Extraction of bioactive primary and secondary Metabolites from Microalgae by atmospheric pressure Plasmas and pulsed Discharges in Water

I n a u g u r a l d i s s e r t a t i o n

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Greifswald

vorgelegt von

Maria Katja Zocher

geboren am 04.08.1984

in Meißen

Greifswald, den 23.09.2019

Dekan:	Prof. Dr. Werner Weitschies
1. Gutachter:	Prof. Dr. Michael Lalk
2. Gutachter:	Prof. Dr. Jürgen F. Kolb
3. Gutachter:	Prof. Dr. Timo Niedermeyer
Tag der Promotion:	03.12.2019

Index

Index III

Introduction (P1-P3) 4
1.1 *Chlorella vulgaris* 5
1.1.1 Metabolites 5
1.1.2 Extraction methods 5
1.2 Atmospheric pressure plasmas in liquids 7
1.3 Aim of this work 8

Plasma: A feasibility study (P1) 10
2.1 Plasma Sources and Reference Methods 10
2.2 Detection parameters for cell wall rupture 13
2.3 Determination of effective extraction methods 13

Plasma effects: Proteins as Targets (P2) 17
3.1 Overall content and semi-quantitative protein yields 17
3.2 Protein yield of selected groups 20
3.3 Oxygen and nitrogen modifications 23

Plasma effects: Mechanism of Cell wall Disintegration (P1-P4) 26
4.1 Shockwave pressure 26
4.2 Reactive species impact 30
4.3 Electric field effects 30
4.4 Temperature effects 31
4.5 Ultraviolet light emission 32
4.6 Cell morphology alterations 32

Summary and concluding Remarks 34

References 36

Original Publications 41

Eigenständigkeitserklärung 111

Curriculum Vitae 112

Publications and Conference Contributions 113

Acknowledgment 116

Chapter 1

Introduction (P1-P3)

Microalgae are unicellular or simple multicellular photosynthetic microorganisms, which are ubiquitously found in nature. They possess a fast growth rate and generally double their biomass within 24 hours (Chisti 2007) Of economic and biotechnological interest are their high amount of valuable primary and secondary metabolites, e.g. lipids, proteins, pigments or carbohydrates. Moreover, these compounds have also shown pharmacological effects, which makes them come to the fore for pharmaceutical applications. For instance, extracts from these metabolites have shown antimicrobial, antitumor, and anti-inflammatory activity. High amounts of pigments, especially carotenoids, have shown antioxidative activity, which can be applied against neurodegenerative diseases, atherogenesis, or macular degeneration (Skjånes et al. 2013).

Most microalgae are frugal in their living condition and can be grown in nearly any environment. For cultivation, only a light source, water and some elementary salts and vitamins are necessary. Microalgae are aquatic organisms that live in fresh, saline or brackish water, but can also be found in any form of soil (Richmond 2008) (Kose and Oncel 2015). These uncomplicated cultivation conditions result in a lower water and land footprint than found for land plants (Günerken et al. 2015). Sufficient growth, however, strongly depends on a constant supply of carbon dioxide and light to conduct photosynthesis (Mata et al. 2010).

The ability of microalgae to grow ubiquitously is also their major disadvantage. For survival, microalgae excrete often a strong and rigid cell wall with layers of glycoproteins and complex polysaccharides. This circumstance results in a notable chemical and mechanical resistance and thus, requiring strong forces to disrupt the cell wall to extract the aforementioned compounds (Kim et al. 2013). Albeit several chemical and mechanical extraction methods are available, they are not without shortcomings and disadvantages, which can be harmful to the extractives. For instance, mechanical methods, such as sonication, microwave exposure or bead milling may develop heat, which can result in thermal degradation of thermolabile compounds. Chemical methods, e.g. classical solvent extraction, pressurised liquid extraction or cell disintegration with enzymes require long extraction times and again, sensitive compounds may be degraded or inactivated by the solvent (Kim et al. 2013). Moreover, solvents are often environmentally harmful, toxic or are not suitable for sensitive compounds, which is another drawback of solvent extraction (Mubarak et al. 2015). An alternative extraction technique, which is effective in cell wall rupture and yet, gentle enough towards sensitive compounds, is therefore necessary. With plasma and its physical and chemical effects, such as shockwaves, electric fields, UV-light emission and reactive oxygen and nitrogen species may address the aforementioned problems and might be stablished as alternative extraction method.

1.1 *Chlorella vulgaris*

1.1.1 Metabolites

Microalgae host a vast variety of primary and secondary metabolites, such as lipids, polyunsaturated fatty acids, proteins, polysaccharides, pigments, carotenoids, and vitamins. These compounds gained more and more interest, and a systematic examination of algae for biologically active substances began in the 1950's (Borowitzka 1995). One of the best-characterised alga is *Chlorella vulgaris* and was first described by Beijerinck (Beyerinck 1890). It has been used in the Far East as alternative medicine since early times and contains a wide spectrum of substance classes (Sousa et al. 2008), which contribute to a broad range of positive health effects. Amongst others, three groups of substances with their respective bioactivity will be described in more detail.

Polysaccharides, especially β-(1,3)-glucane, which seems to be one of the most important substance in *Chlorella vulgaris*, have shown several effects. Remarkably, immune-stimulating effects, antitumor activities and a positive influence on blood lipids have been reported for β-(1,3)-glucane (Spolaore et al. 2006). A regulation of blood glucose levels and insulin response have been shown is several studies, as well (Pignolet et al. 2013) (Mundt S 2014).

Chlorella vulgaris comprises a rather high amount of proteins as well, which ranges between 48–58%(w/w dry weight biomass) (Postma et al. 2015). This makes it an interesting alternative protein source for human nutrition and as animal feed in comparison to other proteins sources, e.g. soybeans, rice bran, or poultry. Moreover, the protein profile compares favourably with that of the reference and the other food proteins, recommended by WHO/FAO (Becker 2007) because the cells synthesise essential and non-essential amino acids. In addition, the proteins of *Chlorella vulgaris* have shown outstanding emulsifying capacity, which increases their value for additional valorisation in the industry, especially on the food market (Safi et al. 2014).

In the thylakoids of the chloroplast, high amounts of pigments are found, which are responsible for the colour of the cell and that are an important part of the photosynthesis. Besides a remarkable chlorophyll *a* content (1-2 % of dry weight, (Safi et al. 2014)), carotenoids play also an important role as accessory photosynthetic pigments. Their ability to scavenge reactive oxygen species during photosynthesis contributes significantly to the total antioxidant capacity of microalgae (Goiris et al. 2012). Due to this quenching-abilities, carotenoids have shown to be active against cardiovascular and neurodegenerative diseases, e.g. Alzheimer, Parkinson or macular degeneration (Goiris et al. 2012) (Zhang et al. 2014) (Luengo and Raso 2016).

1.1.2 Extraction methods

All compounds from *Chlorella vulgaris* are protected by a robust and sturdy cell wall, which consists of three thick layers and was thus, chosen as model organism in this study.
The layers are, amongst others, made of cellulose, hemicellulose, glycoproteins, and lipids (Staehelin 1966) (Kim et al. 2013). For down streaming and thus, extracting the compounds, it is necessary to overcome this hurdle, which is often associated with several shortcomings, e.g. high energy consumption and long processing times. Numerous extraction techniques are available, which are distinguished between mechanical and chemical methods.

Mechanical methods are, for instance, high-pressure homogenisation, bead milling, sonication, or microwave exposure. Advantage of these methods is that they are applicable to a wide range of species (Kim et al. 2013). However, due to shear forces, cavitation effects or dipole rotation, heat development cannot be avoided and hence, conversion or degradation of heat-sensitive compounds may occur. An additional cooling of the system is therefore obligatory, which increases the energy consumption.

With the help of chemicals and solvents, algal cell walls can also be disrupted. The use of acids, bases, surfactants and other solvents holds the risk of unwanted modifications of the extracted compounds. Moreover, especially acids and bases can corrode the extraction system constantly and need to be neutralised after use (Kim et al. 2013). Organic solvents, in addition, are often harmful for the environment, need special deposition and require high amounts during the extraction process. Solvent extraction, for example Soxhlet and its derivatives are highly efficient through a constant recycling of amounts of solvent, but requires several hours or days of extraction and subsequent evaporation of the exploited solvent. It was also found that this type of extraction was found to be only applicable to a small number of algal strains (Kim et al. 2013) (Ranjith Kumar et al. 2015), presumably depending on the cell wall composition.

Another biochemical way of disintegrating algal cell walls is the application of enzymes, such as snailase, lysozyme, or cellulase. Those enzymes decompose the aforementioned cell wall components without degrading the desired extractives and can be seen therefore as a gentle method for valuable compounds. Nevertheless, a major disadvantage of this technique are long processing times (> ten hours) and a stringent operating temperature range. Constant temperatures are necessary because the administered enzyme are only active at certain temperature span (Zheng et al. 2011). This increases extraction costs and makes this method gentle but economically unsustainable.

Within the last decades, pulsed electric fields (PEF) were also established for the extraction of microalgae. PEF are known for charging the membrane of a cell, resulting in a temporary or constant pore formation inside the membrane when exceeding a certain threshold. Through the formed pores, substances can be transported inside the cell and vice versa. Several studies have shown an enhancement of the extraction yield of several compounds, for instance pigments or carbohydrates. However, this technique has no effect on the algal cell wall itself, which limits their application. It was found that PEF cannot be recommended for large molecules, such as proteins and lipids because a subsequent intermittence after PEF treatment is often necessary before further down streaming to gain a reasonable extraction yield for these molecules (Eing et al. 2013) (Goettel et al. 2013) (Luengo et al. 2014) (Postma et al. 2016).

It is evident that alternative methods are necessary, which are not only effective to overcome the cell wall stiffness, but also to be gentle enough to not to destroy these valuable compounds, especially heat-sensitive ones. Atmospheric pressure plasmas with their physical and chemical properties may offer such an alternative to the currently applied methods. The application of plasmas for extraction purposes is introduced in this study with a particular focus on spark discharges and their effects on *Chlorella vulgaris*.

1.2 Atmospheric pressure plasmas in liquids

Physical plasmas can occur in nature and are often referred to as the "fourth state of matter" (Eliezer and Eliezer 2001) to indicate the similarities to other phase transitions. In 1928, Irving Langmuir described his observation as follows: "Except near the electrodes, were there are *sheaths* containing very few electrons, the ionized gas contains ions and electrons in about equal numbers so that the resultant space charge is very small. We shall use the name *plasma* to describe this region containing balanced charges of ions and electrons."(Langmuir 1928). An example of the phase transition from ice to plasma is shown in Figure 1. Naturally occurring plasma phenomena are for instance lightning bolts and northern lights, so-called *aurora borealis* (at the northern hemisphere) or *aurora australis* (at the southern hemisphere).

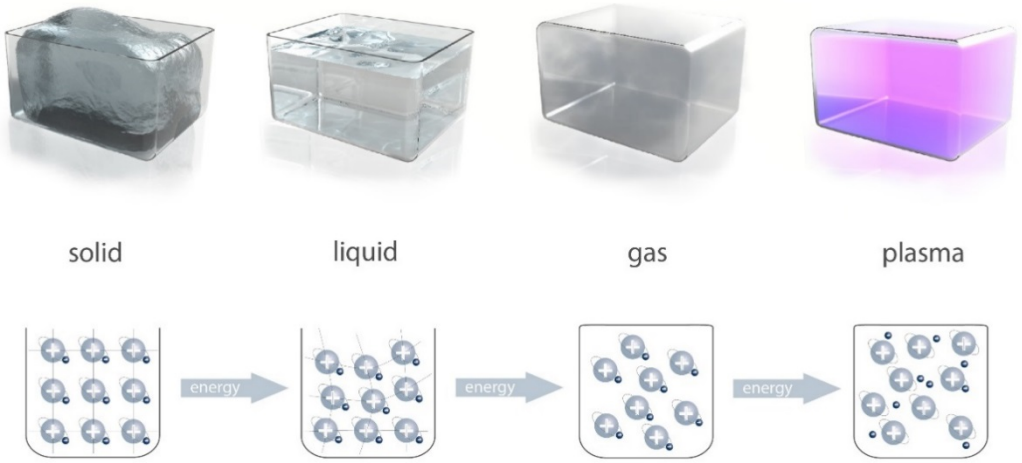

Figure 1 Water as an example for phase transition from solid to liquid and gaseous, eventually (panel 1-3, from left to right). Further application of energy enables the transition from the gaseous-state to the plasma-state (last pan-el). Picture reference by INP, Greifswald.

In general, the plasma state is described as a certain amount of ionised particles in a gas (Fridman et al. 2012), were the particles are a mixture of neutral particles, positive ions and free, negatively charged electrons. When the plasma state is attained, the moving free particles (i.e. electrons and positive or negative charged ions) are highly electrical conductive and can achieve higher conductivities than metals (Fridman et al. 2012). The operating and ambient conditions determine the degree of ionisation of a plasma, i.e. partial or full ionisation. The individual energies of electrons, ions and neutrals, determine whether plasma is in equilibrium (thermal plasma), or non-equilibrium state (non-thermal plasma).

To generate a technical plasma in general, electrical energy is required. The electrical energy can be provided by direct current (dc) or alternating current (ac), radiofrequency, microwaves, or pulsed dc high-voltage. Beside the generation of plasmas in gases, it is also possible to ignite a plasma in liquids. The advantage is to apply the plasma directly to the desired target, without the interaction of the ambient gas phase. The plasma channel itself is developed in the liquid, when a voltage pulse is applied. However, to produce a pulsed discharge, for instance in water, it is necessary to have a high electric field with several hundred to thousand megavolt at the tip of the electrode (Locke et al. 2006). To generate these high electric fields, it is usually concentrated as an inhomogeneous field, e.g. on the

tip of a wire, a needle electrode or the edges of a rod electrode. The formation of the high electric field eventually results in the formation of a narrow conductive channel, which expands over time and allows the plasma development.

