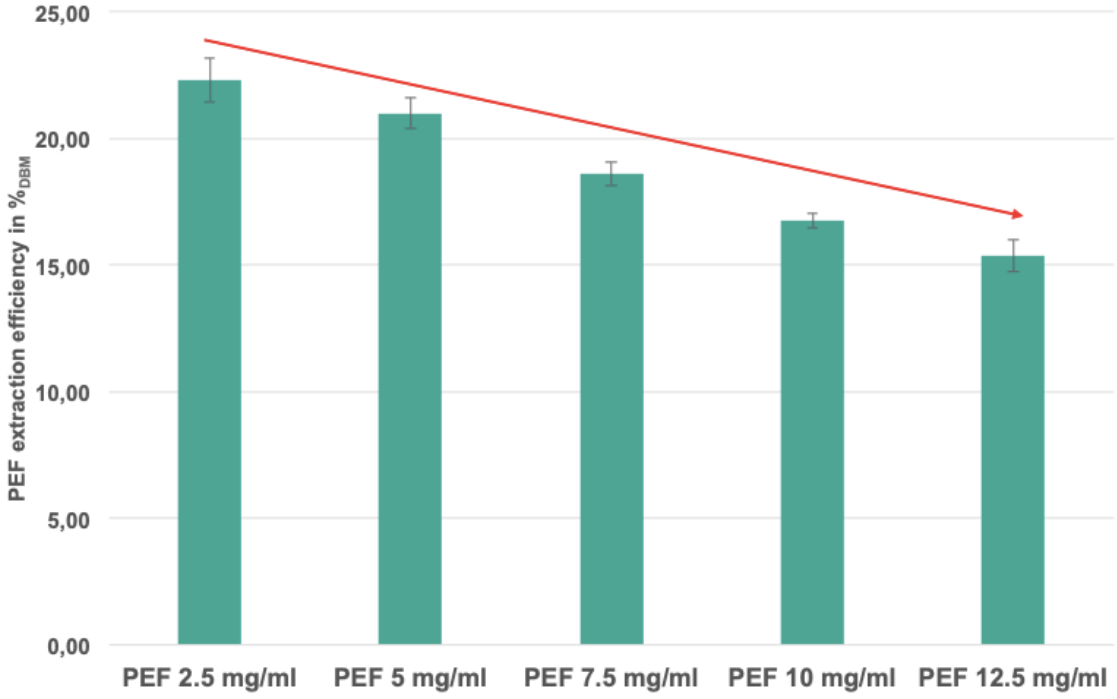


Figure 10: Protein recovery efficiency from *C. vulgaris* in dependence of biomass concentration. *C. vulgaris* suspensions of various concentrations were pulsed (40 kV·cm^{-1}, 1 µs, 150 J·g$^{-1}_{suspension}$), diluted and incubated for 24 h at room temperature. Data are shown as average with standard deviation, n = 3. Published in (Scherer *et al.*, 2019)

In order to see whether protein extraction efficiency is dependent on the biomass concentration, a serial dilution of the algae suspension to the according biomass concentrations (2.5, 5, 7.5, 10, 12.5 mg·ml^{-1}) was done and the algae subsequently pulsed. The results show a gradient within a range of 2.5 to 12.5 mg·ml^{-1}: at 2.5 mg·ml^{-1}, 22 %$_{DBM}$ are extracted, and at 12.5 mg·ml^{-1}, only about 15 %$_{DBM}$ (Figure 10)

Given that the algae have an average protein content of 45 %$_{DBM}$, this means that the biomass concentration can decide between extracting half of the proteins or one third of the proteins that are present in the cell.

4.3 Impact of incubation temperature on protein recovery

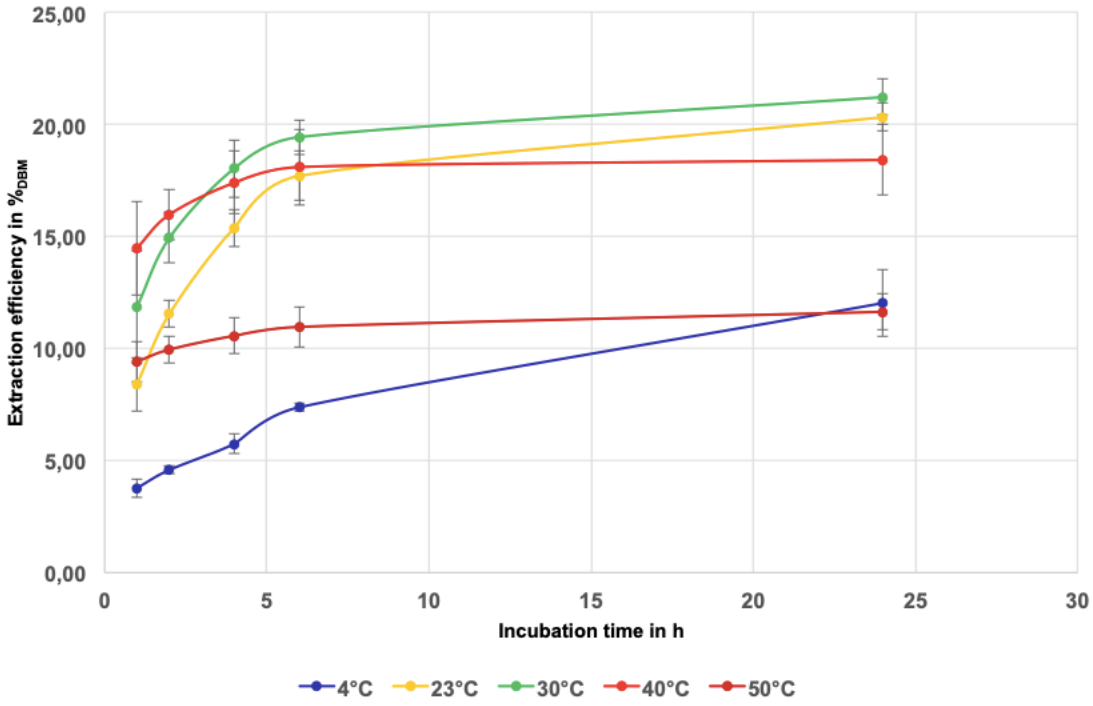


Figure 11: Time course of protein extraction efficiency from *C.* vulgaris in dependence of extraction temperature. The algae suspension was pulsed (40 kV·cm^{-1}, 1 µs, 150 J·kg$^{-1}_{suspension}$) and then incubated at different temperatures. Data are shown as average with standard deviation, n = 3. Published in (Scherer *et al.*, 2019)

To determine the influence of incubation temperature on the protein yield after PEF treatment, aliquots of PEF treated samples (5 mg·ml^{-1}) were incubated at various temperatures (4, 23, 30, 40 and 50 °C) and the amount of protein released into the supernatant was sampled after 1, 2, 4, 6 and 24 h. The kinetics of the extraction varies between the different temperatures. The extraction seems to have an optimum around 30 °C at which the best extraction efficiency can be seen, although the differences between 23, 30 and 40 °C are statistically insignificant, with the end points for each temperature clustering together. 4 and 50 °C however show an inhibitory effect on the extraction and are significantly different to 23, 30 and 40 °C. For 4 and 50 °C, the

extraction efficiency does not exceed 12 $\%_{DBM}$ in the period of 24 h, with yields far below the ones obtained at 23, 30 and 40 °C (Figure 11). It has to be pointed out that there is no detectable amount of protein in the supernatant directly after the PEF treatment, at t = 0 basically.

4.4 Impact of alkaline pH and protease inhibitor on PEF extraction efficiency

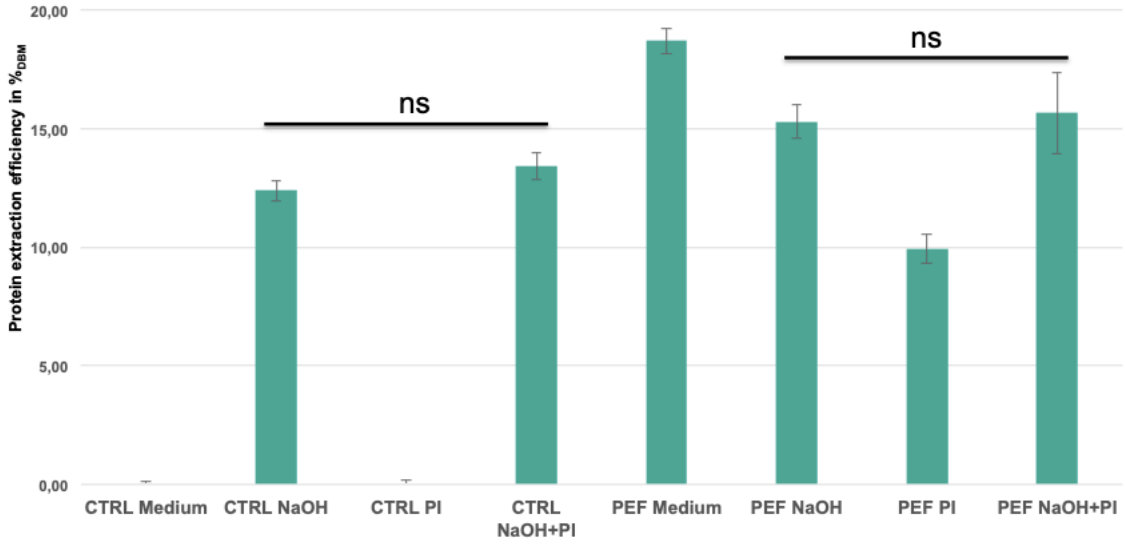


Figure 12: Protein recovery efficiency from *C. vulgaris* under the influence of 0.1 M NaOH and protease inhibitor. The algae suspension (10 $mg \cdot ml^{-1}$) was pulsed (40 $kV \cdot cm^{-1}$, 1 µs, 150 $J \cdot g^{-1}_{suspension}$) and afterwards incubated for 24 h at room temperature under the influence of 0.1 M NaOH and/or protease inhibitor. Data are shown as average with standard deviation, n = 3. CTRL = control, PEF = pulsed electric field treated, NaOH = sodium hydroxide, PI = protease inhibitor. Published in (Scherer *et al.*, 2019)

The results from the temperature variation suggest that there must be a process that goes beyond diffusion which is influencing protein release, because protein release seems to work best in "physiological" temperatures but not at extreme temperatures. Therefore the effect of alkaline pH and the impact of protease inhibitor on the extraction yield were tested, because these could interfere with the protein release under the assumption that enzymes such as proteases support this release. Extraction in 0.1 M NaOH (pH 13) yields slightly less protein (just around 3 % less) compared to extraction in plain medium (Figure 12, PEF Medium vs. PEF NaOH). However, it is obvious that 0.1 M NaOH already has an effect on the control cells per se as it is corrosive (Figure 12, CTRL NaOH). When the cells were incubated with protease inhibitor, the protein yield decreased even further to around 10 % (Figure 12, PEF PI). There is no additive effect of 0.1 M NaOH and protease inhibitor; the differences between the extractions in NaOH and NaOH combined with protease inhibitor are insignificant, whereas the other differences are significant ($p < 0.05$).

4.5 Impact of pH on PEF extraction efficiency

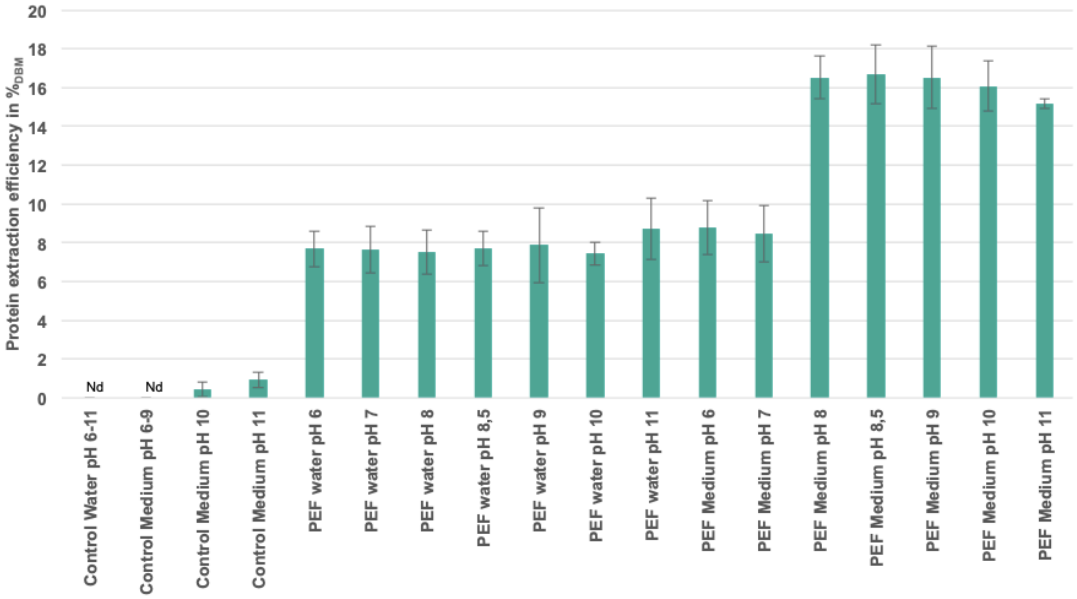


Figure 13: Protein recovery efficiency from *C. vulgaris* under the influence of pH value. The algae suspension (10 mg·ml^{-1}) was pulsed (40 kV·cm^{-1}, 1 µs, 150 J·g$^{-1}_{suspension}$) and afterwards incubated for 24 h at room temperature under the influence of different pH values in water or leftover medium conditioned to defined pH values. Data are shown as average with standard deviation, n = 3.