There are several theories how a plasma channel in liquid is generated. One of the most accepted theories, the approach of partial and full discharges (Sato et al. 1996) (Akiyama 2000) (Sugiarto et al. 2003). If the gap between the electrodes is wide enough and thus, the discharge does not reach the counter electrode, a partial discharge or a so-called corona discharge is developed. In contrast, when the discharge reaches the opposite electrode due to a shorter distance between the electrodes, a conductive channel develops which is called arc or spark and is usually referred to as full discharge. The difference between spark and arc are only their durations. After several hundreds of nanoseconds, the current inside the spark channel decreases to sub-ohm values and subsequently, after some microseconds, the electric field in the spark discharge channel is very low, where an arc is then established. (Locke et al. 2006).

In partial discharges, the current is transferred by slow ions, which generate corona discharges as non-thermal plasma. Corona discharge generate rather weak shockwaves, moderate UV light emission, and radicals and reactive species are formed in the narrow region near the electrodes. In full discharges, i.e. spark or arc discharges, the current is provided by electrons, which means a small plasma volume is heated by a high current. Due to a high breakdown electric field of water, a small gap between the electrodes is necessary. The discharge current heats a small volume of plasma, which results in the generation of a quasithermal plasma with almost equal temperatures of electrons and heavy particles, such as atoms or molecules (Locke et al. 2006) (Fridman et al. 2012). Sparks and arcs generate strong shockwaves and UV light emissions. The UV light and the high radical density were found to be short-lived in the cavitation zone of the plasma channel (Locke et al. 2006).

Plasmas combine physical effects, such as shockwaves, high temperatures, light emissions and the formation of reactive species. These properties together with the high electric field enables plasma to be used in various fields of application. Corona discharges and spark or arc discharges in liquids have been used for inactivation of microorganisms or environmental and biotechnological applications, such as pollutant degradation and for surface modifications of materials (Sato et al. 1996) (Sunka et al. 1999b) (Sugiarto et al. 2003) (Lukes et al. 2008) (Banaschik et al. 2015) (Sava et al. 2018). It has to noted that the intensity of the before mentioned properties strongly depends on the applied plasma source and the employed electrical parameters.

1.3 Aim of this work

In this work, the application of atmospheric pressure plasmas for the extraction of compounds from microalgae was evaluated. Ideally, the plasma should be able to address the aforementioned shortcomings of the described standard extraction techniques. As indicated in the previous section, different types of plasmas have different unique properties. Accordingly, plasmas suitable for cell wall disintegration as well as a reliable reference method had to be identified. The results and development of these techniques are presented in Chapter 2. It could be shown that spark discharges, instigated in the algal suspension, was most effective and therefore studied in detail.

Chapter 3 describes how spark discharges affect extracted compounds. Proteins were identified as potential targets for plasma effects and with proteomic analysis (proteomics), the differences

and commonalities of the plasma method versus reference method could be elucidates. Obstacles and their solution concerning the evaluation of the data will be given in detail. Additionally, with proteomics the effects from reactive species could be determined and compared to the reference technique.

In Chapter 4, the underlying mechanism of successful cell wall disintegration with spark discharges is elucidated. Possible plasma effects are evaluated towards their contribution to the observed effects. Microscopic imaging, atomic force microscopy and Schlieren diagnostics were applied to answer the question how cell wall disintegration was achieved when applying a plasma to the cells.

Eventually, summary and concluding remarks are given in Chapter 5.

Chapter 2

Plasma: A feasibility study (P1)

Whether plasma and its properties is able to disintegrate the cell walls of *Chlorella vulgaris*, is object of this chapter. Classical applied methods that are also used for other plant materials were studied to establish a reference method. For example, sonication has shown to enhance the yield of ginseng saponins compared with Soxhlet extraction (Wu et al. 2001) due to cavitation forces. Microwaves have shown to be effective to enhance lipid yield from olives (Amarni and Kadi 2010). Cell wall rupture is achieved by heating the *in situ* water within the cells, which results in a burst of the cells. A reference method was necessary because the results obtained with plasma had to be equal or better than those achieved with the respective classical method were. As indicator for successful cell wall rupture, two metabolite classes were studied.

2.1 Plasma Sources and Reference Methods

In this study, five different plasma sources, pulsed electric fields and three different classical extraction methods were examined regarding their effect on *Chlorella vulgaris*, which was establishes as model organism. The plasma sources were either directly ignited in the algal suspension or at the surface of the suspension.

As reference methods, classic techniques that have shown to be effective on plant cells, were applied (Wu et al. 2001) (Amarni and Kadi 2010) (Safi et al. 2014). Methods of choice were sonication (ultrasound), high-pressure homogenisation and microwave exposures. The technical settings of these methods are given in Table 1.

Plasma sources that interact with the liquid or the liquid interface were chosen because different plasmas develop different characteristics.

For corona discharge treatment, a coaxial wire-cylinder setup was applied, which is described in more detail in Banaschik et al. (Banaschik et al. 2016). The plasma is ignited along a tungsten wire and a metal mesh at the cylinder wall worked as counter electrode. Corona discharges are known for their strong generation of reactive species, but rather weak shockwaves and are preferentially applied for water disinfection (Sunka et al. 1999a) (Malik et al. 2001) (Locke et al. 2006).

Another direct treatment of the algal suspension was conducted with a DC-Plasma jet, were the plasma is lit within a microhollow cathode geometry. The system was applied according to Kolb et al. and Kredl et al. (Kolb et al. 2012) (Kredl et al. 2014). The application of plasma jets are widely used in the field of plasma medicine and is known for its generation of various reactive species (Weltmann et al. 2010) (Hao et al. 2014)

An example of plasma interacting with the liquid can be observed when a volume DBD (dielectric barrier discharge) is applied. It was thus examined for its ability to affect the microalgal cell wall. The system was set up as described in Koban et al with a petri dish as dielectric (Koban et al. 2011). That plasma source has proved to be effective for bacterial decontamination and is characterised by a strong formation of reactive species (Oehmigen et al. 2010) (Ikawa et al. 2010).

As further plasma source with interaction of the interface of the algal suspension, a pin-to-liquid discharge was established. In this system, the surface of the liquid is seen as counter electrode and was described by Chen et al (Chen et al. 2008).

The last applied plasma source were spark discharges, directly ignited in the liquid. The spark was generated in a rod-to-rod configuration with tungsten as electrode material (diameter 2.4mm) and a repetition rate of 4 Hz. The gap distance between the electrodes was set at 0.5 mm, a pulse length of 100 ns and a voltage of 20-25 kV. The high-voltage pulses were generated with a Blumlein line generator in an unmatched mode. During the treatment, the suspension was moved in a continuous mode to avoid settling of the algae. The system is shown in Figure 2. Spark discharges are characterised by high UV-light emission, high radical density, strong electric fields and strong shockwaves (Sun et al. 2006) (Locke et al. 2006).

To consider pulsed electric fields (PEF) as effective for cell wall disintegration, a setup was build according to Eing et al and Goettel et al (Eing et al. 2013) (Goettel et al. 2013). PEF have shown to increase the extraction yield of various compounds from plants and microalgae (López et al. 2008) (Sack et al. 2010) (Luengo and Raso 2016).

All applied parameters of the aforementioned plasma and PEF sources are summarised in Table 2. All examinations were conducted with 50 ml of *Chlorella vulgaris* with an adjusted optical density (OD_{750}) of 0.8.

Table 1 Technical settings of applied standard extraction methods. Reprinted with permission from P1, (Zocher et al. 2016)

STANDARD METHOD	PARAMETERS	TREATMENT TIME
Microwave	600 Watts	20 seconds
Ultrasound (sonication)	20 kHz	20 minutes
Homogenisation	5,000 psi	8 minutes

Table 2 Technical settings of tested plasma sources and PEF setups. Reprinted with permission from P1, (Zocher et al. 2016)

PLASMA	SETUP	TREATMENT TIME
Corona discharges	Coaxial, directly in suspension; recirculation system	30 min.
Plasma jet	dc-jet; operated with air; submerged in suspension	30 min
Volume DBD	above suspension (15mm)	10 min.
Pin-to-liquid discharge	From pin to suspensions; distance 3 mm	3 min
Spark discharges	Rod-to-rod discharge (gap distance 0.5 mm) inside suspension; recirculation system	30 min.
PEF	1µs, 35 kV/cm, 5.5Hz	1,000 pulses

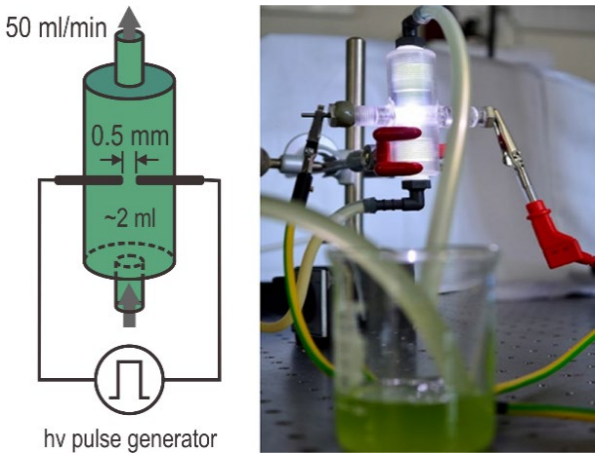

Figure 2 Electrical setup of the spark discharge chamber, described in section 2.1 (left: schematic image, right: setup). The system is operated in a continuous flow system and 100 ns high-voltage pulses from a Blumlein line generator. Reprinted with permission from P1, (Zocher et al. 2016).

2.2 Detection parameters for cell wall rupture

To detect successful cell wall rupture, two metabolite classes were analysed with UV/vis measurements. Additionally, scanning electron microscopy (SEM) imaging was used to visualise successful cell wall rupture

Protein yield in the supernatant is a good indicator for broken cell walls because proteins from the wall as well as from inside the cell elute into the adjacent solvent. An increase in protein yield can thus be considered as marker for effective cell wall rupture. The yield was detected with Bradford assay at 595 nm (Bradford 1976). The most effective standard method and the most suitable plasma source were determined with this assay as first step.

The second marker for successful cell wall rupture are pigments because they are located inside chloroplasts, which are well protected by the cell wall. Hence, an elevated pigment yield can be therefore seen as indicator for successful cell wall disintegration. Besides, pigments are highly heat sensitive and an alteration of the extract colour may indicate negative side effects from the treatment. Chlorophyll *a* and *b*, and total carotenoid content were determined according to Lichtenthaler et al and Luengo et al (Lichtenthaler and Buschmann 2001) (Luengo et al. 2014) (Luengo et al. 2015).

2.3 Determination of effective extraction methods

As mentioned in the last sections, a reference method had to be found as first step. The standard method was necessary to compare the results obtained with the plasma sources. At first, the protein yield was determined, followed by the pigment yield.

In Figure 3, the results from the protein content in the supernatant from the standard methods are shown. Ultrasound and homogenisation achieved 0.4 % and 2.5 %, respectively, but microwave treatment extracted 5.9 % proteins in relation to the total dry weight. Hence, microwave treatment achieved the highest protein yield and was set as reference method.

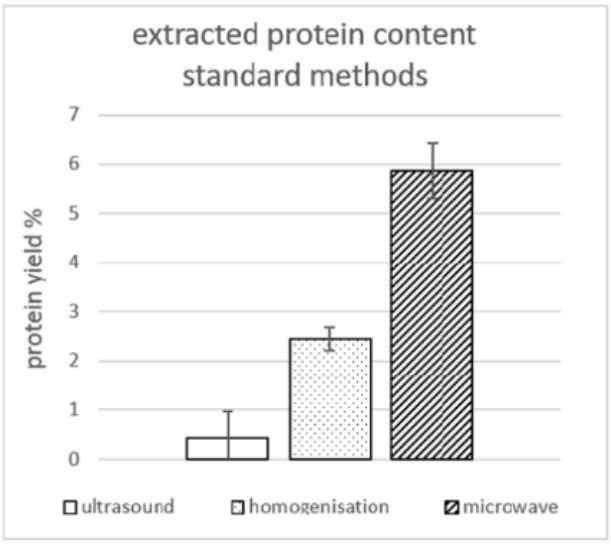

Figure 3 Protein concentration in the supernatant (in %) related to dry weight after ultrasound, homogenisation, and microwave exposure. Reprinted with permission from P1, (Zocher et al. 2016).

When comparing the protein yield after the respective plasma treatment, spark discharges gained not only the highest amount (4.35 %) but also similar yields as was found for microwave exposure. The results are summarised in Figure 4. All other plasma sources and PEF treatment showed only minor effects on the cell wall, with protein yields less than 1 %.

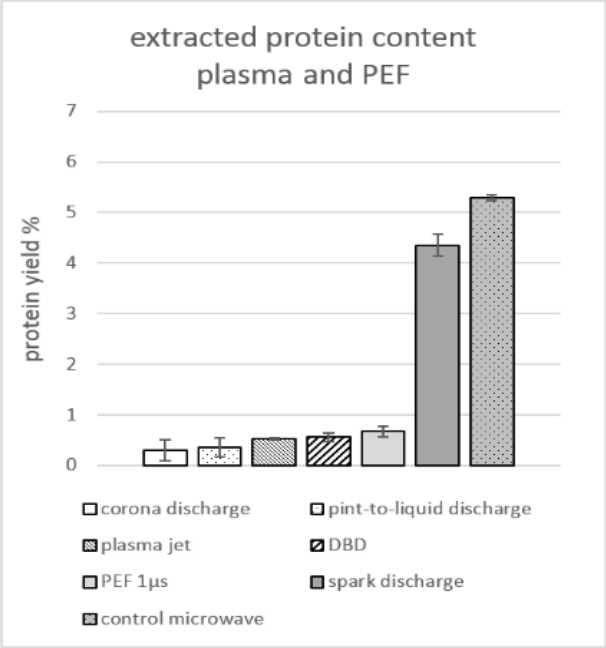

Figure 4 Protein content (in %) in the supernatant related to dry weight after treatment with five different plasma sources and PEF. Reprinted with permission from P1, (Zocher et al. 2016).