The results from the protease inhibitor and NaOH experiment suggested that pH is also a factor that plays a role, and therefore the treated biomass was immediately separated and resuspended in water or medium that was conditioned to a certain pH with HCl or NaOH respectively. The results show that water per se is generally worse than culture medium at same pH levels, whereas the spent culture medium conditioned to a certain pH exhibits better extraction efficiencies (Figure 13, compare PEF water with PEF medium). It is remarkable that the extraction yield in medium is mediocre at pH 7 but then shows an extraction optimum around pH 8-9 and decreases slightly with further rising pH, which also is in line with the previous experiment.

4.6 Visualization of extracted proteins by SDS-PAGE and Coomassie staining

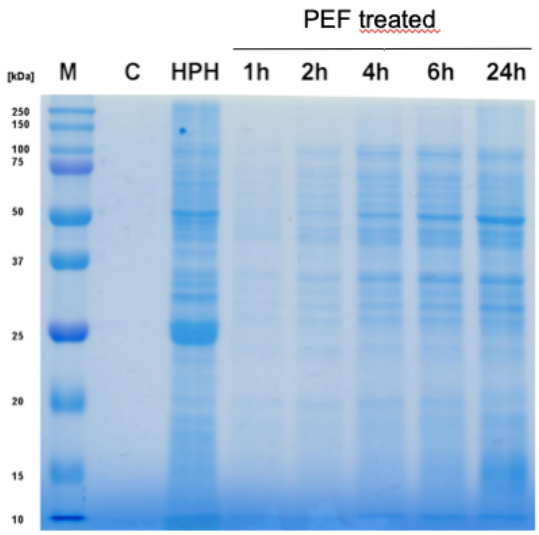


Figure 14: Visualization of the proteins extracted by HPH and PEF treatment via SDS-PAGE and subsequent Coomassie staining. PEF samples of various time points and a HPH extract from a *C. vulgaris* suspension (5 mg·ml^{-1}) were loaded and separated on a 12% polyacrylamide gel. Published in (Scherer *et al.*, 2019)

To see qualitative differences in the protein fractions obtained by HPH and PEF treatment, samples of PEF extracts of a time course experiment (which were incubated at 23 °C) were loaded onto a polyacrylamide gel together with an extract generated by HPH. The bands of the PEF time course experiment get more pronounced and intense over time (Figure 14, PEF treated 1-24 h). The HPH extract (Figure 14, HPH) represents a total protein extract to which the PEF extracts only show minor differences. The only striking difference would be the very pronounced band at 25 kDa in the HPH sample.

4.7 Localization of PEF extracted proteins from *C. vulgaris*

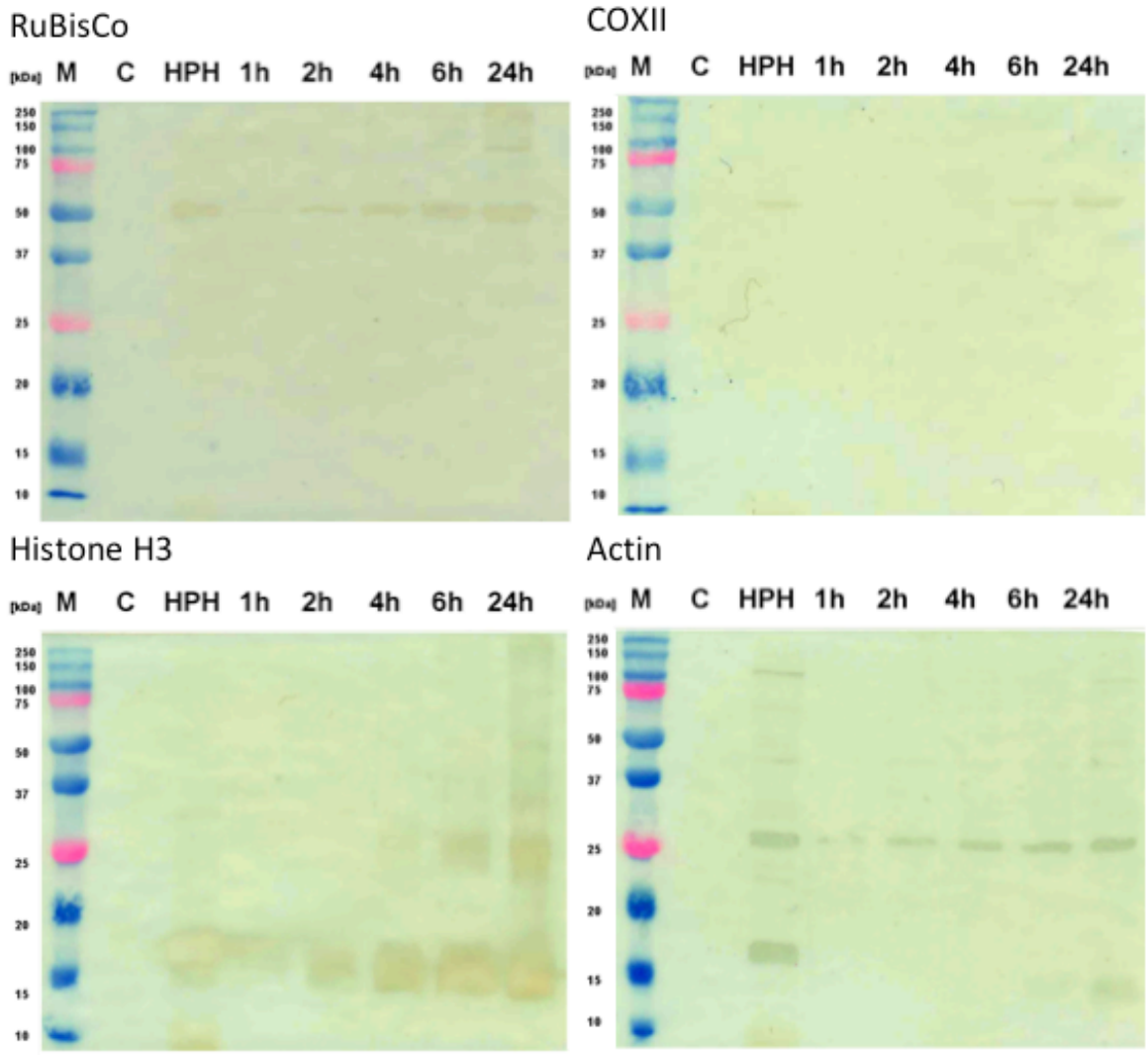


Figure 15: Western blot detection of organelle-specific proteins RuBisCo (chloroplast), COXII (mitochondrion), Histone H3 (nucleus) and Actin (cytosol). The sample size of these blots is n = 3, and the most representative ones for each antigen are shown here. Published in (Scherer *et al.*, 2019)

Western blot detection of organelle-specific proteins reveals that marker proteins from all the organelles can be detected in the supernatant after PEF treatment. A signal for RuBisCo, Histone H3 and Actin can be detected within 1 h after PEF treatment (Figure 15, RuBisCo, Histone H3 and Actin). The signal for COXII on the other hand shows up only after an extended period of time, 6 h after the PEF treatment (Figure 15, COXII). It has to be pointed out that the size of the signals for actin and COXII do not line up with the sizes predicted by the manufacturers of the antibodies, so it is questionable whether the signals really correspond to the correct proteins, but in the PEF extracts there is only a single band visible for each protein, indicating specificity of the antibodies. The western blots reflect that the signals for the proteins intensify over time,

confirming the time-dependent manner of protein release after PEF once more. The band for Histone H3 exhibits a size shift and seemingly becomes smaller over time. It has to be explicitly pointed out that in western blots of samples incubated with protease inhibitor, the size shift in the histone band could not be observed and the COXII band was not detectable. The signals for the other proteins are slightly weaker under the influence of protease inhibitor (these blots can be found in the appendix).

4.8 Neutral Red staining

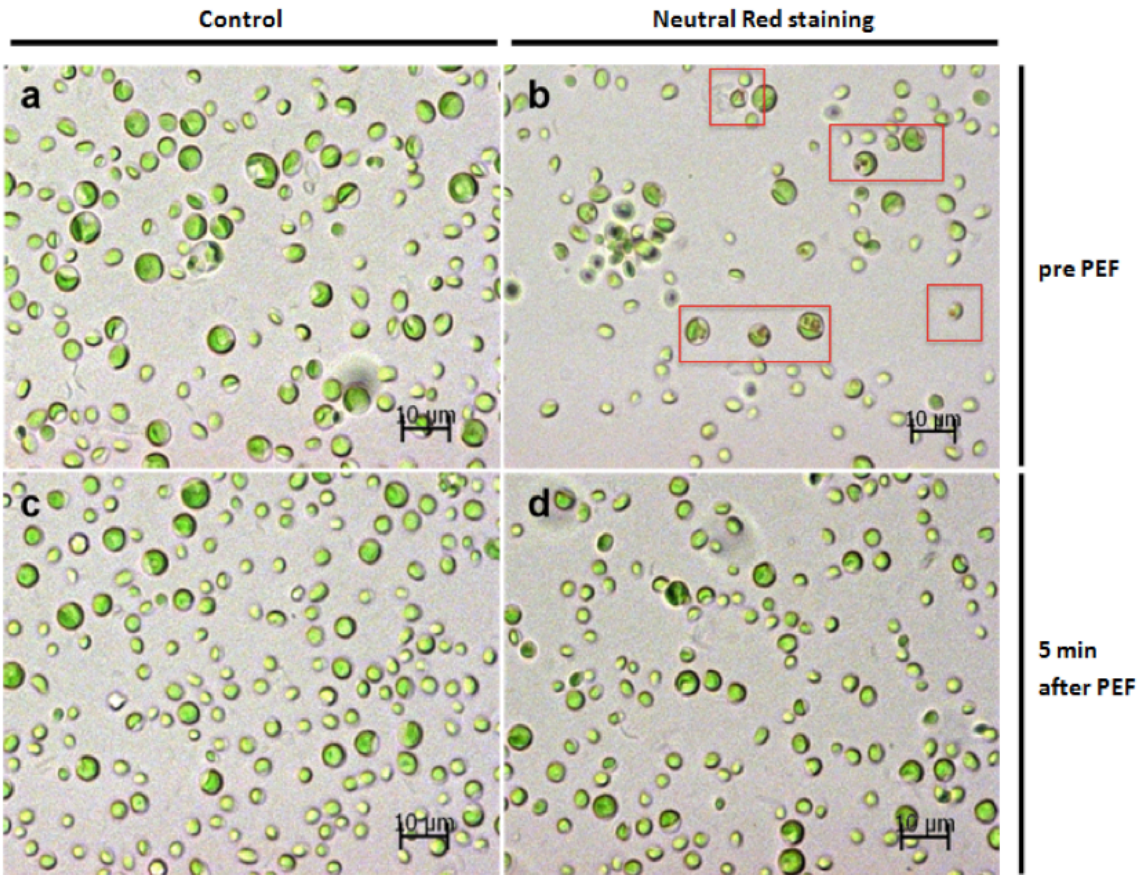


Figure 16: Neutral Red staining of *C. vulgaris* reveals acidic compartments of the cell as the dye accumulates in a ion trapping mechanism there and turns red. Cells with captured Neutral red are indicated by red boxes. a = control cells before PEF treatment; b = Neutral Red stained cells before PEF treatment, stained cells are indicated by red boxes; c = control cells after PEF treatment, d = Neutral Red stained cells after PEF treatment

In order to investigate vacuole integrity, cells were stained with Neutral Red. Acidic compartments such as the vacuole are indicated by the red dye (Figure 16). The staining efficiency lays around 16 % and drops to less than 1 % post-PEF treatment, indicating that the signal disappears upon PEF treatment and does not recover. This has also been quantified by manual cell counting (Figure 17).

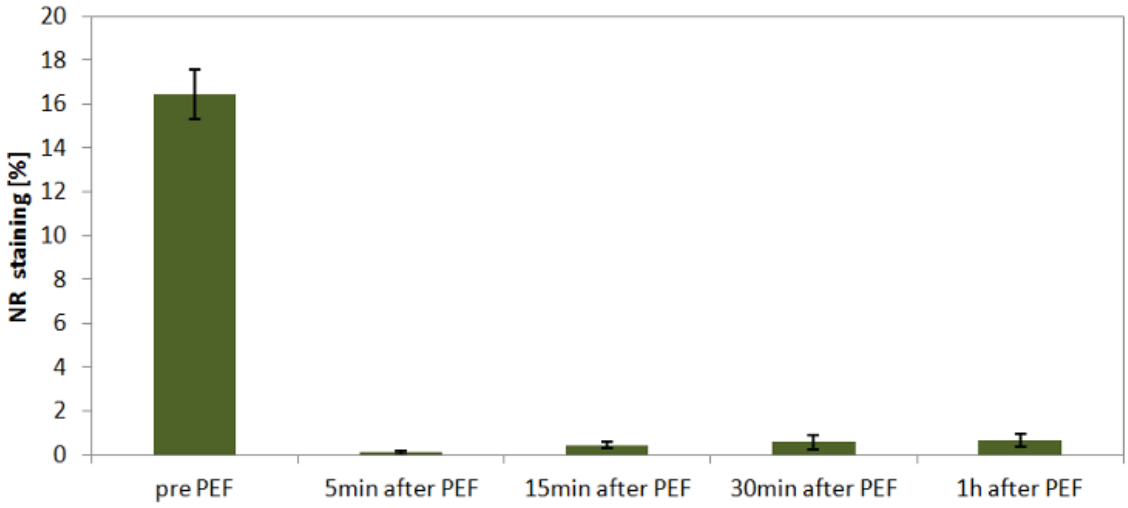


Figure 17: Neutral Red staining efficiency quantified in % of cells before and after different time points after PEF treatment.

4.9 PEF energy variation

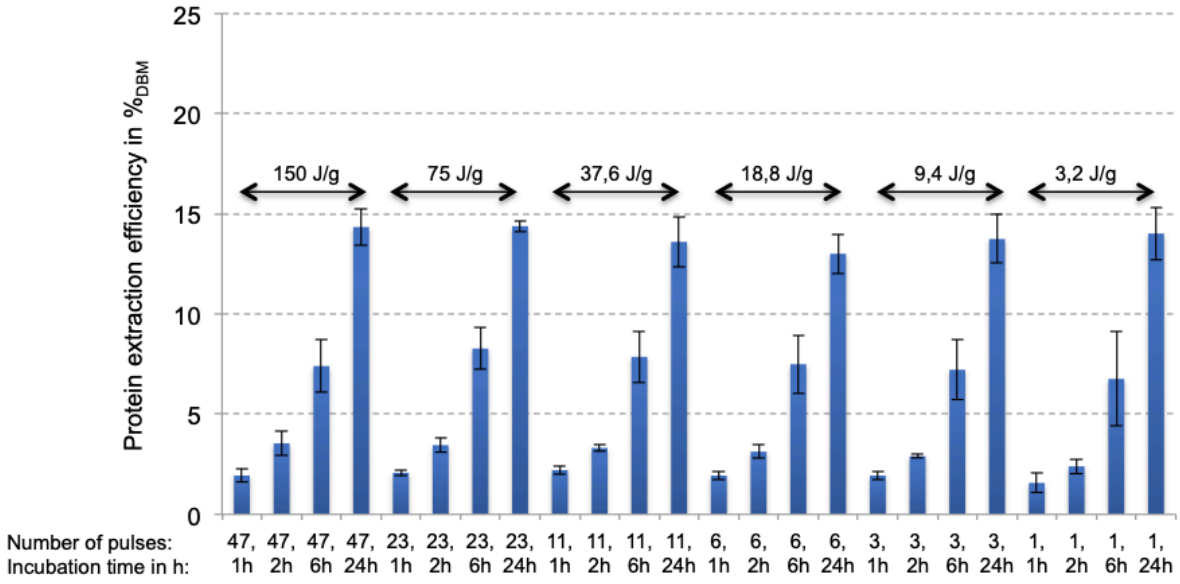


Figure 18: Protein recovery efficiency from *C. vulgaris* in dependence of energy input. *C. vulgaris* suspensions were pulsed (40 kV·cm^{-1}, 1 µs) and sampled at various time points (1, 2, 6 & 24 h). The specific treatment energy is dependent on the number of pulses applied. Data are shown as average with standard deviation, n = 3.

To determine the minimum energy required to extract proteins, the energy (i.e. the number of pulses) was gradually reduced and the protein extraction efficiency over time was monitored. This variation of the specific treatment energy shows that the same effect can be achieved with basically any amount of pulses all the way down to one single pulse, which is roughly one fiftieth of the standard energy usually applied (Figure 18).

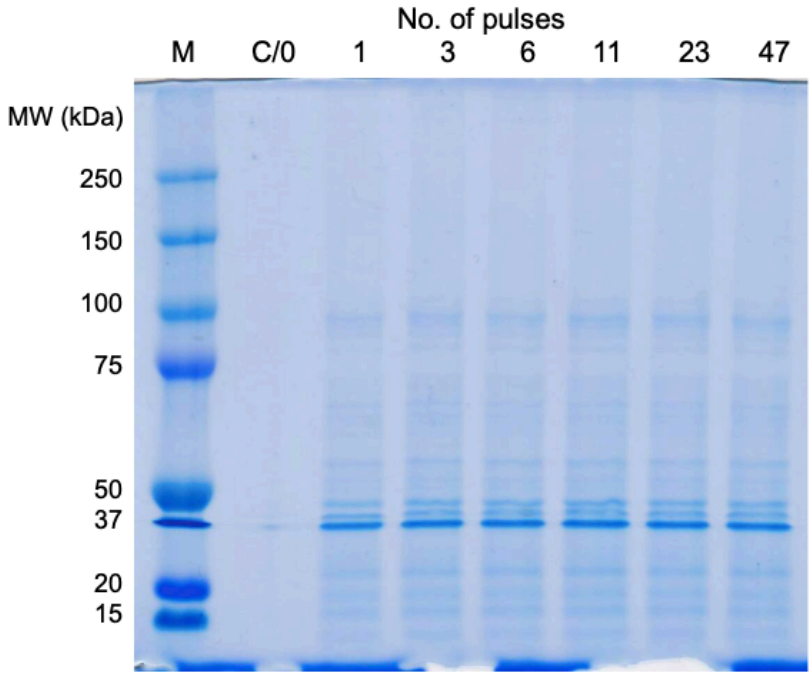


Figure 19: Visualization of the extracted proteins of the energy variation experiment via SDS-PAGE and Coomassie staining. The protein fractions look the same for every energy applied. (C/0 = Control, 0 pulses, M = Marker, MW = molecular weight in kDa)

The extracted proteins were also visualized via SDS-PAGE and Coomassie staining to see whether there are differences between the different energies that were applied. The pattern looks the same for every amount of pulses/energy applied (Figure 19).

4.10 Upscaling of the protein extraction from flasks to 25 l PBR

In order to obtain significant amounts of protein powder, the experiment was scaled up from flask to 25 l indoor PBRs. The 25 l were concentrated to 2 l, and 1 l was treated by HPH, and the other 1 l was subjected to PEF. The pH value was adjusted back to 8.5 using NaOH, then the algae were incubated at 30 °C for 24 h. Afterwards the treatments, the supernatants were separated via centrifugation and the proteins precipitated with Acetone 4:1. The precipitate was collected using coffee filters and transferred to glass petri dished to dry and evaporate the acetone (Figure 20).

Figure 20: Protein precipitation of extracts obtained by HPH and PEF. The protein fraction obtained from HPH is greener than the fraction obtained via PEF.

It was possible to obtain protein powder via acetone precipitation from both HPH and PEF processed samples on a bigger scale. This experiment was repeated three times, and this triplicate was submitted to the LUFA in Speyer for further analysis.

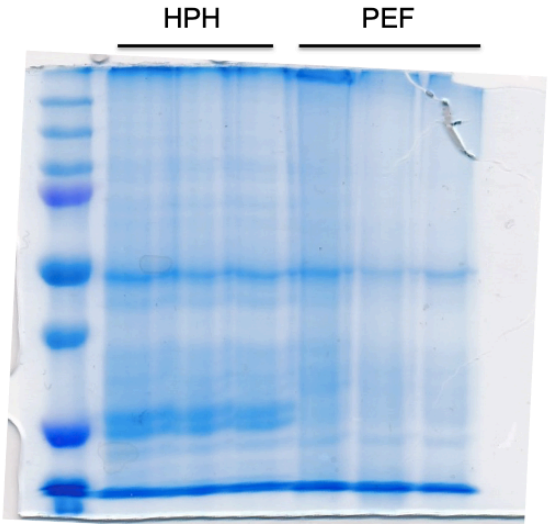


Figure 21: Visualization of protein precipitates of extracts obtained by HPH and PEF via SAS-PAGE and Coomassie staining. The protein fractions exhibit a similar pattern as observed earlier in the study, but at a worse quality, i.e. more blurry.

SDS-PAGE of the triplicates shows similar differences as pointed out in the previous Coomassie-staining, namely the pronounced and band at 25 kDa in the HPH protein fraction that is absent in the PEF protein fraction. The quality and resolution of the proteins is worse (i.e. they appear more blurry on the gel, see Figure 21) due to them going through a precipitation step and having to be re-dissolved by boiling in Lämmli buffer.

4.11 Properties of the protein precipitate

The protein content for the PEF precipitates was on average 32.87 % ± 1.88 and 56.9 % ± 1.73 for the HPH precipitates related to their dry weight as determined by Dumas method (total nitrogen x 6.25). The amino acid content was also given in % related to dry weight. These values were used to calculate the following amino acid contents in % normalized to total protein content determined by Dumas method:

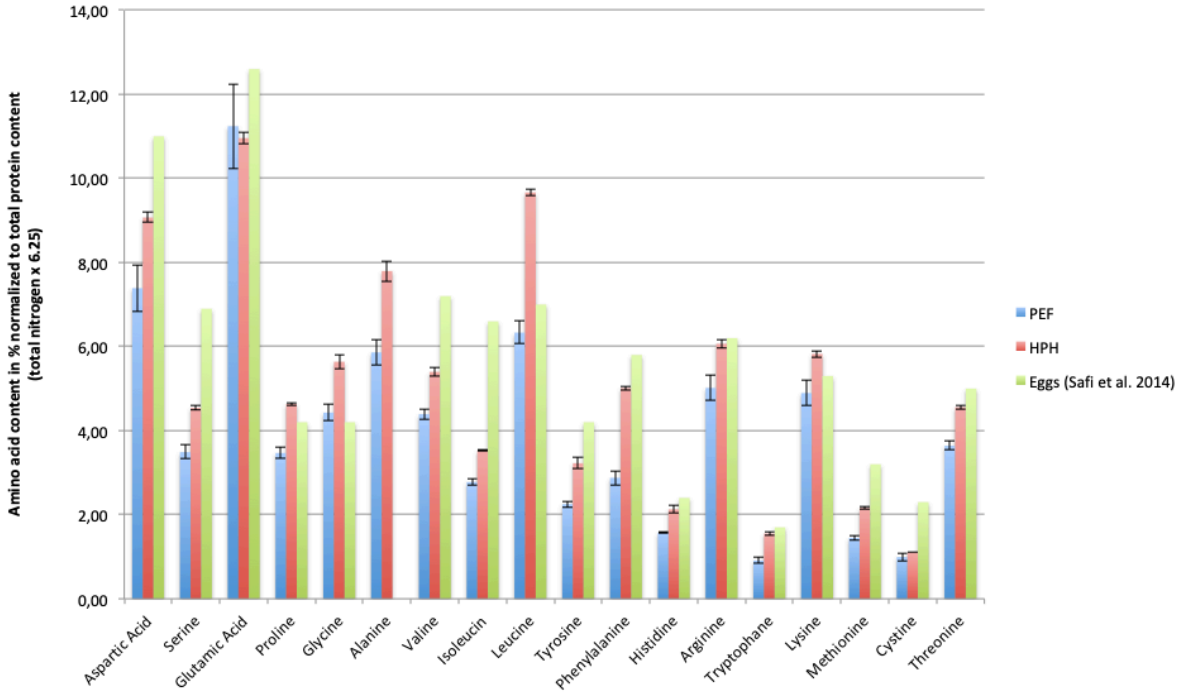


Figure 22: Amino acid content of various amino acids normalized to total protein content (total nitrogen x 6.25) in PEF and HPH extracts. The amino acid composition of egg is taken from (Safi *et al.*, 2014) for comparison.

The comparison between PEF and HPH extracts show that the protein content of the precipitates differ from each other by roughly 24 %, and when looking at the individual amino acids, it is apparent that the precipitates obtained by PEF treatment contain less of each individual amino acid (with the exception of glutamine which is the same) than the precipitates generated by HPH. The values for the amino acid composition of eggs was taken from (Safi *et al.*, 2014) for comparison. The protein fraction obtained by HPH is closer to eggs than the one obtained by PEF treatment (Figure 22). It is also noteworthy that the sum of the individual amino acids doesn't add up to the determined

total protein content. In the PEF precipitates, a greater portion of protein than in the HPH precipitates is not characterized: in the PEF precipitate, 26.99 % ± 3.86 of the total protein is not resolved in amino acids while this portion is only 7.14 % ± 0.97 in the HPH precipitate (exact numbers can be seen and checked in the appendix, Table 1 and 2).

4.12 Summary of results

From the results of this work it is possible to say:

- PEF treatment permeabilizes the *C. vulgaris* cells immediately and irreversibly (4.1)

- PEF treatment triggers DNA laddering (4.1)

- Proteins are released slowly in a time-dependent manner (4.3)

- Up to half of the proteins present in the cells can be extracted (4.2)

- The protein release is influenced by biomass concentration (4.2), temperature (4.3), pH (4.4 and 4.5) and can also be inhibited by protease inhibitor (4.4)

- The higher the biomass concentration is during the incubation step, the less efficient the extraction efficiency becomes (4.2)

- The extraction works well at temperatures between 20 and 40 °C, but cold (4 °C) and hot (50 °C) temperatures show an inhibitory effect (4.3)

- The inhibitory effect of "extreme" temperatures can be mimicked by protease inhibitor at room temperature (4.3 and 4.4 combined).

- The extraction has a pH optimum around 8.5 - 9 (4.5) and is worse at neutral pH

- Unlike HPH, PEF treatment cannot extract membrane proteins and is thus selective towards "free-floating" proteins that are not membrane-bound or membrane-associated (4.6)

- PEF treatment affects the cell as whole and has an effect on various organelles: On the western blot, proteins from the chloroplast, mitochondrion, nucleus and cytosol are detectable (4.7) and Neutral red staining confirms that the vacuole is damaged as well (4.8)

- The treatment energy can be reduced to one fiftieth of the standard parameters and yields the same effect (4.9)

- Differences of the protein fractions obtained by HPH and PEF are also evident from the amino acid profiling of the protein precipitates (4.10). While the amino acid profile of the *C. vulgaris* protein is similar to eggs, the protein content of the PEF protein precipitates is lower than in the HPH protein precipitates, and there is generally less of each amino acid present in the PEF fraction (4.11)

5 Discussion

5.1 Recapping the initial situation of this work

The aim of this work was primarily to characterize and optimize protein extraction from microalgal biomass for food and feed purposes, for which the already certified *C. vulgaris* was chosen. In the works of (Postma *et al.*, 2016; 't Lam *et al.*, 2017; Safi *et al.*, 2017), PEF treatment is usually compared to mechanical disruption methods such as bead-milling. In this comparison, PEF for one cannot directly compete with the extraction efficiency of mechanical extraction methods but was also reported to show poor extraction efficiencies in general. The consensus of these works is that PEF treatment is not suitable as an extraction method. But these works did not identify which parameters influence PEF-assisted extraction even though there were clues for it already. (Coustets *et al.*, 2014) have demonstrated that it is possible to extract proteins from *C. vulgaris* with PEF treatment, and that an incubation step after PEF treatment is necessary, which the works of (Postma *et al.*, 2016; 't Lam *et al.*, 2017; Safi *et al.*, 2017) neglected (they often measured within 1 h after PEF treatment, a time point at which no significant amount of protein is detectable). The pulse parameters used in this work are different from the ones used in (Coustets *et al.*, 2014), so it is necessary to identify and characterize the effect of these parameters first and then optimize the extraction process in terms of yield and energy consumption. The first questions that have to be addressed based on these works are quite simple and boil down to:

- Are the established PEF parameters (Eing *et al.*, 2013; Frey *et al.*, 2013; Goettel *et al.*, 2013) also suitable for protein extraction?