The pigment yield after spark discharge treatment and microwave exposure is displayed in Figure 5. Chlorophyll *a* and *b* content (19.204 mg/g culture $_{(dw)}$ and 9.583 mg/g culture $_{(dw)}$) are even higher than the yields after microwave treatment (12.207 mg/g culture $_{(dw)}$ for chlorophyll *a* and 4.058 mg/g culture $_{(dw)}$ for chlorophyll *b*). Total carotenoid contents were nearly equal for spark discharges and microwave treatment (3.952 mg/g culture $_{(dw)}$ versus 4.386 mg/g culture $_{(dw)}$). The pigment yield gained with other reference methods, plasma sources, and PEF stayed in the same range as the untreated control and proofed the findings from the protein yield.

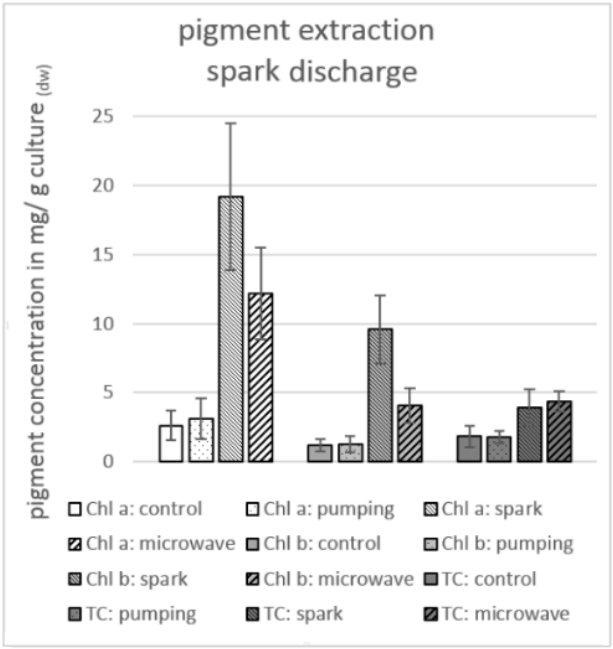

Figure 5 Pigment concentration (in mg/g culture $_{(dw)}$) after spark discharge treatment in comparison to microwave exposure, untreated control, and pumping of the suspension alone. Reprinted with permission from P1, (Zocher et al. 2016).

Plasma and microwave exposure are associated with elevated temperatures and might be associated with detrimental effects on extractives. In Figure 6, a degradation of pigment colour after microwave exposure is visible (*b*), whereat spark discharge-treated pigments kept their lush green colour (*a*). Thus, spark discharges seem to be effective and yet, gentle enough for heat sensitive compounds without degrading them.

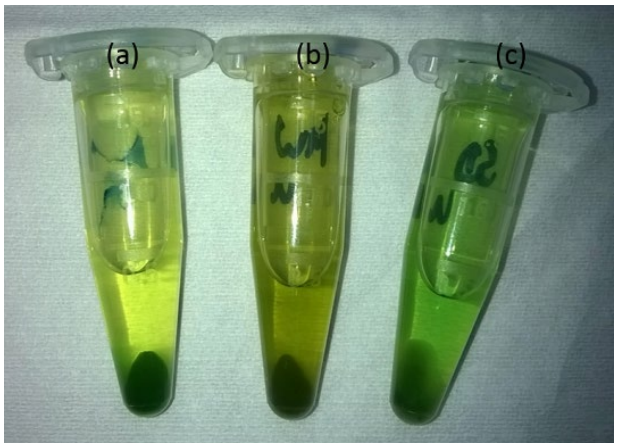

Figure 6 Colour image of the pigment extracts from untreated control (a), microwave exposure (b), and spark discharge treatment (c). Reprinted with permission from P1, (Zocher et al. 2016).

Eventually, the cell wall disintegration was visualised with SEM. Microwave-related cell wall damage is depicted in Figure 7, panel *b*. A single hole can be seen, which is most likely due to boiling of the intracellular liquid. Microwaves heat the *in situ* water within the cells, which accelerates cell wall rupture by sudden temperature rise. Consequently, the components are released into the solvent (Amarni and Kadi 2010). In contrast, the mechanism behind the spark discharges seem to be different

when observing the images in Figure 7, panel *c*. The cells look deflated or wizened and a mechanic force might be the reason for the observed effects.

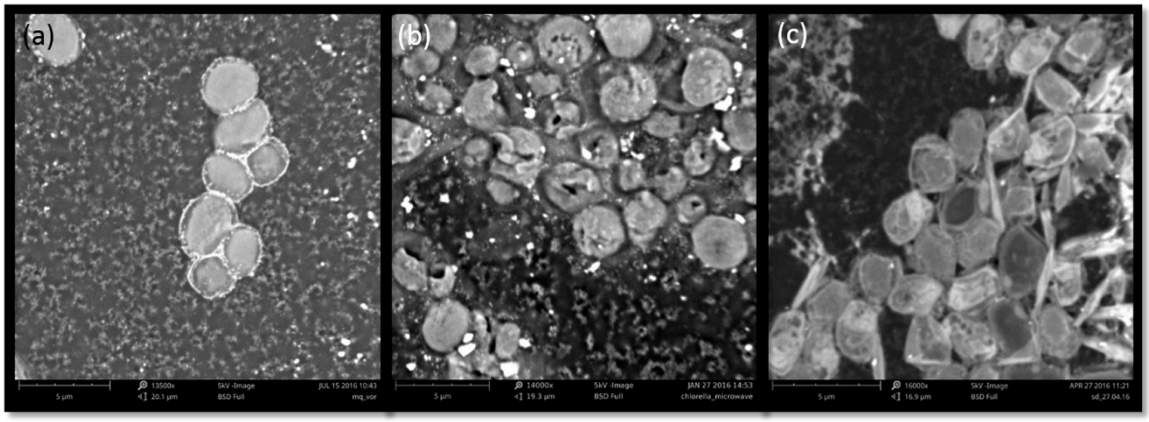

Figure 7 Scanning electron images of Chlorella vulgaris before treatment (panel a), after microwave exposure (panel b), and after spark discharge treatment (panel c). Reprinted with permission from P1, (Zocher et al. 2016).

In this study, microwave exposure has shown to be most effective for cell wall rupture of *Chlorella vulgaris*, and was therefore, applied as reference method for further studies. Among all analysed plasma sources have spark discharges proofed to be the most effective method. The protein amount was comparable to the microwave-extracted yields. For pigment extraction, spark discharges have shown to be even more effective and yet gentle towards thermolabile components. Therefore, this plasma source was found be an alternative technique to address the aforementioned shortcomings of currently available methods and were further analysed with regard to their effects and mechanism.

Chapter 3

Plasma effects: Proteins as Targets (P2)

After a suitable reference method and an effective plasma source were identified, several research questions occurred. Although the protein assay revealed comparable protein amounts, no statement concerning possible differences could be made. Spark discharges are known for the generation of reactive species, UV-light, electric fields and shockwaves (Šunka 2001) (Sun et al. 2006) that may contribute to an effective extraction. In addition, the different cell wall rupture mechanism in comparison to microwave radiation might favour different substances, for instance proteins. Proteins are sensitive for plasma exposure and are a known target for plasma effects (Lackmann et al. 2018) (Wende et al. 2018). Due to the heat development during microwave exposure, possible heat-related differences in the protein pattern may have been conceivable as well. To address these questions, label-free shotgun proteomic analysis (proteomics) was applied as a reliable tool for protein studies. However, the lack of a full proteome database for *Chlorella vulgaris* had to be solved first, to administer proteomic analysis.

3.1 Overall content and semi-quantitative protein yields

To examine the nature of the microalgal proteins, a gel-free proteomic protocol was established. Before precipitation, the samples after the respective treatments were divided. Two samples types were processed immediately and two sample types were stored for two hours on ice before further treatment. The process was conducted with the supernatant proteins and the proteins that stayed inside the pellet. The procedure of a time lag of 2 hours was chosen to evaluate protein content and quality without significant degradation of proteins. All samples were measured with a nano-LC system (UltiMate 3000), coupled to a QExactive conventional mass spectrometer, with a nanoFlex source (all Thermo Scientific Inc., MA; USA), by employing a steel emitter. Data analysis was conducted with Proteome Discoverer ™.

Major issue of for data analysis is the lack of a full proteome for *Chlorella vulgaris*. Although it is a well-studied microalga, it is not fully characterised on proteome level. This problem is applicable for all microalgae, which still hinders a sufficient protein analysis (Guarnieri et al. 2011) (Reijnders et al. 2014). The approach in this study was to apply the proteome of the higher order *Chlorellales*, which comprises all microalgae from the *Chlorellaceae* family. The *Chlorellales* database was transferred from Uniprot® protein database (UniProt Consortium 2016).

In Table 3, an overview of all extracted proteins is given. The shared amount of proteins found for both extractions varied only minimally with direct processing and the two hour time lag, in the supernatant and inside the pellets. The total number of proteins in the supernatant decreased slightly from 1,022 to 792 and the number of proteins in the pellet raised from 968 to 986. These results confirm the observed similar protein yields, gained with the Bradford assay.

Table 3 Number of proteins in the supernatant (a) and pellet (b) after microwave and spark discharge treatment. Numbers account for direct processing and two hour time lag. Reprinted with permission from P2, (Zocher et al. 2019).

	Method		
a) number of proteins in supernatant	microwave (unique amount)	shared amount	spark plasma (unique amount)
directly after processing	65	1,022	4
2 hours' time lag	288	792	3
b) number of proteins in pellet			
directly after processing	58	968	83
2 hours' time lag	24	986	102

Whether there are differences in protein yield and types between both extraction techniques, semi-quantitative analysis was conducted. The area under the curves (AUCs) for all plasma-extracted proteins were set into relation with their corresponding AUCs from microwave-treated proteins. Ratios higher 2 or smaller 0.5 were set as statistically significant, i.e. a twofold higher or smaller yield after spark discharge treatment in comparison to microwave exposed proteins. The resulting sigmoidal curves are displayed in Figure 8; the limits of 2 and 0.5 are marked with horizontal lines. For instance, 278 proteins, detected in the supernatant directly after processing, had ratios higher than 2. Conversely, for 204 proteins decreased the amount after plasma treatment in comparison with microwave exposure, i.e. ratios were smaller than 0.5. A similar sigmoidal curve was observed for proteins, which were processed with a delay of two hours. For the ratios, calculated for proteins that were kept inside the pellet, the results are shown in Figure 8b. Directly after processing, a ratio higher than 2 was calculated for 174 and below 0.5 for 485 proteins. After a time lag of two hours, 263 proteins achieved a ratio higher than 2 and 345 were smaller than 0.5.

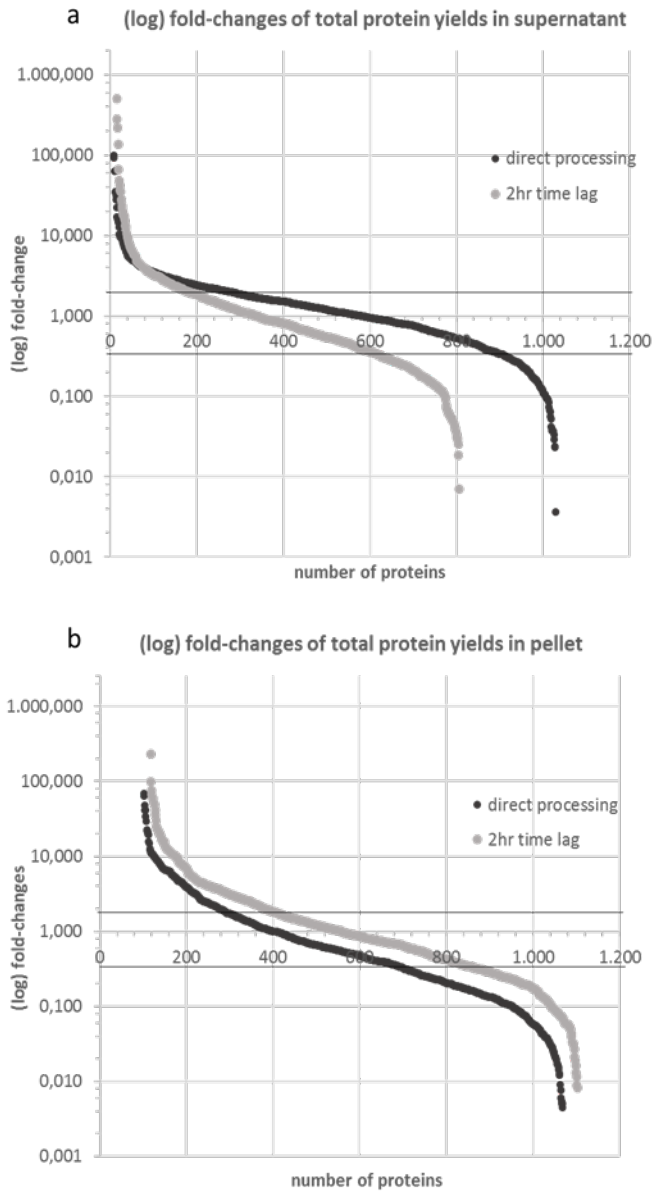

Figure 8 Fold-changes of areas under curve (AUCs) for proteins identified with LC-MS/MS, after spark discharge treatment in comparison with microwave exposure. Panel a represents identified proteins in the supernatent and panel b proteins that were kept inside the pellet. Black symbols represent direct processing (dp) and grey symbols the two hour time lag. Horizontal lines indicate the threshold of ratios 2 or 0.5. Reprinted with permission from P2, (Zocher et al. 2019).