- Which parameters have to be considered to achieve better PEF extraction yields?

In order to understand how the process can be optimized, it is also necessary to think about biological processes that might influence the protein extraction by PEF, just so that a model can be established that helps to understand which factors have to be considered in this process. In the following parts of this discussion, the results gathered are interpreted to formulate a model/mechanism that shall describe PEF-assisted protein extraction.

5.2 PEF treatment under these pulse parameters is lethal to *C. vulgaris*

One of the first things investigated in this work is the effect of the PEF treatment parameters established in previous works of this lab on *C. vulgaris*. As suggested by (Eing *et al.*, 2013; Frey *et al.*, 2013; Goettel *et al.*, 2013), the treatment parameters and amount of energy applied should ensure irreversible electroporation that is considered

to be necessary for any kind of extraction in general. Evans blue staining was therefore used to check for the electroporation efficiency and to validate that the cells are irreversibly electroporated. The results show that the cells are stained by Evans blue at any point after the PEF treatment, and the treatment electroporated 99.92 % of the cells. This suggests that membrane integrity is lost and does not recover. This can already be seen as a hallmark for cell death since membrane integrity is vital to the life of a cell in general (Crutchfield *et al.*, 1999). During the Evans blue staining it can also be noticed that the cells don't change in size or shape (which supports the idea of extracting things from the cells without shredding them to smaller bits), but their organelles do. The chloroplast is sickle- or cup-shaped in control cells but seems to bloat in PEF treated cells so that they look uniformly green. Since Evans blue is strictly speaking only an indicator of membrane integrity, it is necessary to find additional evidence for cell death, so it was decided to check for DNA laddering which is usually seen as a hallmark for PCD (Elmore, 2007). DNA extracts from PEF treated cells reveal that DNA laddering kicks in after PEF treatment and progresses over time until the genomic DNA is completely fragmentized. These DNA fragments also start to leak out of the cell and are thus detectable in the supernatant. The laddering of the DNA is typical as PCD-induced nucleases cleave the DNA into fragments that are multiples of approx. 180 bp. However, it is reported by other publications that DNA laddering can also happen during necrosis (Dong *et al.*, 1997; Kuthanova *et al.*, 2008), so it might not be safe to say that it is really PCD based on this result alone. This phenomenon also cannot be investigated by mechanical disruption methods since they are based on shearing forces that essentially shred the DNA into pieces as proven by the HPH processed sample: it only shows a slight smear on the TAE agarose gel. Lastly, experiments with protease inhibitor shows that DNA laddering still takes place but is inhibited to the point where genomic DNA is still visible after 24 h. It has to be taken into account that the protease inhibitor is not EDTA-free, and EDTA chelates divalent cations (Ca^{2+}, Mg^{2+}) that are essential for nucleases to work properly. At the same time it might interfere with signaling that is necessary to induce PCD.

From these results it is possible to say that the PEF treatment parameters permeabilize the cells immediately and irreversibly, and that this is lethal for the cells.

5.3 PEF extraction is enzyme-mediated and is influenced by environmental factors

Early in the experimentation of this work, it could observed that proteins are released in a time-dependent manner, confirming that an incubation step is necessary as concluded by (Coustets *et al.*, 2014) and neglected by others. However, there were also fluctuations in extraction efficiency. Parameters such as biomass concentration, temperature and pH value during the incubation were not normalized initially. The first parameter investigated was the influence of biomass concentration. It turned out that the extraction yield decreases the higher the biomass concentration is. In the

concentration range between 2.5 and 12.5 $mg \cdot ml^{-1}$ of algae it can be seen that the concentration can decide between extracting one half of the proteins or one third of the proteins that are present in the cells. This might explain the low extraction efficiencies reported by other studies that use higher biomass concentrations: (Postma *et al.*, 2016) for example used 25 $mg \cdot ml^{-1}$ algae. One reason for this phenomenon could be diffusion gradients that drive proteins from the inside to the outside of the cell. This circumstance can be an obstacle for attempts to scale up this process in industrial applications. A major task will be to figure out how to maintain proper extraction efficiencies at higher biomass concentrations. After normalizing the biomass concentration (typically 5 $mg \cdot ml^{-1}$) the next thing to check for was the influence of temperature on the extraction efficiency. The extraction shows the best efficiencies in a range between 20 °C and 40 °C. At 4 and 50 °C respectively, the maximum yield over the 24 h period lays around 12 $\%_{DBM}$, half of what can be obtained in the range of 20 - 40 °C. This indicates that there must be a process beyond diffusion - perhaps an enzyme-driven one - to be involved in the release of proteins, since enzymes are prone to extreme temperatures: they don't work efficiently when it's cold and might denature at temperatures of 50 °C and higher. To verify the assumption that enzymes/proteases take part in the release of proteins, the samples were then incubated with a protease inhibitor cocktail: if the decreased extraction efficiency is caused by thermal inhibition of enzymes at extreme temperatures, protease inhibitor should exhibit a similar extraction efficiency at room temperature. This is exactly what the results reflect: the extraction efficiency of samples incubated with protease inhibitors at room temperature is as low as the one of samples incubated at 4 or 50 °C respectively (10 $\%_{DBM}$ for protease inhibitor and 12 $\%_{DBM}$ for 4 and 50 °C). The last parameter to be checked for is the pH value. In (Ursu *et al.*, 2014) it is suggested that a high pH (12 in their case) helps to solubilize proteins. Against initial expectations, a slight decrease in the extraction yield by 3 % could be seen when 0.1 M NaOH was used. While this effect is statistically significant, a difference of 3 % can be considered irrelevant. However, the assumption was that the slight decrease is also caused by an inhibition of proteolytic enzymes by the extreme pH, but 0.1 M NaOH is also corrosive and has an effect on the cells by itself, independent of PEF treatment. This is also a reason why the protease inhibitor and 0.1 M NaOH experiment went hand-in-hand initially: the protease inhibitor was supposed to mimic protease inhibition without using corrosive substances on the cells. To gain more resolution of the effect of the pH value during the incubation, spent culture medium was calibrated to certain pH values to see at which pH values the extraction works best. It can be seen that the extraction efficiency works well at alkaline pH around 8.5 - 9 in a buffered environment. At pH values higher than 9 the extraction seems to slightly decrease again. This explains why our TAP medium is well-suited for PEF-assisted protein extraction: it contains TRIS, a buffering substance that is alkaline by nature and which is supposed to hold an equilibrium around pH 7 with acetate or CO_2 (this depends on the mode and scale of cultivation). The pH value of the medium alkalinizes as the algae culture growths due to the consumption of acetate and/or CO_2/carbonate. This adds another possibility to the biomass concentration problem: the algae are at an ideal pH

range when they are PEF treated, and the buffer capacity of TRIS can compensate the ion release/acidification from the algae upon PEF to a certain point. But once the algae concentration increases, the ions released might override the buffer capacity of TRIS and significantly acidify the environment to the point where the extraction doesn't work properly.

To summarize, proteins are released in a time-dependent manner and the release of proteins is affected by environmental factors such as biomass concentration, temperature and pH during this incubation step, factors that have not have been taken into account in previous works such as (Postma *et al.*, 2016; ’t Lam *et al.*, 2017; Safi *et al.*, 2017).

5.4 The cell is affected in its entirety

From previous results it can be concluded that protein extraction via PEF is in principle possible and can possibly be optimized in scaled up industrial applications. But from a biological point of view it is unknown which proteins are extracted and what the properties of these extracted proteins are. (Coustets *et al.*, 2014) claim that only cytosolic proteins are extracted, and that the PEF treatment leaves the vacuole intact.

The first step in this work was to extract proteins via PEF treatment and HPH to compare and visualize the extracted proteins via SDS-PAGE and Coomassie staining. The pattern is roughly similar, but there is one key difference: the protein extract obtained by HPH shows a strong band around 25 kDa, and despite this certain protein being so abundant the signal in the PEF extract is absent. The protein is very likely chlorophyll a-b-binding protein, a largely abundant protein in the thylakoid membranes of chloroplast *(*Jackowski *et al.*, 2001; Caffarri *et al.*, 2004). The reason why this band can be seen in the HPH processed sample might be due to the principle of HPH: the process creates shearing forces that shear membranes and turns them into vesicles that behave like lipid droplets in an emulsion. This is also the reason why supernatants look different and why the protein precipitates obtained by HPH are greener due to residual membrane lipids and chlorophyll (see Figure 20 for example).

This SDS-PAGE analysis of the protein extracts suggests that PEF treatment cannot extract proteins that are membrane-bound (i.e. transmembrane proteins). It might also be possible that PEF treatment does not affect the chloroplast in a way that proteins are extracted from it, although its shape changes significantly and another work by (Straessner *et al.*, 2013) shows that the photosynthetic apparatus of the chloroplast is damaged by PEF treatment. In consequence, to really test the claim made by (Coustets *et al.*, 2014), the logical step was to do western blots of the extracted protein fractions and probe for hallmark proteins of some organelles (Histone H3 for nucleus, Actin for cytosol, RuBisCo for chloroplast and COXII for mitochondria). And as the western blots show, there is a signal for every protein in the PEF extracts. The signals intensify over time, confirming the time-dependent release of proteins even further. The signal for RuBisCo also confirms that the chloroplast is affected and that "free-floating"

proteins can be extracted from it but not membrane-bound proteins such as chlorophyll-a-b-binding protein. The band for actin doesn't run at the predicted size that is predicted by the manufacturer, and there are multiple bands detectable in the HPH extract. In the PEF extract there is only one precise band. It is possible that this signal is an actin-related protein and/or multimers of it in the HPH extract, but these are cytosolic as well, so the signal should still be usable as a proxy to confirm extraction of cytosolic proteins. Furthermore the detection of actin implies that the cytoskeleton is dissipating, and this has also been reported to happen in (Berghöfer *et al.*, 2009) upon nanosecond PEF treatment of *Arabidopsis thaliana*. This could also explain why the chloroplast seems to "bloats" as internal structures of the cell are likely not held in place by an intact cytoskeleton anymore after PEF. Interesting in these results are Histone H3 and COXII. Histone H3 exhibits a size shift and seemingly becomes "smaller" over time. There can be two reasons for that: Histones are DNA-associated proteins, and DNA is negatively charged, as are SDS molecules that are essential for the working principle of SDS-PAGE. The SDS molecules are repelled by the DNA (both are negatively charged), leading to reduced mobility of the Histones in the gel. As the DNA degrades gradually over time, this shielding effect wears off and the mobility of the Histones increases as they can be better carried by the SDS molecules. Another possibility is degradation of the Histones themselves through which they lose some molecular mass. However, the degradation of DNA is also in agreement with the observations from the DNA laddering experiment. In a PEF-treated sample incubated with protease inhibitor, there is no size shift and the Histone then runs at the same height as in the HPH treated sample, most likely because the protease inhibitor prevents degradation of proteins and to some degree also degradation of DNA, also visualized in the DNA laddering experiment. COXII is perhaps the most interesting protein in this case as it is the only one that shows a signal after a prolonged time, after 6 h of incubation. After 24 h there is a clean signal detectable for COXII. All other already show a signal after 1 h of incubation post-PEF. And when protease inhibitor is involved, there is no signal of COXII. This might be due to the circumstance that COXII is a membrane-associated protein, and the protein detected on the western blot might be a domain of COXII that has to be released into the environment by a proteolytic activity. For the vacuole, a different approach was chosen as it was difficult to find a suitable marker protein as a read-out. V-ATPases are considered to be hallmark proteins of plant vacuoles but are membrane-bound, so they might not be detectable by western blotting in PEF extracts. So as an alternative approach, it was chosen to check for vacuolar integrity via Neutral Red staining. Neutral Red is a yellow dye that can pass cell membranes and accumulates in acidic cell compartments such as the vacuole via an ion trapping mechanism. It gets protonated and can't pass cell membranes as a charged molecule, and it also turns red. If PEF treatment damages the vacuole, the red staining should disappear, which is clearly what can be seen. These results are important since they stand in contrast to the claim made by (Coustets *et al.*, 2014) that only cytosolic proteins are extracted without damaging the vacuole. However, it is important to keep in mind that their pulse parameters are very different

from the ones used in this work. This might open up the possibility to play around with the pulse parameters to selectively affect specific organelles.

5.5 Amino acid composition is similar to egg, but not in the PEF extracts

When looking at the pattern of the proteins on the polyacrylamide gel, more significant differences in amino acid composition of the protein extracts obtained by PEF and HPH treatment were expected. However, it can be seen that basically every amino acid is diminished in the PEF extract compared to the HPH extract. It was expected that e.g. some hydrophobic amino acids would be more abundant in the HPH extract, but looking at the pattern it seems that simply the amino acid content is simply scaled down, as is reflected in the total protein content of the protein precipitates. Of the two extracts, the HPH protein extract is closer to the amino acid profile of eggs than the PEF protein extract. The values determined here for the HPH extract are also in agreement with the values indicated in (Safi *et al.*, 2014). But what this exactly means for the technofunctional properties of the PEF protein extract has still to be elucidated. It is noteworthy that adding up all the amino acid content leaves you with a bigger portion of non-identified amino acids in the PEF extract than in the HPH extract. This is likely to be accounted to the intrinsic flaws of the Dumas method: some portion of the nitrogen detected is non-protein-nitrogen and artificially increases the protein content as all the nitrogen is included in the calculation with the conversion factor of 6.25. This conversion factor is well established for crops but might not apply for algae. The true protein content in the acetone precipitates might therefore be even lower, and the values for protein content determined by the Dumas method have to be seen as apparent values.

5.6 PEF treatment seems to trigger many processes associated with PCD

After summarizing the previous results and observations, it can be concluded that PEF treatment kills the *C. vulgaris* cells, and that the protein release observed is a consequence of an enzyme-mediated process after cell death. As mentioned in the first part of the discussion - and to reframe this discussion - the assumption is that PCD is this biological process. An indicator beside DNA laddering and damage to the cytoskeleton, vacuole and chloroplasts lies can be seen on the western blot in the release of COXII. The release of COXII seems innocuous at first but could be caused by formation of the mitochondrial permeability pore that is associated with cell death (Crompton, 1999; Scott *et al.*, 2007; Scott and Logan, 2008; Halestrap, 2009). Another hallmark of cell death is leakage of Cytochrome c itself (Balk *et al.*, 1999; Balk and Leaver, 2001; Yao *et al.*, 2004; Vacca *et al.*, 2006). This could be tested using an antibody against Cytochrome c. But, if COXII and Cytochrome c are only released due

to the mitochondrial permeability pore, this means that the pore formation in the mitochondria is happening quite late. The first signs of cell death can be seen within 1 h post-PEF as this is the point where DNA laddering is visible. So, if the release of Cytochrome c behaves just like the one of COXII, this can mean that the release of those mitochondrial proteins is a post-mortem effect and doesn't contribute to triggering the PCD. A similar phenomenon is actually discussed in (Yao *et al.*, 2004), because the release of Cytochrome c as a trigger of PCD is well characterized in yeast but in plants the release of Cytochrome c happens on a different timescale, much later so that the assumption is that its release is more of a post-mortem effect, caused by the cell death but not the cause of the cell death itself. Similar to the mitochondrion, the chloroplast can also be used as a read-out for PCD as it is just as capable of producing ROS bursts and can leak Cytochrome f that acts as in a similar fashion as Cytochrome c. Telling from the results it is apparent that the chloroplast is disrupted by the PEF treatment, it deforms and the leakage of RuBisCo happens at an earlier time point as the leakage of COXII. Furthermore (Straessner *et al.*, 2013) also observed a decrease in chlorophyll auto fluorescence that scales proportionally with the energy applied. This means that PEF treatment has the ability to damage the photosynthetic apparatus. Caspases - on in the case of plants, metacaspases or caspase-likes - also play a crucial role in cell death and subsequent degradation of the cell. They are produced in an inactive form and activated by proteolytic cleavage after release of cytochrome c for instance (Liu *et al.*, 1996; Skulachev, 1996; Zou *et al.*, 1997). The protease inhibitor used in this study might inhibit this activation - and of course activity of the metacaspases themselves - so that most breakdown processes come to a halt. These caspase-like proteins can also be activated by Ca^{2+} which can be sequestered by the EDTA in the protease inhibitor (which is also the reason why DNA laddering is inhibited/nuclease activity is decreased as Ca^{2+} is an important co-factor for nucleases).

All these findings and degradation symptoms observed in this work lead to the assumption that PEF treatment triggers (potentially multiple) signaling cascades that result in PCD, and that the protein release is basically a post-mortem effect. To put this model to the test, this should mean that any treatment energy could be used as long as it's lethal to the cells. This would open up the potential to save treatment energy, which is always a desired thing in industry. For this reason a series of pulse experiments were performed in which the amount of pulses was reduced by half until the cells were pulsed with just a single pulse. If the hypothesis is true, and that single pulse is enough to kill the cells, the protein extraction should behave exactly the same. And in fact, the extraction behaved the same for every energy used, whether it was 47 pulses or a single one, so the same result can be achieved with about one fiftieth of the energy that is used in the standard protocol. This greatly contributes to the design of an energy-efficient processing cascade to efficiently fractionate microalgae biomass into its valuable compounds. Telling from the data, a 1 µs pulse with a field strength of 40 $kV \cdot cm^{-1}$ is enough to achieve the same effect. The question remains whether PCD

signaling can be induced with milder, sub-lethal pulses that mimic appropriate stress signals on the algae.

5.7 Conclusion: the working model established in this work

In summary, the model that is proposed in this work based on the results is that PEF treatment under these parameters induces PCD in *C. vulgaris.* The protein release is basically a post-mortem effect, facilitated by autolytic processes associated with PCD, which has been briefly described for other microorganisms such as yeast as well (Simonis *et al.*, 2017; Martínez *et al.*, 2018). This model would explain the kinetics of the protein release that cannot be explained by diffusion alone.

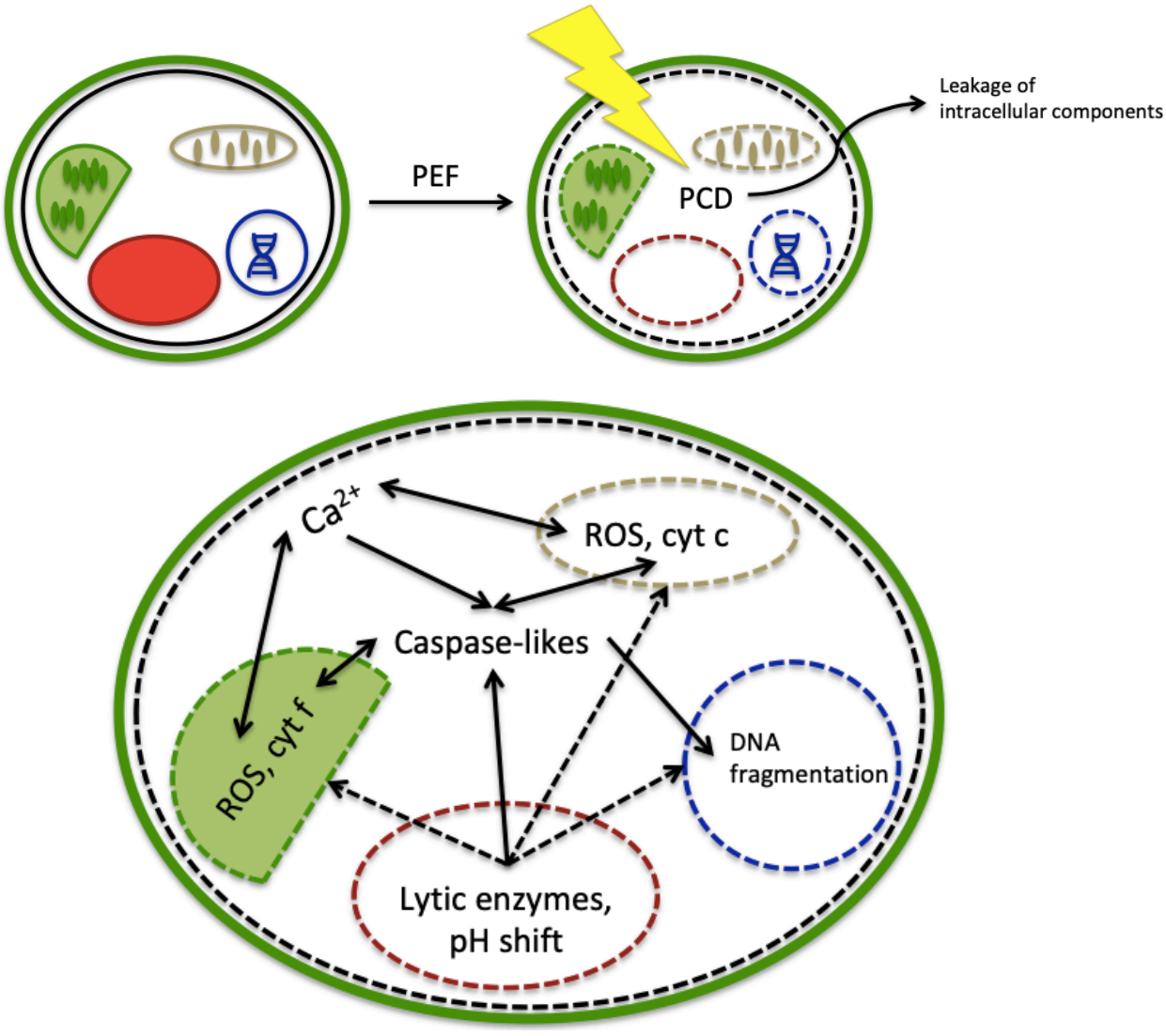


Figure 23: A brief scheme of the events that might happen upon PEF treatment of *C. vulgaris.* PEF permeabilizes the cell membrane (black) and also damages chloroplast (green), mitochondria (dark yellow), vacuole (red) and nucleus (blue). A rough signaling map is illustrated below and shows the convoluted signaling that can emerge from each organelle, and how they might impact each other. All these events result in PCD and leakage of intracellular compounds.

Parallel to this work, this concept has been utilized to extract mannoproteins from baker's yeast (Martínez *et al.*, 2016) and pigments from red algae and yet other yeast species (Martínez et al. 2018; Martínez et al. 2019). These autolytic processes are influenced by environmental factors such as incubation temperature and pH, and these factors have to be considered in the design of a bioprocess cascade and other industrial scale applications. But with the right conditions it allows for extraction of valuables with a low energy effort, which is in agreement with the works from (Luengo *et al.*, 2014, 2015) who utilized this phenomenon to extract pigments from *C. vulgaris.* In the context of lipids and using *A. protothecoides* (Silve *et al.*, 2018b) show that it is possible to trade off treatment energy for incubation: the lipid yield after 24 h of incubation after PEF treatment with low treatment energy gave off the same yield as right after the PEF treatment with 150 $J \cdot g^{-1}$. It is very likely that there are similar PCD-associated processes that make the lipids of the cell more accessible to solvent extraction, although it is still hard to explain why the lipid yield scales with the treatment energy when the algae are treated with solvents afterwards. While PEF treatment might not be not as efficient as mechanical cell disruption methods in direct comparison, it is still possible to obtain better yields than reported by (Safi *et al.*, 2013; Postma *et al.*, 2016; ’t Lam *et al.*, 2017). And based on this proposed mechanism (and evidence from the western blots), the claim of (Coustets *et al.*, 2014) that PEF treatment only extracts cytosolic proteins can also be rebutted. From a bioprocess-engineering point of view, this model is sufficient to help in designing an energy-efficient bio refinery concept in which PEF parameters and incubation parameters are optimized. But from a biological point of view, there is a grain of salt to be taken in regards to the model, which can be found in the work of (Kuthanova *et al.*, 2008): in this work, it is proposed that all the degradation symptoms that speak for PCD might as well be caused by the myriad of lytic enzymes that are unleashed upon rupture of the vacuole (which is clearly happening as shown per NR staining). These lytic enzymes essentially do what the caspase-like proteins would do upon induction of PCD. This is to say that it is recognized that further studies and more in-depth analysis on this topic are necessary to really confirm that all the clues observed in this work really lead to "real" PCD and are not merely an artifact caused by the lytic enzymes of the vacuole. An outlook for things to investigate from the point of this work is discussed in the next section.

6 Open questions / perspectives

6.1 How to purify the proteins from the extract?

After characterizing the mechanism that leads to protein release and determining the optimal conditions for the post-PEF incubation, protein purification or enrichment also had to be briefly addressed. The SDS-PAGE of the *C. vulgaris* proteins show that the proteins released make up for a rather complex mixture of proteins. Precipitation methods involving acids or ammonium sulfate (Burgess, 2009; Wingfield, 2010) typically precipitate only portions of the protein and are used to fractionate protein mixtures. These procedures also need neutralization or de-salting afterwards by dialysis, which is not feasible for industrial scale applications, and probably are also not necessarily suitable for food and feed applications as there are thresholds for ammonium sulfate in formulations for instance. Solvent precipitation with acetone was the choice for this work, as this method typically precipitates most of the proteins in bulk. Acetone precipitation was preferred over methanol 9:1 precipitation (Bychkov *et al.*, 2011) or 50 % ethanol/50 % acetone 8:1 precipitation (Grossmann *et al.*, 2018a; Grossmann *et al.*, 2018b) because acetone 4:1 is the most compact precipitation method in terms of volume-to-solvent ratio and given the boiling point of acetone is also the solvent that should be the easiest one to remove by evaporation. Solvents like Methanol are toxic and are therefore not an option to generate food-grade bulk protein. There is the risk that this way of protein enrichment might be not feasible as it requires large amounts of solvent, space (incubation tanks) and energy (solvent recovery and perhaps also cooling of the acetone). Spray drying of the extract could be considered as an alternative to obtain protein powder, but this method will also dry other substances that are extracted from the cells. In one attempt to freeze-dry the protein-containing supernatant after PEF treatment, the residue in the cups was sticky and honey-like, so it is questionable whether spray drying will yield better results. Freeze-drying is also very costly, as it requires a lot of energy to maintain the vacuum and to cool the sample. It is also very time consuming and hard to scale up. The way of protein purification might heavily influence the technofunctional properties of the isolate since solvent precipitation is denaturing to the proteins most of the time. Spray drying also exposes the proteins to heat and thus denatures them. Acid precipitation with HCl and citric acid was also tested briefly, but acid precipitations usually rely on hitting the isoelectric point of a protein (at which it is basically not charged and will likely precipitate). In a complex mixture of proteins it's not possible to precipitate all proteins. In the works of (Grossmann *et al.*, 2018a; Grossmann *et al.*, 2018b) it is also described that some algal proteins stay soluble even at very acidic pH around 2, basically making it impossible to precipitate all proteins by acid. Furthermore the samples processed by us developed an unbearable, sulfurous stench, most likely from the breakdown of

sulfur-containing compounds by the acid. Inspired by tofu, salting-out with MgCl was also considered, but this again would have the same issues as with ammonium sulfate.