Considering the results presented, it was shown that a delay in processing is not necessary. Delays after processing are often required, for instance, for pulsed electric fields treatment. Moreover, a delay in further handling might be detrimental for the proteins, increases the processing time, and would raise economic cost during industrial application. Applying the *Chlorellales* database enabled an insight to the extracted proteins and helped to observe protein modifications. This more generalised approach is well applicable for industrial application. However, for a more in-depth analysis a more defined database would be necessary and is a recommendation for future research in this field.

3.2 Protein yield of selected groups

Based on the results mentioned before, a more detailed analysis of five selected protein groups was conducted to determine possible differences of the respective protein yield. The following proteins of choice were observed: antifreeze proteins, chlorophyll *a–b* binding proteins, photosystem-related proteins (e.g. reaction centre or light harvesting complex proteins), histones, and heat shock proteins. The groups enclose rather heat sensitive proteins, e.g. photosystem-related proteins and less thermolabile proteins, such as heat shock proteins. Moreover, some of these groups are of industrial interest and their extractability was paid special attention. Two of those groups, i.e. antifreeze proteins and photosystem-related proteins are presented in this chapter in detail.

An example for rather heat resistant proteins are antifreeze proteins, which are found in plants, fish, fungi, and algae. They modify the growth of ice inside the cell, which stabilises the ice crystals and inhibit the recrystallization of ice (Griffith and Ewart 1995). Below 4 °C, antifreeze proteins are up regulated in *Chlorella vulgaris*. Figure 9 a+b shows the ratios calculated for the three identified antifreeze proteins. For proteins found in the supernatant, the ratios were not significant. Contrarily, the ratios for proteins inside the pellet were over 3.15 and over 1.50 after plasma treatment. Therefore, antifreeze proteins seem to be equally extractable with plasma and microwave exposure. Similar results as were found for the antifreeze proteins were also observed for heat shock proteins. Temperature robust proteins seem to be extractible with both methods likewise.

Antifreeze proteins are of great industrial interest, especially in food industry to improve the quality of frozen food. They are furthermore recommended for cryosurgery, cryopreservation or for transgenic technologies. However, their application strongly depends on costs since large quantities of highly pure proteins are needed (Venketesh and Dayananda 2008). With spark discharges and microwave exposures, a promising contribution towards this issue can be offered.

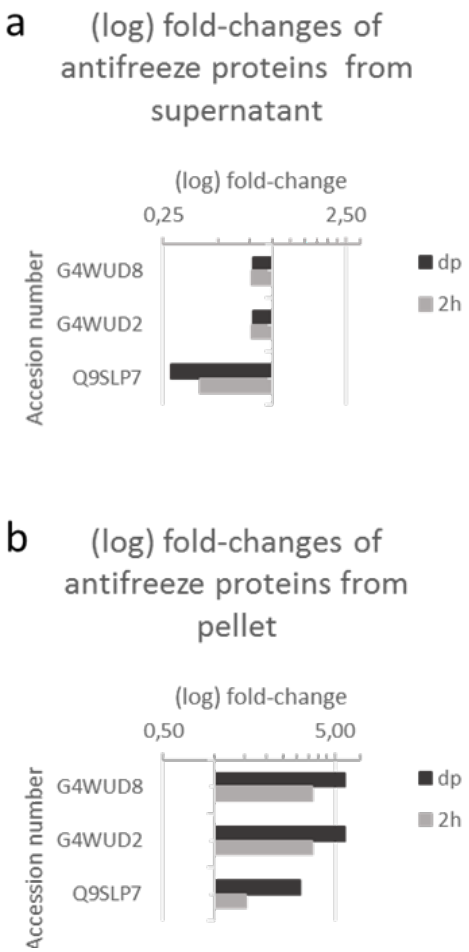

Figure 9 Fold-changes calculated from AUCs for respective signals from LC-MS/MS spectrographs for antifreeze proteins after spark discharge treatment in comparison to microwave exposure. Panel a represents proteins identified in the supernatant whereas panel b displays proteins identified in the remaining pellet directly after processing (black bars, dp) and with a two hour time lag (grey bars, 2h). Accession numbers were applied according to Uniprot database®. Reprinted with permission from P2, (Zocher et al. 2019).

Rather heat sensitive proteins are in the group of photosystem-related proteins. The compiled group amounts altogether 20 proteins, which are associated with the photosystems II and I (PSI and PSII), e.g. reaction centre subunits, assembly proteins, or apoproteins were collected in this group. Chlorophyll *a-b* binding proteins were studied in a separate group. The resulting ratios are depicted in Figure 10 a+b. Similar to the chlorophyll *a-b* binding proteins, the amount of extracted photosystem proteins was higher after spark discharge treatment than after microwave exposure. For example, 12 of the 20 proteins in the supernatant had a ratio higher than 2 after direct processing. This number decreased only slightly from 12 to 10 after two hours, but two proteins had a ratio smaller than 0.5. Remarkably, the protein *photosystem I reaction centre subunit XII* (accession number C7BEU1) was no longer detected after two hours in the microwave-exposed group, but had the highest ratio of 9.76 after plasma treatment (directly after processing). Comparable results were observed for chlorophyll *a-b* binding proteins and histones. This proofs that spark discharges are effective and yet gentle for extracting highly heat sensitive compounds from *Chlorella vulgaris*.

Photosystem-related proteins have recently gained interest for industrial purposes. Since these proteins are known for a sufficient charge transfer during photosynthesis, they can function as

energy carrier for the production of molecular hydrogen. As an example, PSI proteins have been successfully integrated as electron transfer vehicle into a bio-electrochemical tool, which is seen as key step towards a semiartificial hydrogen production (Badura et al. 2006) (Esper et al. 2006).

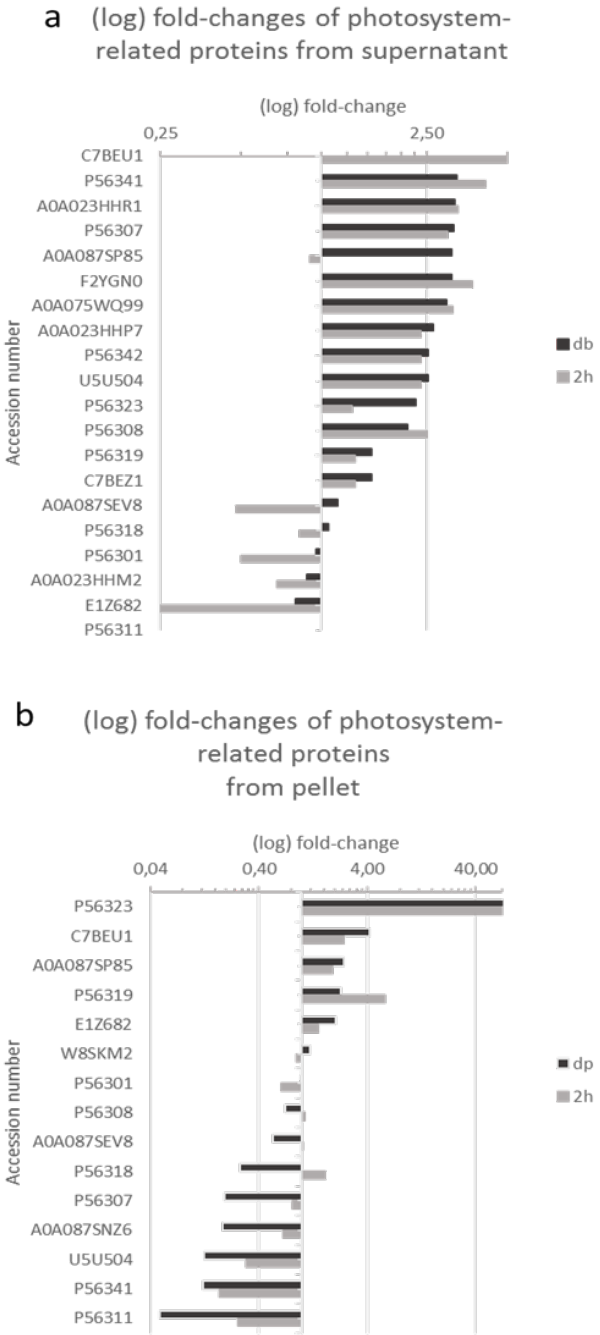

Figure 10 Fold-changes calculated from AUCs for respective signals from LC-MS/MS spectrographs for photosystem-related proteins after spark discharge treatment in comparison to microwave exposure. Panel a represents proteins identified in the supernatant, whereas panel b displays proteins identified in the remaining pellet directly after processing (black bars, dp) and with a two hour time lag (grey bars, 2h). Accession numbers were applied according to Uniprot database®. Reprinted with permission from P2, (Zocher et al. 2019).

Based on the proteomics results, it could be demonstrated that spark discharges and microwave exposures achieve similar amounts of extractable proteins, as was found for the Bradford assay. Nevertheless, a notable difference regarding the extracted protein classes could be shown. Heat sensitive proteins are better extracted with spark discharges, whereas proteins that are more robust showed no preference for the applied extraction method. This of importance for industrial application because it allows a preselection for proteins of interest by executing the respective extraction technique. That spark discharges reach not only the cell wall but also intracellular components, such as chloroplasts, was shown by the extracted photosystem-related and chlorophyll binding proteins. These results confirm the findings from the pigment extraction studies. Eventually, it could be affirmed that spark discharges address the shortcomings of the standard methods concerning efficacy and temperature development.

3.3 Oxygen and nitrogen modifications

Plasmas are known for the generation of reactive oxygen and nitrogen species (RONS) that are well-known to interact with biological material (Lackmann et al. 2015) (Lu et al. 2016). Whether reactive species generated by spark discharges affect proteins from *Chlorella vulgaris*, was also part of this study. Peptides with a false discovery rate of 5 % or less were permitted and thus, 7,777 modifications were available for examination.

Oxidation patterns, for example on cysteine, are often caused by ROS. While single oxidations are quite unstable and already occur during the sample handling, trioxidations (e.g. cysteine sulfonic acid) are highly stable and hence, simply detectable with mass spectrometry (Lackmann et al. 2018). In the supernatant, large amounts of threefold oxidation were found. All results are shown in Figure 11 and Figure 12. Directly after processing, 132 oxidised peptides were detected, whereas only 19 peptides were modified by microwave exposure. After two hours, still 56 triple oxidised peptides were found in the plasma samples. Although the number of modification seem to decrease, the amount stayed constant over time because the total number of proteins after 2 hours decreased by 30 % as well. The number of altered proteins in the microwave samples stayed constant over time; nevertheless, the modified peptides varied between direct processing and the two hour time lag.

Proteins that remained inside the pellet were modified as well, but with a lower degree and varied for different sample regimes. In the plasma-treated samples, 25 (directly after processing) and 43 (after 2 hours) modified peptides were found. For microwave-exposed samples, six oxygen modifications were found immediately and 14 modifications after two hours. It is conceivable that components inside the cell have protective properties against ROS. ROS recombine to the more stable H_2O_2, which can be subsequently decomposed again into O_2 and H_2O (Banaschik et al. 2017). Generation, recombination and degradation procedures will eventually establish a dynamic equilibrium, which depends on operating parameters. The high amount of triple oxidation, which are presumably due to long-lived H_2O_2, was confirmed by the photometrical-determined yield of 3.00 mg/l after 30 minutes treatment time. Plants and microalgae undergo photosynthesis, were superoxide radicals and eventually, H_2O_2 are produced. To prevent themselves from intoxication, superoxide dismutase (SOD) and catalase are part of the photosynthetic pathway to scavenge these radicals (Scandalios et al. 1997). Gebicki et al. have demonstrated that active SOD present during γ-irradiation decreased the yields of

protein peroxide that is otherwise generated by reactive oxygen species (Gebicki and Gebicki 1993). On the one hand, it is possible that SOD and catalase scavenge the ROS from plasma generation inside the algal cell. On the other hand, it might be conceivable that these enzymes are a preferred target of ROS and other proteins are protected indirectly. Other protection systems in microalgae are carotenoids. They have a high antioxidative activity and scavenge ROS from the photosynthesis pathway (Goiris et al. 2012). *Chlorella vulgaris* is rich in carotenoids and it is likely that the carotenoids contribute strongly to the observed antioxidative effects inside the pellet.

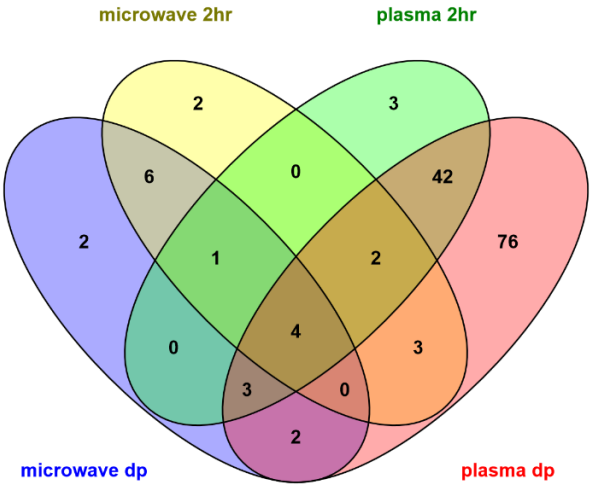

Figure 11 Number of trioxidised proteins in the supernatant after microwave exposure (blue and yellow ellipses) and spark discharge treatment (green and red ellipses). Directly after processing, 19 modifications were found for microwave-treated samples (microwave dp) and 132 after spark discharge treatment (plasma dp). Within the 2-hour time lag, 19 modifications, different from the ones for direct processing, were detected in microwave samples (microwave 2hr), but 56 remained after spark plasma treatment (plasma 2hr). Reprinted with permission from P2, (Zocher et al. 2019).