6.2 PEF induces PCD - does it?

The results of this work open up biological questions on the cell death of *C. vulgaris*. It is shown that one pulse with 40 $kV \cdot cm^{-1}$ and duration of 1 µs is sufficient to kill the cells and achieve the same extraction efficiency usually obtained with 150 $J \cdot g^{-1}$, showing great energy saving potential. So the question is whether it is possible to further reduce and save energy by just eliciting PCD reactions with sub-lethal pulses that don't cause irreversible electroporation? Or is it the way in which the energy is delivered to the cell, i.e. does field strength and duration of the pulse play a greater role? To investigate this question, it would be necessary to vary the pulse energy by changing the field strength and/or the pulse duration. Signaling to look out for besides DNA laddering would be ROS bursts, induction/expression of caspase-like proteins and other elicitors of PCD like cytochrome c and f. The gene expression of caspase-like proteins would in this case be an important thing to investigate as it would rule out the scenario stated in (Kuthanova *et al.*, 2008) that all the signs that speak for PCD are merely caused by the lytic enzymes of the vacuole. The inclusion of inhibitors of these pathways might also modulate cell mortality to further strengthen the hypothesis. It has recently been shown that nsPEF treatment causes ROS bursts in *Chlamydomonas reinhardtii* (Bai *et al.*, 2017), although in that context they didn't cause PCD but changes in development and formation of palmella stage cells. These ROS bursts can be inhibited by diphenyleneiodonium (DPI) and thus modulate cell death or developmental changes of the cells. To specifically quench ROS bursts in the mitochondria, there is a so-called Q_{10}-peptoid that could be used to achieve that. It localizes to the mitochondria by itself and carries a ROS scavenger (Asfaw *et al.*, 2019). Also, now that sequencing data is available for *C. vulgaris* (Guarnieri *et al.*, 2018) it is possible to do more in-depth genetic analysis.

Another marker to look for in the western blots could be Cytochrome c. COXII can be detected in the supernatant after a prolonged incubation time, indicating that parts of it have been released into the environment by proteolytic activity and possibly loss of membrane integrity of the mitochondria. In yeast, Cytochrome c itself is reported to be released from the mitochondrial membrane under stress and acts as a trigger for PCD (Ludovico *et al.*, 2002; Giannattasio *et al.*, 2008). Detection of Cytochrome c on western blots could therefore also be used as a read-out for PCD in *C. vulgaris*, although (Yao *et al.*, 2004) have observed that Cytochrome c is released later in plants to the point where it could be simply a post-mortem effect. This would rule out Cytochrome c as the trigger of PCD. Analogous to Cytochrome c of the mitochondrion, Cytochrome f from the chloroplast could also be investigated in a similar fashion. In summary, it is highly interesting to explore how the cell basically dies after PEF treatment and which signaling is induced that orchestrates cell death. Using BY-2 cells, (Eggenberger *et al.*, 2017) have established a model in which auxin, the actin

cytoskeleton and ROS act as a integrity sensor for membrane integrity. According to their model, a perturbation of the membrane leads to a ROS burst via RboH, which then in consequence leads to "freezing" of the cytoskeleton, a state that is cytotoxic to the cell on the long run and could thus lead to PCD. This pathway is partially antagonized by auxin as ROS are also used in auxin signaling. These two signaling circuits (auxin-actin and RboH-actin) could therefore serve as a sensor to measure the severity of membrane distuption. In yeast, it has been shown that reduced actin dynamics lead to ROS production which in turn causes cell death (Gourlay *et al.*, 2004), so it is possible that severe membrane damage (as per PEF treatment) can induce a ROS burst that ends up amplifying itself in a positive feedback loop via actin bundeling to cause a definitive PCD signal. These findings could help to determine which pulse parameters and energies are still in agreement with life and at which point they are starting to push the cells towards PCD.

So, the disruption of actin can lead to cell death, and in the work of (Akaberi *et al.*, 2018) it is shown that GLVs have a similar effect that causes the cell to commit suicide. The consensus from these works is - roughly - that prolonged actin freezing leads to a PCD signal resulting in cell death. It is also the mode of action of the toxin phalloidin (Cooper, 1987). The induction of PCD via GLVs can be considered since *C. vulgaris* smell a little bit like freshly cut grass after PEF treatment. This is a good hint at the capability of *C. vulgaris* to produce GLVs upon damage of the membrane via conversion of membrane fatty acids to GLVs (Figure 24).

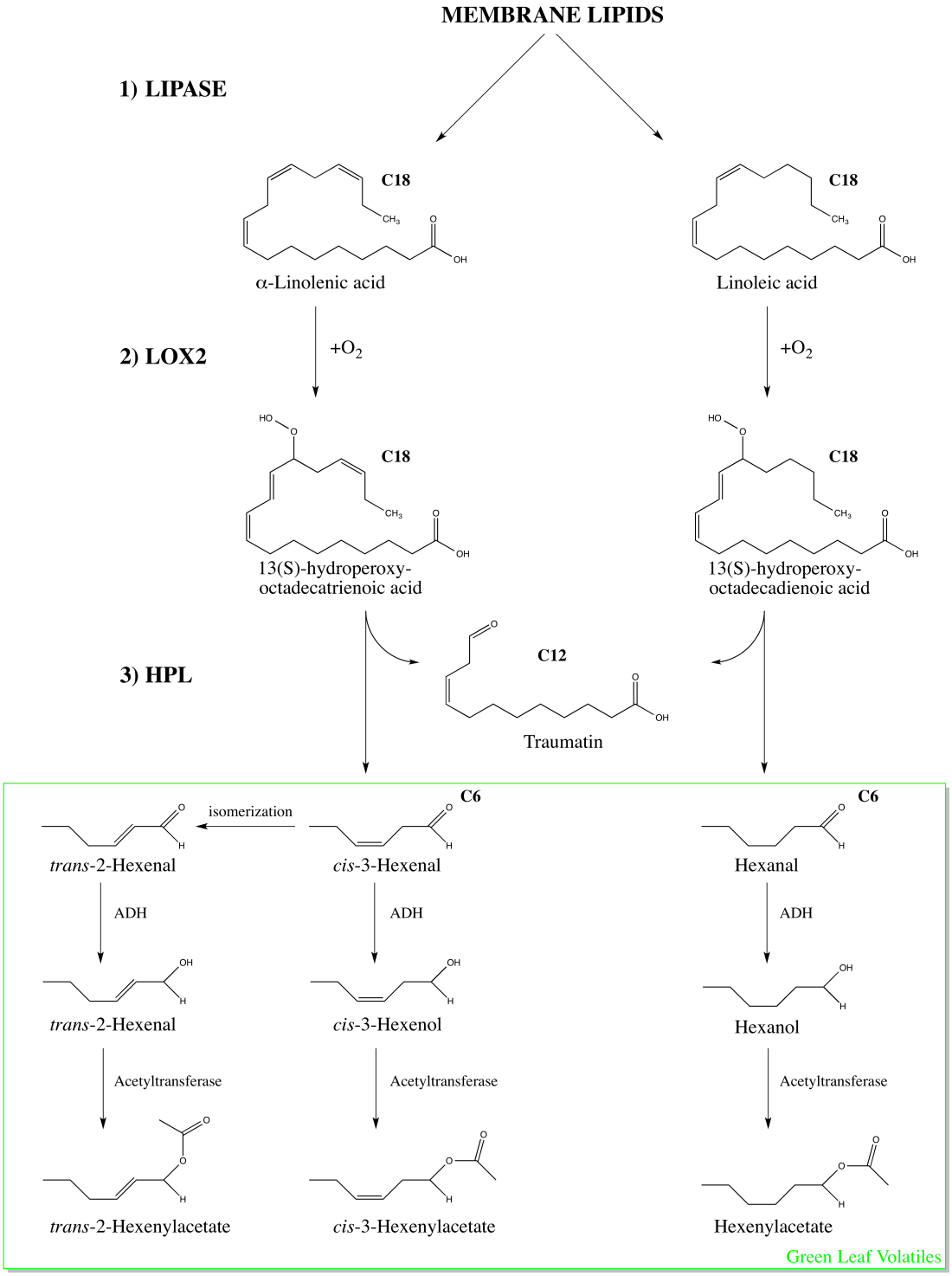


Figure 24: GLV biosynthesis pathways from alpha-linolenic acid (ALA) and linoleic acid (both C_{18}). Both fatty acids are rapidly released from membrane lipids by yet unknown glycerolipases and are peroxidated by a specific lipoxygenase (LOX2 in tobacco species) at position 13. The resulting 13-hydroperoxides are cleaved by hydroperoxide lyase (HPL) into the C_{12} fatty acid traumatin and the C_6-aldehyde *cis*-3-hexanal, the latter being substrate for isomerization, reduction to alcohol derivatives and esterifications. The set of commonly produced GLVs in plants is highlighted by the green box. Modified from (Hatanaka, 1993) and (Matsui, 2006).

This could be investigated by volatile trapping with solid phase microextraction (SPME) fibers or capturing of the headspace of pulsed cells with filters and subsequent gas chromatography with coupled mass spectrometry (GC-MS) analysis. Investigating whether *C. vulgaris* is capable of producing GLVs can also be interesting because GLVs can have anti-bacterial (Croft *et al.*, 1993; Nakamura and Hatanaka, 2002) and anti-fungal properties (Major *et al.*, 1960; Zeringue, *et al.*, 2002). This could help to inhibit microbial contaminations during the processing and incubation of the algae. Similarily to GLVs, a phytohormone screening upon PEF could be performed to identify which phytohormones *C. vulgaris* can produce and which ones might act in PCD signaling. This is in so far interesting because with a mobile signal that causes PCD, it might be possible to trigger PCD in the whole cell population without needing a 99.92 % efficiency of the PEF treatment. It might be efficient enough to kill a critical mass of cells that will basically drag the survivors to death along with them. One way to assay this would be to mix pulsed cells with untreated cells and observe the mortality in the untreated cells somehow. An example could be to have a 30 %/70 % ratio of live to dead cells an monitor whether these 30 % of living cells start dying or whether they thrive on the dead biomass. Another way to assay whether there is a mobile signal that causes PCD could be to introduce untreated cells into the supernatant of PEF treated cells. If they have also released signals that induce PCD, the untreated cells should show signs of PCD.

As pointed out in the introduction, cell size might affect the impact of PEF treatment as cells with a larger diameter are more affected than cells with a smaller diameter. Cell size also correlates with the developmental stage of a cell, and in the experimentation of this work, mostly non-synchronized cells were used. It would be interesting to switch to synchronized cells that have a stable day-night rhythm that also translates into a synchronized cell division and cell growth (i.e. size increase) phase. Pulse treatments at different times (e.g. morning, afternoon, night) might reveal when the cells are most vulnerable to PEF treatment in their developmental stage. This circumstance could then be exploited in industrial scale processes by determining the best timing for harvest and processing of the cell to optimize the yield and reducing treatment energy.

6.3 What are the advantages of the protein fraction obtained by PEF?

As the amino acid determination confirms the amino acid profile of *C. vulgaris* is fairly close to the one of eggs in principle. However, the bioavailability of *C. vulgaris* protein can vary depending on the processing of the algae between 58 - 77 % as reported by (Neumann *et al.*, 2018), whereas the one of whey or egg protein is higher, 92 % and 94 % respectively according to (Hoffman and Falvo, 2004). The amino acid profile of the protein fraction obtained by PEF however is quite different, it essentially contains less of every amino acid, likely a scale-down effect of the reduced protein content. But what this means for technofunctional properties has still to be elucidated. One starting point

would be to conduct experiments on the *C. vulgaris* proteins like (Buchmann *et al.*, 2019) did for foaming properties and foam stability on protein extracts of *Spirulina,* or like (Ebert *et al.*, 2019) did on emulsifying properties for protein extracts from the algae *Chlorella sorokiniana* and *Phaeodactylum tricornitum*. In this work, the foaming properties and zeta-potential were determined to determine whether the protein extract could be useful as a food additive. (Dai *et al.*, 2019) have a different approach of mobilizing insoluble/membrane-bound proteins by acid hydrolysis, and using *A. protothecoides* they have observed that some proteins are quite stable under acidic conditions and that they still exert good emulsion properties. These protein properties could be further explored for *C. vulgaris* protein as well. But what the amino acid analysis confirms once again is that all essential and conditionally essential amino acids are present in the algae, making *C. vulgaris* protein extracts a potential alternative to eggs and whey as a non-animal protein source. But due to the flux in bioavailability this need to be studied more in-depth. At this point it is not clear which advantages the protein fraction obtained by PEF can offer compared to the protein fraction obtained by HPH. However, what can clearly be shown is that it the PEF protein fraction can be obtained with very low effort, energy-wise. The precipitation methods that are available to isolate the extracted proteins are usually denaturing, so in the future it is possible to think about methods to re-nature the proteins prior to technofunctional analysis even though this process would not be feasible for industrial purposes, but would certainly give a deeper insight into this matter. There are fairly novel methods that allow re-folding of denatured proteins such a Vortex Fluidic Device (Britton *et al.*, 2017). This method has been demonstrated to be able to unboil boiled egg white and is also used to re-fold proteins that are of interest in medical applications.