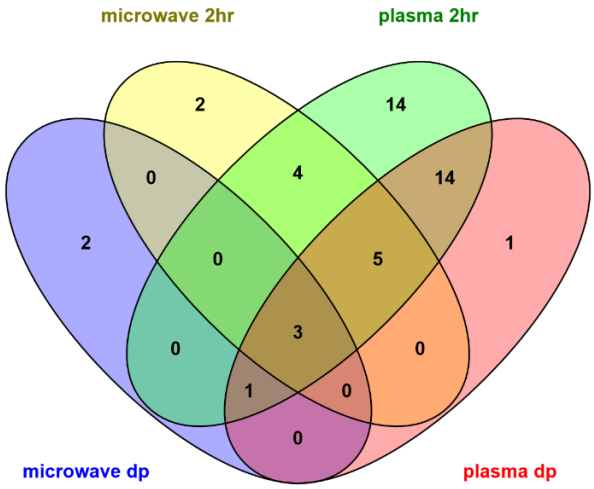

Figure 12 Number of trioxidised proteins that were kept inside the alga pellet after microwave exposure (blue and yellow ellipses) and spark discharge treatment (green and red ellipses). Directly after processing, 6 modifications were found for microwave treated samples (microwave dp) and 25 after spark discharge treatment (plasma dp). Within the 2-hour time lag, 14 modifications were detected in microwave samples (microwave 2hr) and 43 after spark plasma treatment (plasma 2hr). Reprinted with permission from P2, (Zocher et al. 2019).

The occurrence of nitration on peptides would be a sign for reactive nitrogen (RNS) generation during the spark discharge treatment. Whether RNS affect the extracted proteins as well, was therefore ascertained as well. In contrast to elevated trioxidation, no increase of nitration was found for plasma treatment in comparison to microwave exposure. For microwave treatment, 21 modifications and 15 for spark discharge-treated samples were found directly after processing. After two hours, 25 modified peptides were found in the microwave samples. A decrease down to eight was observed in the spark discharge-exposed sample. Inside the pellet, the alterations stayed constant over time; here, 12 (microwave) and 18 (spark) modified peptides were detected. Remarkably, although the numbers stayed constant, the protein types differed depending on treatment and processing conditions, indicating no main target for RNS. The low amount of RNS was confirmed by photometric measurements. Nitrite yield was 0.13 mg/l (±0.01 mg/l) and nitrate concentration ranged below the detection limit. A generation of RNS during spark propagation seems therefore unlikely.

With proteomics, oxygen and nitrogen modifications could be determined. ROS have an effect on the extracted proteins in the supernatant, but inside the cell, there seem to be protective properties, which attenuate the ROS influence. Associated modifications may alter the function of the proteins, but modifications might not always be detrimental. It is also likely that the modified proteins may open new pharmaceutical or other industrial applications. Oxygen modifications were not investigated in detail, but it is recommended for further research. For other plasma sources, an impact on amino acids have been shown. Preferable targets are any sulphur or nitrogen functionalities under acidic conditions (Takai et al. 2014) (Smith et al. 2017) (Lackmann et al. 2018). It has to be kept in mind that microwave radiation may also modify peptides during exposure. However, such modifications are currently unknown and thus, cannot be detected by proteomics. It would be beneficial for this research field to perform a more in-depth analysis regarding microwave-related peptide modifications. Again, a more precise proteome database is necessary to answer this question.

Chapter 4

Plasma effects: Mechanism of Cell wall Disintegration (P1-P4)

When a plasma, e.g. a spark is ignited in an aqueous solution, several chemical and physical effects can be observed. The electrical energy, which is dissipated in the spark channel, is released by UV-light emission, formation of reactive species, high electric fields, temperature increases, and strong shock-waves (Sugiarto et al. 2003) (Sun et al. 2006) (Sarkisov et al. 2006) (An et al. 2007) (Lukes et al. 2008). A schematic overview of the expectable effects is given in Figure 13.

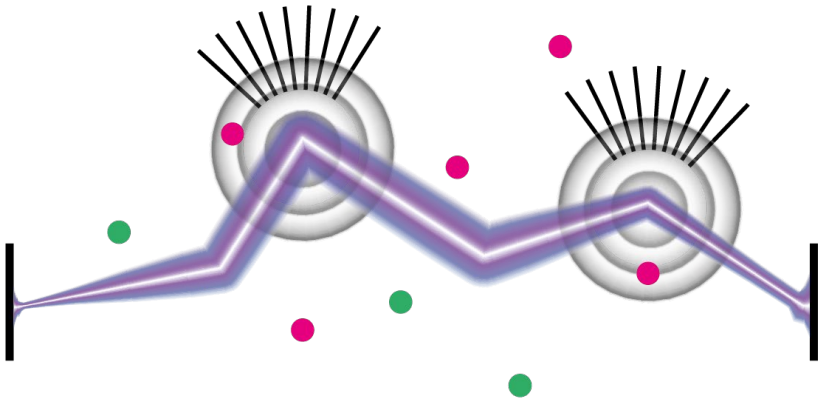

Figure 13 A spark propagating from one electrode to the counter electrode inside a liquid. Consequently, strong electric fields, ultra-violet radiations, shockwaves and, chemical reactive species are produced. Image courtesy by INP Greifswald

Each of these phenomena may contribute to the observed cell wall rupture Figure 7. To categorise the possible impact on the cell walls, all chemical and physical effects were determined either by experiments or, when not possible, by a literature review. In the following sections, the effects are described and weighed for their contribution towards the cell wall disintegration.

4.1 Shockwave pressure

Especially spark discharges are known for their strong pressure shockwaves, due to their fast energy input. When a submerged discharge takes place, an excessive amount of electrical energy is consumed on the formation of a plasma channel. When the channel expands, the stored energy is dissipated by radiation, conduction, and mechanical work. High-pressure gradients give rise to a shockwave, which travels with a higher speed than the speed of sound (Fridman et al. 2012). The destructed mechanical energy imparted on the cell is arguable the main reason for the observed cell wall rupture.

The cell walls of *Chlorella vulgaris* is characterised by a remarkable tensile strength and had thus to be compared with the determined shockwave pressure. Although in literature 9.5 MPa are

generally assumed for microalgal tensile strength (Lee et al. 2012), a more accurate value was necessary for the comparison with the shockwave pressure. To obtain a reliable value, atomic force microscopy (AFM) was conducted. For the calculation of the elasticity modulus, 27 viable cells of various size were scanned in contact mode. As can be observed in Figure 7, cells are affected by the spark discharges and hence, a determination of the elasticity modulus on ruptured cells could be omitted. An example of the scanning images from AFM measurements are given in Figure 14. The bright parts on the cell surface were set as basis for calculations. An average elasticity module of 13.95 MPa was determined and is hence, higher than the literature value (9.5MPa). It may be possible that this is a typical value for *Chlorella vulgaris*. However, variations of the moduli were observed that ranged between 8.06 and 22.31 MPa. The reason for these deviations might be explainable with observations made by Lee et al. Their indentation studies on *Tetraselmis suecica*, a green microalga, revealed no identical force curves even on various parts of one single cell. This effect might be described by ultrastructures inside the cell. Ultrastructures can be, e.g. chloroplasts, nucleus, starch or lipid bodies (Lee et al. 2013). The irregular distribution of such structures underneath the cell surface may lead to different pressures spots above the cell surface. Notably, the tensile strength seems to be independent from the cell size because no correlation was found between elasticity module and the associated cell size. This might rather represent the characteristics of the cell wall and thus, the tensile strength credibly depends on the cell wall composition, e.g. amount of cellulose or hemicellulose, or proteins and the number of cell wall layers. The elasticity moduli for other microalgae species might therefore vary in comparison to *Chlorella vulgaris*. With AFM measurements in this study, a precise tensile strength was obtained and the result might contribute to improvements in the field of microalgal extraction.

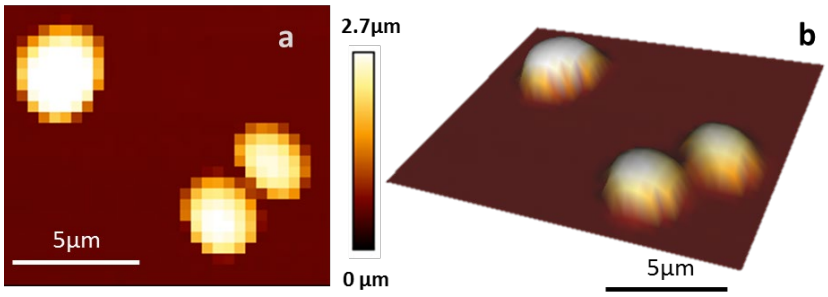

Figure 14 Atomic force microscopy (AFM) images from untreated Chlorella vulgaris cells. Panel a displays the 2D image and panel b the 3 D image. The brightest spots represent the strongest parts of the cell (panel a). Reprinted from P3, submitted.

The shockwave propagation and the resulting pressure were examined with Schlieren photography, by applying a laser system of 532 nm, a lens system, a knife-edge, and a framing camera. When a shockwave travels through a medium, a change in density can be detected. With this change of the refractive index, conclusions on the shockwave speed and pressure can be drawn. For the calculations, the Hugoniot equation was applied (Eq. 1):

$$P = \rho_0 u_s \frac{(u_s - A)}{B} \qquad (1)$$

The mean propagation velocity is u_s and for the density of water, ρ_0, a value of 1 g/cm^3 was assumed. The constant A is the speed of sound in water and was assumed with 1.45 km/s / and B is a proportionality constant that was experimentally determined by Nagayama et al. with 1.99 (Nagayama et al. 2002).

For the study of the shockwaves, a clear shockwave front was necessary to determine the shockwave pressure. Therefore, the setup had to be changed from rod-to-rod configuration to a needle-to-needle setup because in the rod-to-rod configuration overlays of several shockwave fronts were observed. However, the results gained with the altered electrode configuration can be transferred for the cell wall rupture experiments. The propagation pattern of the shockwave in the pin-to-pin setup is shown in Figure 15. The shockwave between the pins propagated from the centre of the discharge radially towards the walls of the reaction chamber. From these images, the radii from the discharge centre to the shockwave front (white dotted line) were determined to calculate the propagation velocity. The propagation pattern is indicated with white arrows in these images. The velocity was then calculated from the pressure by applying Equation (1).

As displayed in Figure 16, the pressure at the close vicinity of the discharge was 500 MPa (±42 MPa, at a 0.6 mm distance) and decreased to 140 MPa (±10 MPa, at a distance of 2.4mm) at the outer boundary of the shockwave front. Thus, the shockwave pressure is much higher than the determined tensile strength of *Chlorella vulgaris*. The pressure decrease is usually not constant and therefore, a fit model, r^{-2}, that is used for nanosecond pulses (Vogel et al. 1996), was applied to predict pressure values at farther distances of the discharge. For instance, by applying this model, an average pressure of ca. 20 MPa would be achieved at a distance of 20 mm from the centre of the discharge and may be therefore still strong enough to disintegrate algae. In Figure 16, the red line displays the fit model r^{-2}. The dash-dotted line along the x-axis displays the pressure region, were algae are able to withstand the pressure. It can be seen that error bars are not within this region and thus, sufficient cell wall rupture is possible throughout the entire volume of the reactor. In summary, the shockwave pressure generated from the spark channel can thus be considered as the main reason for the observed cell wall rupture of microalgae, when comparing the tensile strength and the shockwave pressure.

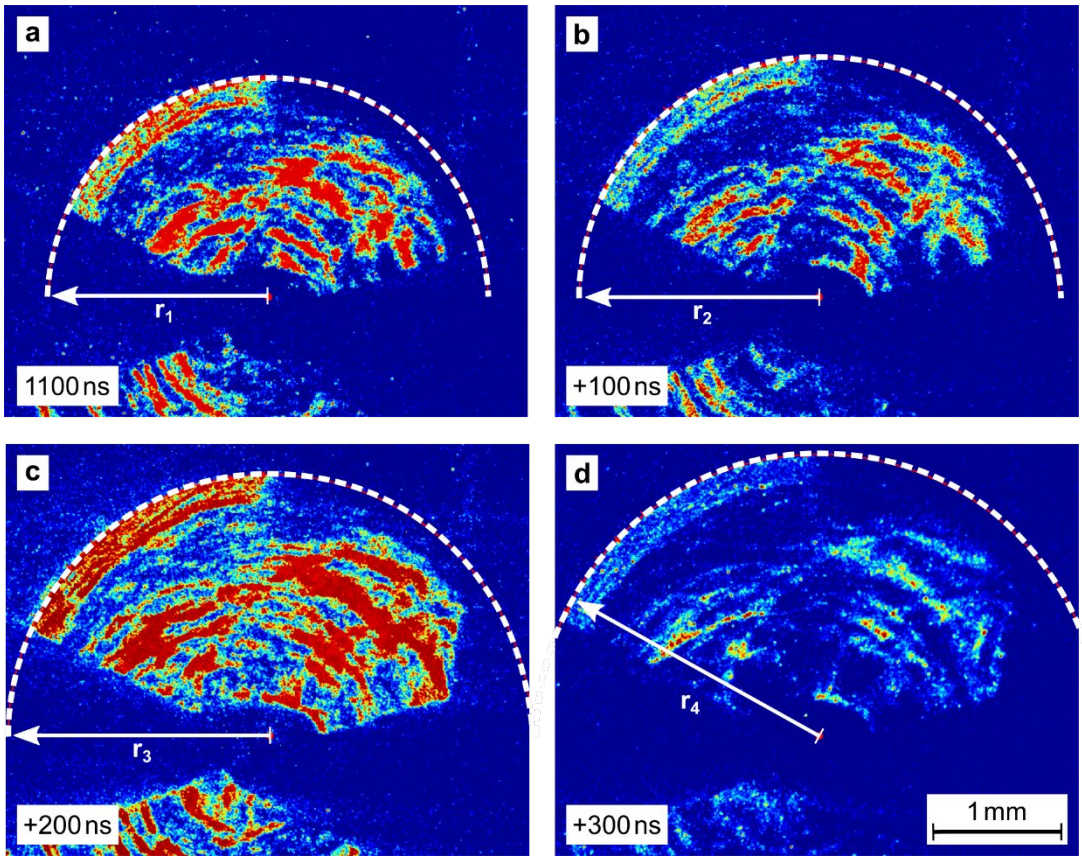

Figure 15 Time-resolved radial propagation of a shockwave front, displayed by white arrows, in a needle-to-needle configuration (panel a-d). In these images, the radii of the shockwave centre and outer shockwave front were defined for calculating the shockwave speed. Coloured areas occurred due to different light sensitivities of the four camera sensors, which were then normalised for pressure calculations. Reprinted from P3, submitted.