6.4 Realizing a bio refinery cascade in the lab

The protein yield of PEF-assisted extraction is roughly half of what can be extracted by HPH protein extraction. It is hard to tell whether this is justifiable for industrial processes based on the yield alone. However, with the findings above it is apparent that this PEF protein fraction can be obtained with a much lower energy effort. The results from the energy variation experiment show that the extraction behaves the same even at 1 / 50 of the energy established in the standard protocols, and in works like (Silve *et al.*, 2018b) it is also shown for *A. protothecoides* that an incubation step can substitute for reduced treatment energy, although the solvent extraction of lipids underlies other mechanisms that are not fully understood. In short, the lipid extraction with ethanol-hexane blends scales with the energy input of the PEF treatment. With 150 $J \cdot g^{-1}$, almost all the lipids can be extracted right after the PEF treatment. With 25 $J \cdot g^{-1}$ the same extraction efficiency is only achieved after a 24 h incubation step after PEF. This phenomenon would be in agreement with the cell death and autolysis consensus, but it remains cryptic why a higher energy input has an immediate effect.

One theory is that the membranes become more porous with higher energy quantities, providing more surface area for the solvents to attack and dissolve lipids.

Either way, the findings in these works should culminate in the establishment of a lab-scale bio refinery cascade. Briefly, extractable proteins should be extracted first by PEF and an incubation step. Then the residual, protein-reduced biomass should be extracted for its lipids by ethanol-hexane blends. And finally, the residual biomass (which should consist of cell wall material at this point) could be used in conversion processes such as hydrothermal liquefaction, or converted by chemical and/or enzymatic treatments. The process can only be feasible if the most possible amount of valuable compounds can be extracted. It has been have observed that protein-reduced *C. vulgaris* can indeed be extracted by ethanol-hexane blends (Ioannis Papachristou, unpublished data), bringing this process one step closer to achieving a process cascade that fractionates the algal biomass into its valuable components with the help of PEF, and this will be a promising feature of this technique for the future.

6.5 How can this process be scaled up?

Despite all these findings, there are still obstacles to overcome for industrial scale production of algal proteins: incubation at high biomass concentrations lead to a decreased extraction efficiency, most likely due to diffusion effects or the limited buffer capacity of the medium as pulse treatment liberates ions immediately including H^+ ions that acidify the environment. This acidification can be buffered to a certain degree but at some point will override the buffer capacity and acidify the environment beyond favorable conditions for protein extraction. One way to address this issue would be to design an extraction buffer that can deal with the acidification by high biomass concentrations or to use an apparatus that externally titrates some alkaline substance (Ammonia, NaOH, KOH) to hold the pH at the determined pH optimum around 8.5 - 9.

Another problem arises during the scale-up is the cultivation itself. As the literature suggests, the most cost-effective way to cultivate large amounts of algae is an open pond system. This by itself comes with a variety of problems and challenges to address. From a technical point of view, an open pond cultivation can accumulate foreign particles like sand, dirt, micro-plastics etc., which limits the options of mechanical disruption methods. Having dirt particles in a sample is definitely not compatible with HPH. However, this would be no problem for the PEF treatment as the algae suspension is simply passed through a chamber. From a biological point of view an open pond is not ideal because the algae won't grow in there alone, there will certainly be bacteria growing in competition or sometimes in symbiosis with the algae. In this case, the microbiome of the cultivation has to be identified and checked for safety. Bacteria also react slightly different to PEF treatment, as they vary in shape and are significantly smaller than algal cells. It is possible that reduced treatment energy will essentially kill the algae but leave a fair amount of bacteria alive, which then could possibly out-grow the algae during the incubation step, especially since the incubation

optimum lies around ambient temperatures like 30 °C. It might be necessary to keep the energy high to also inactivate bacterial contaminations that could ferment the biomass during the incubation step in which the proteins are supposed to be released. But in that case it is inevitable that proteins from bacteria will also be extracted.

Ultimately, these problems and challenges have to be taken into consideration when transferring the findings of this work - that took place at lab-scale - to industrial scale applications, but the PEF technique can be adjusted to fit all these requirements in the future.

6.6 How applicable is the model "protein extraction via PCD" to other organisms?

As mentioned in the conclusion the procedure "PEF + waiting for cell death" has already been applied for *C. vulgaris* and yeasts to extract proteins, pigments and has also shown to improve lipid extractability. It's just that the role of cell death isn't obvious from these works. (Halim *et al.*, 2019) worked on an incubation protocol for *Nannochloropsis* that they call "dark anoxia" in which the algae are concentrated and kept in darkness and under anaerobic conditions. This is supposed to lead to stress, starvation and in consequence autolytic processes that are supposed to make the cells more extractable because they will partially break down their cell wall and other components of the cell, essentially fermenting themselves. If this protocol basically operates on (stress-induced) PCD as well, it could thus be accelerated by PEF. It is likely that this model "PEF + PCD" can be universally applied to (eukaryotic) microorganisms (including all sorts of cell cultures). However, preliminary experiments on other algae such as *A. protothecoides* (Ioannis Papachristou, unpublished data) and *Scenedesmus almeriensis* (Sahar Akaberi, unpublished data) do not show the same behavior as *C. vulgaris. S. almeriensis* have a comparable protein content to *C. vulgaris*, yet the amount of protein detectable in the supernatant after PEF is extremely little, around 2 - 3 $\%_{DBM}$. The protein content for *A. protothecoides* ranges between 10 - 20 % depending on the time of harvest, and the protein yields are just as low. It is possible that for these microalgae, the extracellular matrix must be considered as an additional obstacle for proteins, which telling from the SDS-PAGE and western blot analysis was not a big concern for *C. vulgaris*. So even though this model could be hypothesized for any eukaryotic cell culture, it doesn't mean that this mechanism is suitable to extract proteins from all kinds of cells.

7 Appendix

7.1 Growth of *C. vulgaris* and generation time in flasks

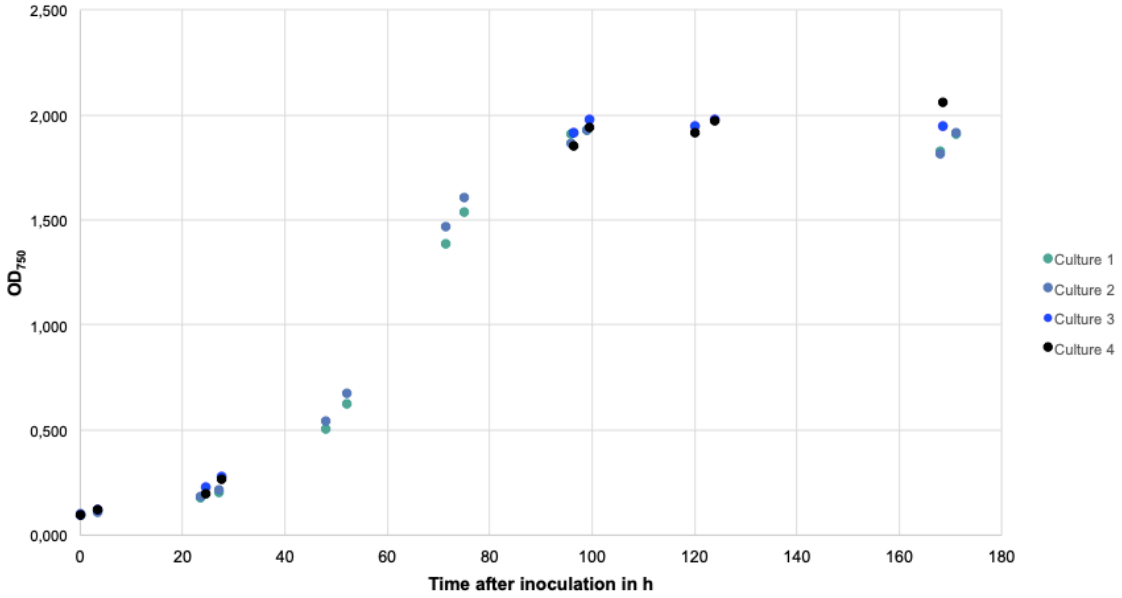


Figure 25: A growth curve of *C. vulgaris* under our cultivation parameters in flasks. The algae are inoculated at an OD_{750} of 0.1 and reach an OD_{750} of approx. 2.0 towards the end of our cultivation between 5 and 7 days, at which point they are stationary.

The growth curve shows that the algae become stationary after 5 days at an OD_{750} of around 2.0 and stay this way until 7 d after inoculation. The average generation time determined using this dataset and under these growth condition is 19.57 h. The end concentration of the algae after 7 d is usually 0.9 $g \cdot l^{-1}$ with a protein content of around 45 $\%_{DBM}$, the conductivity of the medium is approx. 1,4 $mS \cdot cm^{-1}$ and the pH is around 8.3 - 8.5.

7.2 The influence of protease inhibitor on protein extraction and DNA laddering

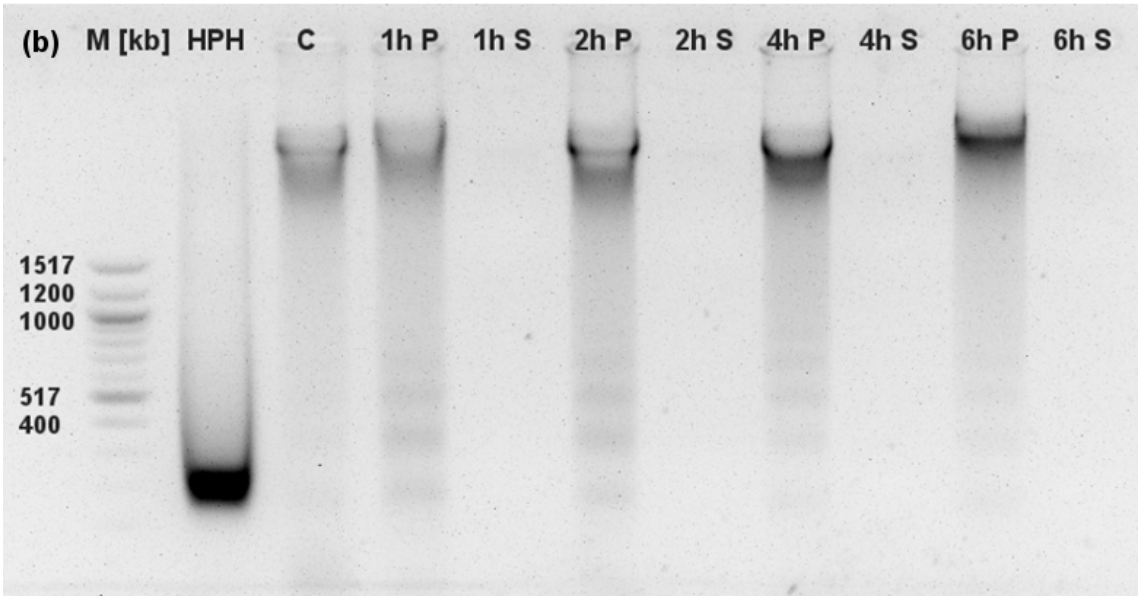


Figure 26: Analysis of DNA-laddering after PEF treatment under the influence of protease inhibitor. Genomic DNA of PEF treated *C. vulgaris* was extracted from freeze-dried pellets (P) and supernatants (S) and visualized on a 1,5 % TAE agarose gel stained with SYBR Green.

The repetition of the DNA extraction after PEF treatment under the influence of protease inhibitor reveals that the protease inhibitor also has an inhibitory effect on DNA laddering. The laddering still takes place but the signal is very weak when compared to the samples what were incubated without protease inhibitor. There is also no DNA detectable in the supernatant in this case.

Furthermore, the protease inhibitor also altered the behavior of some of the proteins during extraction as detected by western blotting. In the PEF extract with protease inhibitor, Histone H3 exhibits a shift upwards and thus runs at the same height as in the HPH sample. The signal of RuBisCo and Actin is slightly reduced and COXII is not detectable under the influence of protease inhibitor.