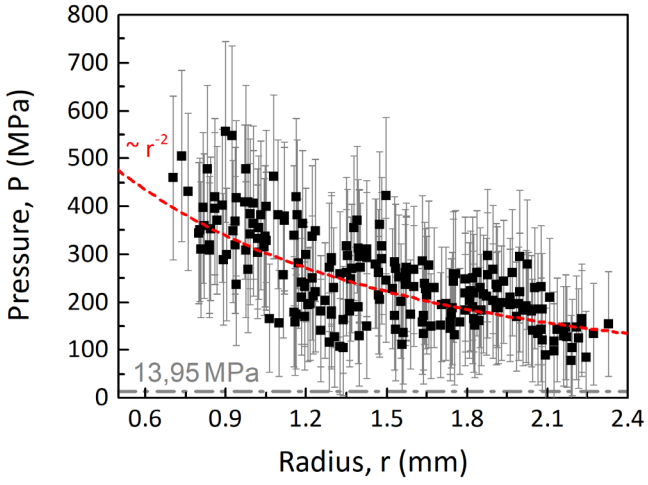

Figure 16 Shockwave pressure in relation to the distance from the electrode gap (needle-to-needle configuration). The shaded area along the x-axis indicates the pressure range, in which algae are supposedly able to withstand the respectively applied pressure. The red curve demonstrates the fit model to describe the pressure profiles, p_r, proportional inverse square to the distance radius, r. Reprinted from P3, submitted.

4.2 Reactive species impact

During spark propagation in water, reactive oxygen and nitrogen species are generated (RONS), which are formed by electron collision with water molecules, subsequent electrolysis, and by UV-light driven photolysis (Sun et al. 2006).

Reactive oxygen species recombine to more stable compounds, such as H_2O_2 and decompose finally to O_2, and H_2O (Banaschik et al. 2017). The process of generation, recombination decomposition is a dynamic balance and depends on operating parameters. For the spark discharges applied in this study, a high amount of threefold-oxidised proteins was found in the supernatant with proteomic analysis. The trioxidation, apparently due to long-lived H_2O_2, was verified by the photometrical-determined yield of 3.00 mg/l after 30 minutes of treatment time in distilled water. However, as was described in the previous chapter, only proteins in the supernatant were affected by ROS, but not those that remained in the pellet.

Proteomic analysis was also applied to elucidate the formation of RNS. As shown in the previous chapter, no remarkable yield of NO_2- and NO_3- was achieved during the spark discharge treatment. A formation of reactive nitrogen species is hence unlikely and may not affect the cell wall of *Chlorella vulgaris*.

Since reactive oxygen species might have an effect only on proteins in the supernatant, a contribution to the cell wall damage itself seems to be small. Although Smith et al found damage on bacterial cell walls after DBD treatment (which are known for strong radical generation) (Smith et al. 2017), a different observation was made by Laroussi et al., when applying a DBD on bacteria (Laroussi et al. 2003). Lysis was found for gram-negative bacteria, but no effect occurred on gram-positive bacteria. As gram-positive bacteria have a rather strong cell wall, Laroussi et al. concluded that radical formation has no effect on this more robust cell walls. Gram-positive bacteria and microalgae are comparable in their cell wall composition, with regard to protective effects, structure, and strength and thus, results found for bacteria are indeed transferable to microalgae. In addition, a volume DBD was applied for the extraction of microalgal metabolites, as well. No effects regarding cell wall rupture, neither in extraction yield nor in the SEM images, were found. Although RONS might play no role towards cell wall rupture of microalgae, their influence on extracted compounds, however, cannot be excluded and is recommended for further research.

4.3 Electric field effects

High electric fields are also present during spark propagation and are able to play a role for increased extraction yield after spark discharge treatment. Contemplating the described electrical setup, the rod electrodes with their large surface can be seen as plate capacitors. Unless a spark occurs, with the gap distance of 0.5 mm and an applied voltage of 30 kV, a field strength of 600 kV/cm can be estimated in the reactor system.

Chlorella vulgaris was also treated with pulsed electric fields and corona discharges to compare their effects on the microalgal cell wall with the results from spark discharge treatment. Neither for the PEF, nor for the pulsed corona discharges, could an increase in protein yield, and, hence, no cell wall rupture, be detected. For PEF treatment, a pulse length of 1 µs and a field strength of 35 kV/cm

was applied. When treating microalgae with corona discharges, a wire-to-cylinder setup with an applied voltage of 80 kV and a 100 ns pulse width were administered. For this setup, a field strength of 2.7 MV/cm in the close vicinity of the wire was calculated (final field strength at the cylinder wall=8.1 kV/cm). A fast inactivation of the gram-negative *Legionella pneumophila* was achieved by applying the corona discharges and the according field strength. It was found that a complete inactivation could be achieved after 12.5 minutes with corona discharges. With pulsed electric fields alone, the survival rate was still $\log_{10}$ 1 after 25 minutes. The reason might be that the strong electric field along the wire lead to electroporation of the bacterial membrane, which fosters then the uptake of reactive species. Unlike for pulsed electric fields, reactive species are generated in high amounts during corona discharges. The inactivation of these bacteria took double the time with pulsed electric fields alone than with corona discharges. Additionally, SEM images revealed that PEF barely affects the gram-negative bacterial cell wall (Figure 17), compared to the destructed cells after corona discharges.

However, *Legionella pneumophila* is a gram-negative bacterium, with a considerable weak cell wall in comparison to the rigid and robust cell wall of *Chlorella vulgaris*. Thus, electric fields alone have little to no effect on the sturdy cell wall of microalgae in this configuration, as electroporation is known as consequence of the charging of cell membranes. Contrarily, a charging of the already porous cell wall seems unlikely and therefore, a contribution of electric fields to the examined cell wall rupture, in general, seems unlikely. Moreover, increased extraction yields for microalgae are only detectable, when an incubation time of several hours is conducted probably due to an osmotic imbalance in the cells (Goettel et al. 2013) (Luengo et al. 2014).

4.4 Temperature effects

The temperatures in the narrow spark channel itself can be estimated with a few thousand kelvin (Fridman et al. 2012). However, temperatures are rapidly decreasing in the surrounding medium with distance. Still, a heat influence on microalgae needs to be considered.

Most of the electrical energy input for the spark formation is initially stored within the channel, which is filled with high-temperature gas. When formed, the channel starts its expansion and the electrical energy is then dissipated in different mechanisms. Consequently, the temperature decreases due to radiation, and conduction into the surrounding water. The temperature difference between the channel and the water can be assumed with several thousands of ° Celsius (Sun et al. 1998) (Fridman et al. 2012). Locke and Thagard have calculated the temperature distribution inside the channel and the temperature at the channel-liquid-interface. For a discharge in water with an energy of 1 J/ pulse, the temperature in the centre of the plasma channel was calculated with 5,000 K, whereas the temperature at the plasma-liquid-interface was already decreasing to 300 K (Locke and Thagard 2012). With a pulse energy of 0.2 Joule, which was calculated for the setup used in this study, the temperature at the channel-liquid-boundary can be assumed to be in a similar range. Presuming that for the energy per pulse of 0.2 J, applied at a repetition rate of 4 Hz for 30 min, the temperature rise of 50 ml of the suspension would still only be about 7 K. Besides, the spark discharge treatment was operated in a continuous flow system, allowing the suspension to cool down and any losses can be therefore neglected. Hence, the overall temperature increase in the treated suspension was rather determined by operating parameters, especially the spark repetition frequencies.

When administering the sparks with 4 Hz, the temperature of the bulk liquid stays below 25 °C, but exceeds 30 °C when the frequency is 7Hz or more (data not shown here). In general, these bulk temperatures have been found more important for the deterioration of extracted compounds than the highly localised temperatures in the spark channel. Accordingly, spark frequencies have been kept at about 4 Hz.

4.5 Ultraviolet light emission

In pulsed high-voltage discharges, electrical energy is also converted for the formation of excited species, which release energy again by electromagnetic radiation, e.g. light. The discharge channel can be described as a blackbody radiation source with a substantial portion of the radiation, emitted in the ultraviolet (UV) region of the electromagnetic spectrum (Lukes et al. 2008).

It has been shown in several studies that UV-emission can inactivate bacteria (Gilpin et al. 1985) (Bank et al. 1990). Sun et al. examined the effect of UV-light emission, shockwaves, and electric fields effects from pulsed spark discharges in water on the inactivation of *E. coli* (Sun et al. 2018). It was found that for voltages of 16 kV-20 kV, UV-light contributes to an antimicrobial effect by about 28 % of the respective energy, but was not found to be significant, regardless the distance of the samples to the plasma channel. *E. coli* is a gram-negative bacterium with a somewhat weak cell wall. In contrast, *Chlorella vulgaris* is known for its robust cell wall and hence, less sensitive for these impacts. UV-light is known for its severe effects on DNA, but light is also necessary for photosynthesis in higher plants, and algae and an exposure of UV light can thus not be omitted (Batschauer 1993). A direct effect on the cell wall rupture is therefore improbable and can be excluded as responsible plasma effect.

4.6 Cell morphology alterations

Contemplating all described spark effects, the shockwave pressure can be entitled the major mechanism of the successful cell wall rupture from microalgae. This assumption is supported by the microscopic images and SEM images, where a distortion and inflation of cells imply the effect of an outer mechanical force from the spark channel.

A similar effect was shown when *Legionella pneumophila* was exposed to corona discharges in water. Although corona discharges produce weaker shockwaves than spark discharges, a clear shockwave effect was visible Figure 17. No such effect was visible, when *Chlorella vulgaris* or *Legionella pneumophila* were exposed to electric fields. Both cell types showed no obvious difference in their appearance after treatment. Such damage is generally also not associated with PEF, which are known for pores in cell membranes but not for the already porous cell walls. In contrast, heat during microwave exposure caused a cell burst, i.e. the force had to come from inside the cell (see Figure 7, panel b). As stated before, a similar effect due to the bulk temperatures after spark treatment cannot be excluded, but the cells should appear similar to those found after microwave treatment, which was not observed.

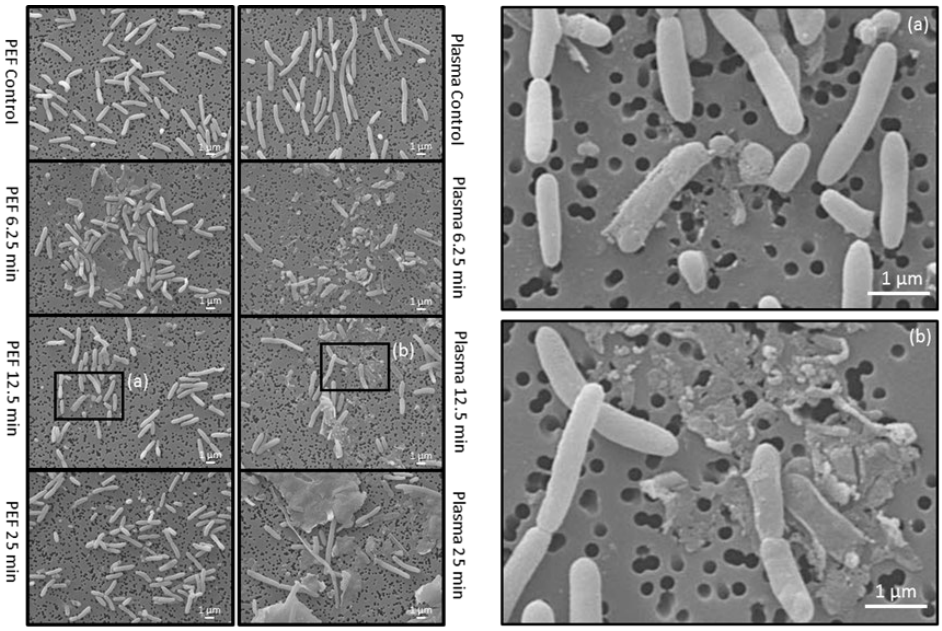

Figure 17 SEM images for Legionella pneumophila after PEF (panel a) and plasma treatment (panel b), respectively. A clear destruction due to the shockwave pressure was found after plasma treatment, whereat only minor damages were observed after PEF treatment. Reprinted from P4 with permission, (Banaschik et al. 2016).

Chapter 5

Summary and concluding Remarks

Microalgae have been an interesting target for biotechnological applications. With regard to their compounds, they are a valuable source for biofuel industry, as fine chemicals, cosmetics, for pharmaceutical, and nutraceutical products.

One of the most challenging step of extracting those compounds is a sturdy microalgal cell wall, accompanied by a small cell size of only a few micrometres. As extraction techniques, various mechanical, chemical and biochemical methods have been established throughout the last decades. However, these methods are associated with several shortcomings, such as high-energy demand, heat development, and alteration of extracted compounds. Consequently, improvements and alternatives to current extraction methods are needed for successful commercialisation of microalgal compounds. Physical plasmas were studied for their potential as alternative extraction technique.

As first step, different plasma sources were examined regarding their ability to rupture the cell wall of *Chlorella vulgaris*, which served as model organism. The alga is well studied and known for a remarkably sturdy cell wall. For comparison, an effective reference method had to be established and microwave exposure was found to be most successful. Submerged spark discharges, instigated directly in the algae suspension, revealed to be comparable to microwave exposure regarding protein content in the supernatant, which was set as indicator for successful cell wall disintegration. Pigment yield was evaluated as well and served additionally as marker for heat sensitive compounds. For pigments, spark discharges gained even higher yields in comparison to microwave-radiated samples. Thus, spark discharges were found to be a promising plasma method and were studied in more detail with respect to plasma effects on extracted compounds and the underlying mechanism of cell wall disintegration.

Proteins are sensitive for plasma effects and were thus, chosen for the analysis of spark discharge-related effects on extracted compounds. To answer research questions in which way the proteins are affected, shotgun proteomics was applied, which is a reliable tool for protein studies. Here, microwave exposure was applied for comparison to evaluate whether differences in the extracted protein pattern were detectable, although the protein yields were similar. Additionally, possible heat-related damages from the microwave irradiation might be detected as well. While *Chlorella vulgaris* is a well-known microalga, no fully sequenced proteome database is available. Therefore, a database from the higher order *Chlorellales* was established for the subsequent analysis. It was found, that the number of extracted proteins are similar for spark and microwave extracted proteins. Nonetheless, especially thermolabile proteins, e.g. photosystem-related proteins or histones were found to be better extractable with spark discharges than with microwave exposure. For more robust proteins, for instance antifreeze or heat shock proteins, no considerable difference was found between both extraction methods. In addition, the effect of reactive oxygen and nitrogen species (RONS) generated by the spark were also determined. Whereas RNS-related modifications were not detected, an elevated amount of oxygen modifications in form of trioxidation could be determined. Remarkably, the trioxidation seems to affect only proteins in the supernatant, which implies that scavenging cell compounds must be present inside the cell.

After the determination of spark discharges as suitable plasma source and the elucidation of plasma-related effects on the extracted compounds, the mechanism of cell wall rupture was studied. Plasma is associated with physical and chemical characteristics that may contribute to the observed

deformation of the cell surface. Whereas UV-light, reactive species, electric fields and heat may only play a minor role, shockwaves are the most likely reason for the cell wall disintegration. To study this assumption, the tensile strength of *Chlorella vulgaris* had to be determined first. With atomic force microscopy, the elasticity module of the microalga could be calculated, which is in the range of values given in the literature. Next, the shockwave pressure was determined with Schlieren photography. The pressure in the close vicinity of the discharge exceeds by far the tensile strength of *Chlorella vulgaris*. Even at farther distances, the shockwave pressure is still higher than the tensile strength. Thus, shockwave pressure seems to be the major contribution towards the cell wall rupture of microalgae when spark discharges are applied.

With spark discharges, a promising alternative extraction method have been established. However, there are still open research questions that could not be answered in this thesis and should be addressed in future research.

An in-depth analysis of the protein modifications may help to improve the understanding of plasma effects on plant material and biological material in general. Additionally, a fully sequenced database for *Chlorella vulgaris* may improve the gained results in this work and could help to raise industrial interest on microalgal proteins. The modifications caused from microwave exposure are also of interest and are recommended for detailed analysis.

The pigments that were extracted are also recommended for comprehensive analyses because modification, as observed for proteins, may occur, as well. It is likely that those possible modifications may also be of industrial interest.

With elucidating the cell wall rupture mechanism, an understanding of how to improve and upscale the technical setup was gained. The system itself could be modified with regard to energy efficiency, throughput volume and/or treatment time, which is necessary for industrial application. An increase in the spark frequency and a simultaneous decrease in treatment time with a careful observation of the temperature could also improve the method. A focused alignment of shockwaves may amplify their effect on the cell walls in a shorter treatment time and thus, enhance the extraction yields Additionally, shorter pulses, i.e. less than 100 ns, could also affect the extraction results positively and might improve the method in general. These results are important to realise a successful industrial upscaling. Nevertheless, in this work it was proved that spark discharges can be considered as a gentle and yet effective extraction method that might be applicable to macroalgae and other plant material as well.

References

Akiyama, H. (2000) Streamer discharges in liquids and their applications. Dielectrics and Electrical Insulation, IEEE Transactions on 7(5), 646-653.
Amarni, F. and Kadi, H. (2010) Kinetics study of microwave-assisted solvent extraction of oil from olive cake using hexane: comparison with the conventional extraction. Innovative Food Science & Emerging Technologies 11(2), 322-327.
An, W., Baumung, K. and Bluhm, H. (2007) Underwater streamer propagation analyzed from detailed measurements of pressure release. Journal of applied physics 101(5), 053302.
Badura, A., Esper, B., Ataka, K., Grunwald, C., Wöll, C., Kuhlmann, J., Heberle, J. and Rögner, M. (2006) Light-Driven Water Splitting for (Bio-) Hydrogen Production: Photosystem 2 as the Central Part of a Bioelectrochemical Device. Photochemistry and Photobiology 82(5), 1385-1390.
Banaschik, R., Burchhardt, G., Zocher, K., Hammerschmidt, S., Kolb, J.F. and Weltmann, K.-D. (2016) Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing Legionella pneumophila as model organism. Bioelectrochemistry.
Banaschik, R., Lukes, P., Jablonowski, H., Hammer, M.U., Weltmann, K.-D. and Kolb, J.F. (2015) Potential of pulsed corona discharges generated in water for the degradation of persistent pharmaceutical residues. Water research 84, 127-135.
Banaschik, R., Lukes, P., Miron, C., Banaschik, R., Pipa, A.V., Fricke, K., Bednarski, P.J. and Kolb, J.F. (2017) Fenton chemistry promoted by sub-microsecond pulsed corona plasmas for organic micropollutant degradation in water. Electrochimica Acta 245, 539-548.
Bank, H., John, J., Schmehl, M. and Dratch, R. (1990) Bactericidal effectiveness of modulated UV light. Appl. Environ. Microbiol. 56(12), 3888-3889.
Batschauer, A. (1993) A plant gene for photolyase: an enzyme catalyzing the repair of UV-light-induced DNA damage. The plant journal 4(4), 705-709.
Becker, E. (2007) Micro-algae as a source of protein. Biotechnology advances 25(2), 207-210.
Beyerinck, M.W. (1890) Culturversuche mit Zoochlorellen, Lichenengonidien und anderen niederen Algen. Botanische Zeitung 47, 725-739, 741-754, 757-768, 781-785.
Borowitzka, M.A. (1995) Microalgae as sources of pharmaceuticals and other biologically active compounds. Journal of applied phycology 7(1), 3-15.
Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72(1), 248-254.
Chen, C.W., Lee, H.-M. and Chang, M.B. (2008) Inactivation of aquatic microorganisms by low-frequency AC discharges. IEEE Transactions on Plasma Science 36(1), 215-219.
Chisti, Y. (2007) Biodiesel from microalgae. Biotechnology advances 25(3), 294-306.
Eing, C., Goettel, M., Straessner, R., Gusbeth, C. and Frey, W. (2013) Pulsed electric field treatment of microalgae—benefits for microalgae biomass processing. Plasma Science, IEEE Transactions on 41(10), 2901-2907.
Eliezer, S. and Eliezer, Y. (2001) The fourth state of matter: an introduction to plasma science, CRC Press.
Esper, B., Badura, A. and Rögner, M. (2006) Photosynthesis as a power supply for (bio-) hydrogen production. Trends in plant science 11(11), 543-549.
Fridman, A., Yang, Y. and Cho, Y.I. (2012) Plasma discharge in liquid: water treatment and applications, CRC press.
Gebicki, S. and Gebicki, J.M. (1993) Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. Biochemical journal 289(Pt 3), 743.

Gilpin, R.W., Dillon, S.B., Keyser, P., Androkites, A., Berube, M., Carpendale, N., Skorina, J., Hurley, J. and Kaplan, A.M. (1985) Disinfection of circulating water systems by ultraviolet light and halogenation. Water research 19(7), 839-848.
Goettel, M., Eing, C., Gusbeth, C., Straessner, R. and Frey, W. (2013) Pulsed electric field assisted extraction of intracellular valuables from microalgae. Algal Research 2(4), 401-408.
Goiris, K., Muylaert, K., Fraeye, I., Foubert, I., De Brabanter, J. and De Cooman, L. (2012) Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. Journal of applied phycology 24(6), 1477-1486.
Griffith, M. and Ewart, K.V. (1995) Antifreeze proteins and their potential use in frozen foods. Biotechnology advances 13(3), 375-402.
Guarnieri, M.T., Nag, A., Smolinski, S.L., Darzins, A., Seibert, M. and Pienkos, P.T. (2011) Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga. PLoS One 6(10), e25851.
Günerken, E., D'Hondt, E., Eppink, M., Garcia-Gonzalez, L., Elst, K. and Wijffels, R. (2015) Cell disruption for microalgae biorefineries. Biotechnology advances 33(2), 243-260.
Hao, X., Mattson, A.M., Edelblute, C.M., Malik, M.A., Heller, L.C. and Kolb, J.F. (2014) Nitric Oxide Generation with an Air Operated Non-Thermal Plasma Jet and Associated Microbial Inactivation Mechanisms. Plasma Processes and Polymers 11(11), 1044-1056.
Ikawa, S., Kitano, K. and Hamaguchi, S. (2010) Effects of pH on Bacterial Inactivation in Aqueous Solutions due to Low-Temperature Atmospheric Pressure Plasma Application. Plasma Processes and Polymers 7(1), 33-42.
Kim, J., Yoo, G., Lee, H., Lim, J., Kim, K., Kim, C.W., Park, M.S. and Yang, J.-W. (2013) Methods of downstream processing for the production of biodiesel from microalgae. Biotechnology advances 31(6), 862-876.
Koban, I., Holtfreter, B., Hübner, N.O., Matthes, R., Sietmann, R., Kindel, E., Weltmann, K.D., Welk, A., Kramer, A. and Kocher, T. (2011) Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro–proof of principle experiment. Journal of clinical periodontology 38(10), 956-965.
Kolb, J.F., Mattson, A.M., Edelblute, C.M., Hao, X., Malik, M.A. and Heller, L.C. (2012) Cold dc-operated air plasma jet for the inactivation of infectious microorganisms. Plasma Science, IEEE Transactions on 40(11), 3007-3026.
Kose, A. and Oncel, S. (2015) Properties of microalgal enzymatic protein hydrolysates: Biochemical composition, protein distribution and FTIR characteristics. Biotechnology Reports 6, 137-143.
Kredl, J., Drache, S., Quade, A., Polak, M., Muller, S., Peglow, S., Hippler, R. and Kolb, J.F. (2014) DC Operated Air Plasma Jet for Antimicrobial Copper Coatings on Temperature Labile Surfaces. IEEE Transactions on Plasma Science 10(42), 2756-2757.
Lackmann, J.-W., Wende, K., Verlackt, C., Golda, J., Volzke, J., Kogelheide, F., Held, J., Bekeschus, S., Bogaerts, A. and Schulz-von der Gathen, V. (2018) Chemical fingerprints of cold physical plasmas–an experimental and computational study using cysteine as tracer compound. Scientific reports 8(1), 7736.
Lackmann, J., Baldus, S., Steinborn, E., Edengeiser, E., Kogelheide, F., Langklotz, S., Schneider, S., Leichert, L., Benedikt, J. and Awakowicz, P. (2015) A dielectric barrier discharge terminally inactivates RNase A by oxidizing sulfur-containing amino acids and breaking structural disulfide bonds. Journal of Physics D: Applied Physics 48(49), 494003.
Langmuir, I. (1928) Oscillations in ionized gases. Proceedings of the National Academy of Sciences of the United States of America 14(8), 627.
Laroussi, M., Mendis, D. and Rosenberg, M. (2003) Plasma interaction with microbes. New Journal of Physics 5(1), 41.