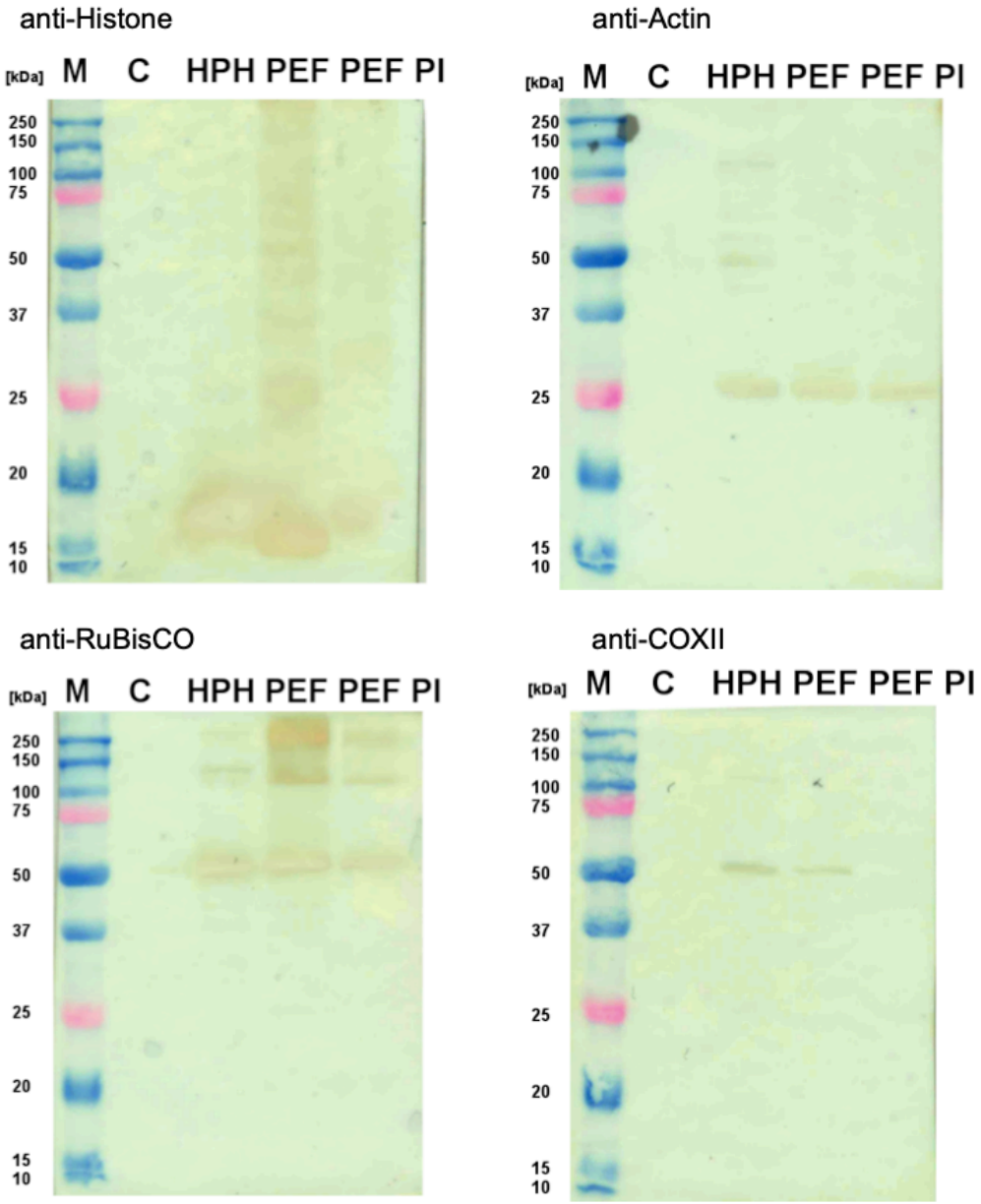


Figure 27: Western blot detection of organelle-specific proteins. Organelle-specific proteins in the PEF extracts were detected after Western Blot using antibodies directed against RuBisCo, COXII, Histone H3 and Actin.

7.3 Amino acid analysis raw data

Table 1: Raw data of the amino acid content in % dry weight of the individual experiments summarized in one table. Essential amino acids are written in bold.

Protein/amino acid content of PEF precipitates in % dry weight

	PEF1	PEF2	PEF3	Average	StdDev
Protein content in % (total nitrogen x 6.25)	34,10 %	33,80 %	30,70 %	32,87 %	1,88
Aspartic Acid	2,38 %	2,71 %	2,20 %	2,43 %	0,26
Serine	1,14 %	1,24 %	1,06 %	1,15 %	0,09
Glutamic Acid	3,49 %	4,14 %	3,45 %	3,69 %	0,39
Proline	1,15 %	1,22 %	1,05 %	1,14 %	0,09
Glycine	1,48 %	1,57 %	1,32 %	1,46 %	0,13
Alanine	1,92 %	2,10 %	1,76 %	1,93 %	0,17
Valine	1,46 %	1,53 %	1,34 %	1,44 %	0,10
Isoleucin	0,94 %	0,97 %	0,83 %	0,91 %	0,07
Leucine	2,14 %	2,24 %	1,87 %	2,08 %	0,19
Tyrosine	0,75 %	0,79 %	0,68 %	0,74 %	0,06
Phenylalanine	0,95 %	1,04 %	0,85 %	0,95 %	0,10
Histidine	0,53 %	0,54 %	0,48 %	0,52 %	0,03
Arginine	1,65 %	1,81 %	1,49 %	1,65 %	0,16
Tryptophane	0,31 %	0,33 %	0,26 %	0,30 %	0,04
Lysine	1,61 %	1,77 %	1,45 %	1,61 %	0,16
Methionine	0,49 %	0,51 %	0,43 %	0,48 %	0,04
Cystine	0,31 %	0,37 %	0,30 %	0,33 %	0,04
Threonine	1,21 %	1,27 %	1,11 %	1,20 %	0,08
Rest	10,19 %	7,65 %	8,77 %	8,87 %	1,27

Protein/amino acid content of HPH precipitates in % dry weight

	HPH1	HPH2	HPH3	Average	StdDev
Protein content in % (total nitrogen x 6.25)	58,80 %	56,50 %	55,40 %	56,90 %	1,73
Aspartic Acid	5,28 %	5,20 %	5,00 %	5,16 %	0,14
Serine	2,64 %	2,57 %	2,54 %	2,58 %	0,05
Glutamic Acid	6,35 %	6,24 %	6,11 %	6,23 %	0,12
Proline	2,71 %	2,64 %	2,56 %	2,64 %	0,08
Glycine	3,20 %	3,26 %	3,16 %	3,21 %	0,05
Alanine	4,42 %	4,51 %	4,37 %	4,43 %	0,07
Valine	3,11 %	3,10 %	3,00 %	3,07 %	0,06
Isoleucin	2,07 %	2,00 %	1,95 %	2,01 %	0,06
Leucine	5,63 %	5,51 %	5,36 %	5,50 %	0,14
Tyrosine	1,98 %	1,80 %	1,73 %	1,84 %	0,13
Phenylalanine	2,95 %	2,86 %	2,75 %	2,85 %	0,10
Histidine	1,24 %	1,16 %	1,23 %	1,21 %	0,04
Arginine	3,50 %	3,47 %	3,38 %	3,45 %	0,06
Tryptophane	0,94 %	0,86 %	0,85 %	0,88 %	0,05
Lysine	3,37 %	3,32 %	3,25 %	3,31 %	0,06
Methionine	1,28 %	1,20 %	1,21 %	1,23 %	0,04
Cystine	0,65 %	0,63 %	0,61 %	0,63 %	0,02
Threonine	2,65 %	2,60 %	2,53 %	2,59 %	0,06
Rest	4,83 %	3,57 %	3,81 %	4,07 %	0,67

Table 2: Amino acid composition of the PEF and HPH protein precipitates in %, normalized to total protein content (total nitrogen x 6.25). The amino acid composition of egg is taken from (Safi *et al.*, 2014) for comparison.

Amino acid content in % normalized to total protein (total nitrogen x 6.25)					
	PEF	StdDev	**HPH**	StdDev	**Eggs** (Safi et al. 2014)
Aspartic Acid	7,39	0,55	9,07	0,12	11,00
Serine	3,49	0,17	4,54	0,05	6,90
Glutamic Acid	11,24	1,01	10,96	0,14	12,60
Proline	3,47	0,13	4,63	0,03	4,20
Glycine	4,43	0,19	5,64	0,17	4,20
Alanine	5,86	0,31	7,80	0,25	n.d.
Valine	4,39	0,12	5,40	0,10	7,20
Isoleucin	2,78	0,08	3,53	0,01	6,60
Leucine	6,33	0,27	9,67	0,09	7,00
Tyrosine	2,25	0,08	3,23	0,13	4,20
Phenylalanine	2,88	0,17	5,01	0,05	5,80
Histidine	1,57	0,02	2,13	0,09	2,40
Arginine	5,02	0,29	6,07	0,10	6,20
Tryptophane	0,91	0,06	1,55	0,04	1,70
Lysine	4,89	0,30	5,82	0,08	5,30
Methionine	1,45	0,06	2,16	0,03	3,20
Cystine	0,99	0,09	1,11	0,01	2,30
Threonine	3,64	0,11	4,56	0,05	5,00

7.4 SDS-PAGE and Western Blot protocols and recipes

7.4.1 SDS-PAGE (Tris-Glycine system after Lämmli)

Material:

- Isopropanol

- 10 % ammoniumperoxodisulfate (APS)

- TEMED

- Acrylamide/Bis-Acrylamide 30 % solution

- Running buffer (pH ≈ 8.3) consisting of 25 mM Tris, 192 mM Glycine, 0.1 % (w/v) SDS

- 4x Lämmli/sample buffer consisting of 200 mM Tris-HCl pH 6.8, 8 % (w/v) SDS, 40 % (v/v) Glycerol, 4 % (v/v) ß-mercaptoethanol, 50 mM EDTA (optional), 0.08 % (w/v) Bromophenol Blue (approximately)

- 4x Separating gel buffer consisting of 1.5 M Tris-HCl pH 8.8, 0.8 % (w/v) SDS

- 2x Stacking gel buffer consisting of 0.25 M Tris-HCl pH 6.8, 0.4 % (w/v) SDS

Example recipes for...

50 ml 12 % separating gel stock:

12.5 ml 4x separating gel buffer, 20 ml 30 % acrylamide solution, ad 50 ml with ddH_2O

Adjust the amount of acrylamide solution to get the desired percentage of the gel.

50 ml stacking gel stock:

25 ml stacking gel buffer, 6.66 ml acrylamide solution, ad 50 ml with ddH_2O

Stacking gel is 4 % usually. Tip: add a little spattle tip of Bromophenol blue if you have trouble to see the pockets.

The gels are polymerized by adding 10 % APS 1:100 and TEMED 1:1000.

Protocol:

Preparation of the separation gel:

3.5 ml (+ 0.5 ml) seperating gel stock per gel are transferred to a clean tube.

Polymerization is initiated by addition of 10 % APS (1:100) and TEMED (1:1000), e.g. to 4 ml of gel stock, 40 µl 10 % APS and 4 µl TEMED are added.

3.5 ml of the gel are poured into the glass plates and then overlaid with Isopropanol to smoothen the surface. The remaining 0.5 ml in the tube is kept as a reference to check for successful polymerization. Discard the Isopropanol after polymerization.

Preparation of stacking gel:

1 ml (+ 0.5 ml) stacking gel stock per gel are transferred into a clean tube.

Polymerization is initiated by addition of 10 % APS (1:100) and TEMED (1:1000)

1 ml of the gel is pipetted onto the polymerized separating gel and the gel combs are inserted immediately.

Sample preparation:

Total protein (e.g. from lysates or extracts) is prepared in 1x sample buffer. Extracts and lysates can be mixed 3:1 with 4x Lämmli buffer after removing the cell debris by centrifugation (e.g. 30 µl of extract with 10 µl of 4x Lämmli buffer).

Proteins are denatured at 90 °C for 5-10 min.

Running the gels:

Samples are carefully loaded into the gel by slowly pipetting them into the pockets. There are special elongated pipette tips for this purpose if necessary. Alternatively, a Hamilton syringe can be used as well.

Samples are stacked at 50 60 V for approx. 30 min and separated at 100 - 150 V for 45 - 60 min

Note: max. 10 - 15 µl for 15 lane gel and 15 - 20 µl for 10 lane gel; samples are easier to load when they are still hot from the denaturing step

Analysis:

The gels can be used for Western blotting or stained, e.g. by Coomassie or silver staining respectively.

This protocol is derived from (Lämmli, 1970) and (Lottspeich and Engels, 2012).

7.4.2 Western Blotting

Material:

- Primary antibody solution: antibody in TBST with 5 % milk powder or BSA and 0.01 % Thimerosal.

Notes:

The appropriate dilution of the antibody has to be determined empirically. In this work, 1:1000 has worked fine.

Milk powder or BSA act as buffer proteins to diminish losses of the antibody to surface adherence in the reaction tubes. BSA is more expensive but milk spoils faster. Milk also interferes with anti-phospho antibodies, so don't use it if your antibody is specific to phosphorylated antigens.

Thimerosal acts as a preservative agent. Sodium azide can also be used but interferes with the horseradish peroxidase (HRP) that is coupled to some secondary antibodies, so don't use it if you are using a HRP-based detection system.

- 10x TBST consisting of Tris-HCl pH 7.4, 1.5 M NaCl, 1 % (v/v) TWEEEN-20

- 2x Transfer buffer consisting of 40 mM Tris, 300 mM Glycine, 40 % (v/v) Methanol, 0.04 % SDS

- Ponceau-S solution consisting of 2 % (w/v) Ponceau-S, 30 % (w/v) Trichloroacetic acid, 30 % sulfosalicylic acid

Protocol:

- The gel holder cassettes have a black and a transparent side. The order of objects from black to transparent is: BLACK SIDE, sponge, Whatman paper, gel, membrane, Whatman paper, spinge, TRANSPARENT SIDE.

Notes: This is the correct way to sandwich gel and membrane between the sponges and filter papers, otherwise the content of the gels will be moved into the wrong direction into nothingness. Remove all air bubbles captured between gel and membrane under any circumstance as this will prevent transfer of the proteins onto the membrane.

- This sandwich is closed and transferred to an ice-cooled blotting chamber

- Blotting: Proteins are transferred onto the membrane at 100 V for 1 h .

- Transfer of proteins can be confirmed by Ponceau-S staining. 10 ml of Ponceau-S solution on the membrane for 1 min is sufficient. The staining can be seen after washing with H_2O.

- Blocking: 10 ml of TBST + 5 % milk powder (Baileys Cream Liquor works as well) is added for 30 min at room temperature under constant agitation.

- Primary antibody: 10 ml of the primary antibody solution is added for at least 1 h at room temperature or overnight at 4 °C under constant agitation respectively

- Wash: The membrane is washed three times with TBST (3x 5 min)

- Secondary antibody: 10 ml of the secondary antibody solution is added for at least 1 h at room temperature or overnight at 4 °C under constant agitation respectively

- Wash: The membrane is washed three times with TBST (3x 5 min)

- Detection: 10 ml of 1-Step TMB-Blotting Substrate Solution (Thermo Fischer Scientific) is added and incubated until color changes in the membranes are visible (it's usually a matter of seconds, within 1 min).

This protocol is derived from (Lottspeich and Engels, 2012).

8 Bibliography

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