Lee, A.K., Lewis, D.M. and Ashman, P.J. (2012) Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements. Biomass and bioenergy 46, 89-101.
Lee, A.K., Lewis, D.M. and Ashman, P.J. (2013) Force and energy requirement for microalgal cell disruption: an atomic force microscope evaluation. Bioresource Technology 128, 199-206.
Lichtenthaler, H.K. and Buschmann, C. (2001) Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. Current protocols in food analytical chemistry.
Locke, B., Sato, M., Sunka, P., Hoffmann, M. and Chang, J.-S. (2006) Electrohydraulic discharge and nonthermal plasma for water treatment. Industrial & engineering chemistry research 45(3), 882-905.
Locke, B.R. and Thagard, S.M. (2012) Analysis and review of chemical reactions and transport processes in pulsed electrical discharge plasma formed directly in liquid water. Plasma Chemistry and Plasma Processing 32(5), 875-917.
López, N., Puértolas, E., Condón, S., Álvarez, I. and Raso, J. (2008) Effects of pulsed electric fields on the extraction of phenolic compounds during the fermentation of must of Tempranillo grapes. Innovative Food Science & Emerging Technologies 9(4), 477-482.
Lu, X., Naidis, G., Laroussi, M., Reuter, S., Graves, D. and Ostrikov, K. (2016) Reactive species in non-equilibrium atmospheric-pressure plasmas: Generation, transport, and biological effects. Physics Reports 630, 1-84.
Luengo, E., Condón-Abanto, S., Álvarez, I. and Raso, J. (2014) Effect of pulsed electric field treatments on permeabilization and extraction of pigments from Chlorella vulgaris. The Journal of membrane biology 247(12), 1269-1277.
Luengo, E., Martínez, J.M., Coustets, M., Álvarez, I., Teissié, J., Rols, M.-P. and Raso, J. (2015) A Comparative Study on the Effects of Millisecond-and Microsecond-Pulsed Electric Field Treatments on the Permeabilization and Extraction of Pigments from Chlorella vulgaris. The Journal of membrane biology 248(5), 883-891.
Luengo, E. and Raso, J. (2016) Pulsed Electric Field-Assisted Extraction of Pigments from Chlorella vulgaris. Handbook of Electroporation, 1-16.
Lukes, P., Clupek, M., Babicky, V. and Sunka, P. (2008) Ultraviolet radiation from the pulsed corona discharge in water. Plasma Sources Science and Technology 17(2), 024012.
Malik, M.A., Ghaffar, A. and Malik, S.A. (2001) Water purification by electrical discharges. Plasma Sources Science and Technology 10(1), 82.
Mata, T.M., Martins, A.A. and Caetano, N.S. (2010) Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews 14(1), 217-232.
Mubarak, M., Shaija, A. and Suchithra, T. (2015) A review on the extraction of lipid from microalgae for biodiesel production. Algal Research 7, 117-123.
Mundt S, B.H., Preisitsc, M, Kretilow S, Bui H.T.N., Pham H.T, Zainuddin E, Le. T. T, Lukowski G, Jülich WD (2014) Microalgae – A promising source of novel therapeutics. JSM Biotechnol. Bioeng 2(1), 1.
Nagayama, K., Mori, Y., Shimada, K. and Nakahara, M. (2002) Shock Hugoniot compression curve for water up to 1 GPa by using a compressed gas gun. Journal of applied physics 91(1), 476-482.
Oehmigen, K., Hähnel, M., Brandenburg, R., Wilke, C., Weltmann, K.D. and Von Woedtke, T. (2010) The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids. Plasma Processes and Polymers 7(3-4), 250-257.
Pignolet, O., Jubeau, S., Vaca-Garcia, C. and Michaud, P. (2013) Highly valuable microalgae: biochemical and topological aspects. Journal of industrial microbiology & biotechnology 40(8), 781-796.
Postma, P., Miron, T., Olivieri, G., Barbosa, M., Wijffels, R. and Eppink, M. (2015) Mild disintegration of the green microalgae Chlorella vulgaris using bead milling. Bioresource Technology 184, 297-304.

Postma, P., Pataro, G., Capitoli, M., Barbosa, M., Wijffels, R.H., Eppink, M., Olivieri, G. and Ferrari, G. (2016) Selective extraction of intracellular components from the microalga Chlorella vulgaris by combined pulsed electric field–temperature treatment. Bioresource Technology 203, 80-88.
Ranjith Kumar, R., Hanumantha Rao, P. and Arumugam, M. (2015) Lipid extraction methods from microalgae: a comprehensive review. Frontiers in Energy Research 2, 61.
Reijnders, M.J., van Heck, R.G., Lam, C.M., Scaife, M.A., dos Santos, V.A.M., Smith, A.G. and Schaap, P.J. (2014) Green genes: bioinformatics and systems-biology innovations drive algal biotechnology. Trends in biotechnology 32(12), 617-626.
Richmond, A. (2008) Handbook of microalgal culture: biotechnology and applied phycology, John Wiley & Sons.
Sack, M., Sigler, J., Frenzel, S., Eing, C., Arnold, J., Michelberger, T., Frey, W., Attmann, F., Stukenbrock, L. and Mueller, G. (2010) Research on industrial-scale electroporation devices fostering the extraction of substances from biological tissue. Food Engineering Reviews 2(2), 147-156.
Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y. and Vaca-Garcia, C. (2014) Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. Renewable and sustainable energy reviews 35, 265-278.
Sarkisov, G., Zameroski, N. and Woodworth, J. (2006) Observation of electric field enhancement in a water streamer using Kerr effect. Journal of applied physics 99(8), 083304.
Sato, M., Ohgiyama, T. and Clements, J.S. (1996) Formation of chemical species and their effects on microorganisms using a pulsed high-voltage discharge in water. IEEE Transactions on Industry applications 32(1), 106-112.
Sava, I., Asandulesa, M., Zocher, K., Kruth, A., Kolb, J.F., Bodnar, W., Witte, K., Ishizaki, T. and Miron, C. (2018) Electrical and mechanical properties of polyimide films treated by plasma formed in water and isopropanol. Reactive and Functional Polymers.
Scandalios, J.G., Guan, L. and Polidoros, A.N. (1997) Catalases in plants: gene structure, properties, regulation, and expression. Cold Spring Harbor Monograph Series 34, 343-406.
Skjånes, K., Rebours, C. and Lindblad, P. (2013) Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. Critical reviews in biotechnology 33(2), 172-215.
Smith, J.B., Adams, I. and Ji, H.-F. (2017) Biomolecule Response to Nonthermal Plasma. 7(4), 427-443.
Sousa, I., Gouveia, L., Batista, A.P., Raymundo, A. and Bandarra, N. (2008) Microalgae in novel food products. Food chemistry research developments, 75-112.
Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A. (2006) Commercial applications of microalgae. Journal of bioscience and bioengineering 101(2), 87-96.
Staehelin, A. (1966) Die Ultrastruktur der Zellwand und des Chloroplasten von Chlorella. Zeitschrift für Zellforschung und Mikroskopische Anatomie 74(3), 325-350.
Sugiarto, A.T., Ito, S., Ohshima, T., Sato, M. and Skalny, J.D. (2003) Oxidative decoloration of dyes by pulsed discharge plasma in water. Journal of Electrostatics 58(1), 135-145.
Sun, B., Kunitomo, S. and Igarashi, C. (2006) Characteristics of ultraviolet light and radicals formed by pulsed discharge in water. Journal of Physics D: Applied Physics 39(17), 3814.
Sun, B., Sato, M., Harano, A. and Clements, J. (1998) Non-uniform pulse discharge-induced radical production in distilled water. Journal of Electrostatics 43(2), 115-126.
Sun, B., Xin, Y., Zhu, X., Gao, Z., Yan, Z. and Ohshima, T. (2018) Effects of shock waves, ultraviolet light, and electric fields from pulsed discharges in water on inactivation of Escherichia coli. Bioelectrochemistry 120, 112-119.
Šunka, P. (2001) Pulse electrical discharges in water and their applications. Physics of Plasmas (1994-present) 8(5), 2587-2594.

Sunka, P., Babický, V., Clupek, M., Lukes, P., Simek, M., Schmidt, J. and Cernak, M. (1999a) Generation of chemically active species by electrical discharges in water. Plasma Sources Science and Technology 8(2), 258.
Sunka, P., Babický, V., Clupek, M., Lukes, P., Simek, M., Schmidt, J. and Cernák, M. (1999b) Generation of chemically active species by electrical discharges in water. Plasma Sources Science and Technology 8(2), 258.
Takai, E., Kitamura, T., Kuwabara, J., Ikawa, S., Yoshizawa, S., Shiraki, K., Kawasaki, H., Arakawa, R. and Kitano, K. (2014) Chemical modification of amino acids by atmospheric-pressure cold plasma in aqueous solution. Journal of Physics D: Applied Physics 47(28), 285403.
UniProt Consortium (2016) UniProt: the universal protein knowledgebase.
Venketesh, S. and Dayananda, C. (2008) Properties, potentials, and prospects of antifreeze proteins. Critical reviews in biotechnology 28(1), 57-82.
Vogel, A., Busch, S. and Parlitz, U. (1996) Shock wave emission and cavitation bubble generation by picosecond and nanosecond optical breakdown in water. The Journal of the Acoustical Society of America 100(1), 148-165.
Weltmann, K.D., Kindel, E., von Woedtke, T., Hähnel, M., Stieber, M. and Brandenburg, R. (2010) Atmospheric-pressure plasma sources: Prospective tools for plasma medicine. Pure and Applied Chemistry 82(6), 1223-1237.
Wende, K., Lackmann, J.-W., Jablonowski, H., Stapelmann, K., von Woedtke, T. and Bekeschus, S. (2018) Can We Achieve Selectivity In Plasma Medicine? Clinical Plasma Medicine 9, 43.
Wu, J., Lin, L. and Chau, F.-t. (2001) Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. Ultrasonics sonochemistry 8(4), 347-352.
Zhang, J., Sun, Z., Sun, P., Chen, T. and Chen, F. (2014) Microalgal carotenoids: beneficial effects and potential in human health. Food & function 5(3), 413-425.
Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X. and Dou, C. (2011) Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Applied biochemistry and biotechnology 164(7), 1215-1224.
Zocher, K., Banaschik, R., Schulze, C., Schulz, T., Kredl, J., Miron, C., Schmidt, M., Mundt, S., Frey, W. and Kolb, J.F. (2016) Comparison of Extraction of Valuable Compounds from Microalgae by Atmospheric Pressure Plasmas and Pulsed Electric Fields. Plasma Medicine 6(3-4), 273-302.
Zocher, K., Lackmann, J.-W., Volzke, J., Steil, L., Lalk, M., Weltmann, K.-D., Wende, K. and Kolb, J.F. (2019) Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures. Algal Research 39, 101416.

Original Publications

The work presented in this thesis is based on the following peer-reviewed publications. The authors' contributions are briefly summarised in the following section:

Publication P1

Comparison of extraction of valuable compounds from microalgae by atmospheric pressure plasmas and pulsed electric fields

Zocher, K., Banaschik, R., Schulze, C., Schulz, T., Kredl, J., Miron, C., Schmidt, M., Mundt, S., Frey, W., & Kolb, J. F. (2016). *Plasma Medicine*, 6(3-4).

Own contribution: KZ designed and performed all experiments and conducted data analysis. The manuscript was written by KZ and edited by all co-authors.

Publication P2

Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures

Zocher, K., Lackmann, J. W., Volzke, J., Steil, L., Lalk, M., Weltmann, K. D., Wende, K. & Kolb, J. F. (2019). *Algal Research*, 39, 101416.

Own contribution: KZ designed and performed the experiments. Data analysis was conducted by KZ with the help of JL for proteomics data. The manuscript was written by KZ and edited by all co-authors.

Publication P3

Mechanism of Microalgae Disintegration by Spark Discharge Treatment for Compound Extraction
Zocher, K., Rataj, R., Steuer, A., Weltmann, K.-D. & Kolb, J. F. (2019), *Journal of Applied Physics: D, (submitted).*

Own contribution: KZ designed and performed the experiments together with RR and AS. Data analysis for atomic force microscopy (AFM) and shockwave pressure calculations were performed by AS and RR, respectively and assisted by KZ. The manuscript was written by KZ and edited by all co-authors.

Publication P4

Comparison of Pulsed Corona Plasma and Pulsed Electric Fields for the Decontamination of Water Containing Legionella Pneumophila as Model Organism

Banaschik, R., Burchhardt, G., Zocher, K., Hammerschmidt, S., Kolb, J.F. & Weltmann, K.-D. *Bioelectrochemistry*, 112 (2016) 83-90.

Own contribution: KZ evaluated the data analysis and performed electric field calculations. The manuscript was written by RB and KZ, and edited by all co-authors.

Confirmed

Date	Katja Zocher
Date	Prof. Michael Lalk

Publication P1

Comparison of extraction of valuable compounds from microalgae by atmospheric pressure plasmas and pulsed electric fields

Zocher, K., Banaschik, R., Schulze, C., Schulz, T., Kredl, J., Miron, C., Schmidt, M., Mundt, S., Frey, W., & Kolb, J. F. (2016). *Plasma Medicine*, 6(3-4).

Plasma Medicine, 6(3–4): 273–302 (2016)

Comparison of Extraction of Valuable Compounds from Microalgae by Atmospheric Pressure Plasmas and Pulsed Electric Fields

Katja Zocher,[a] Robert Banaschik,[a] Christian Schulze,[b] Tilo Schulz,[a] Jana Kredl,[a] Camelia Miron,[a] Michael Schmidt,[a] Sabine Mundt,[b] Wolfgang Frey,[c] & Juergen F. Kolb[a,*]

[a]Leibniz Institute for Plasma Science and Technology e.V. (INP Greifswald), 17489 Greifswald, Germany; [b]Institute of Pharmacy, Department of Pharmaceutical Biology, University of Greifswald, 17487 Greifswald, Germany; [c]Institute for Pulsed Power and Microwave Technology, Karlsruhe Institute of Technology, 76344 Eggenstein-Leopoldshafen, Germany

*Address all correspondence to: Juergen F. Kolb, Leibniz Institute for Plasma Science and Technology e.V. (INP Greifswald), Felix-Hausdorff-Strasse 2, 17489 Greifswald, Germany; Tel.: +49-3834-5543950; Fax: +49-3834-554301, E-mail: juergen.kolb@inp-greifswald.de

ABSTRACT: Microalgae have recently gained interest, not only as source for biofuel, but also as a resource for pharmaceutical and nutritional substances. The bottleneck for extracting these valuable compounds from microalgae is a thick cell wall of high physical and chemical strength. Several extraction techniques are available, but suffer from different disadvantages. Therefore, new technologies are needed, especially those based on processes that will not affect the chemical composition of ingredients. Among these, physical plasma and pulsed electric fields (PEF) might be promising. Three different standard methods, microwave, ultrasound, and homogenization, were compared with plasma treatment and PEF. The plasma sources investigated were corona discharges, a plasma jet, a dielectric barrier discharge, spark discharges, and pin-to-liquid discharges. *Chlorella vulgaris* was chosen as a model organism. To detect successful cell wall rupture, the protein content of the supernatant and pigment concentration after treatment were determined. Scanning electron microscope images were taken to visualize cell wall damage. Microwave and spark discharge treatment were the most successful methods with comparable extracted total protein content in the supernatant. However, spark discharges achieved higher pigment yield than microwave extraction without the thermal degradation of the pigments observed for microwave extraction.

KEY WORDS: extraction, plasma, pulsed electric field, spark discharge, microwave, microalgae

I. INTRODUCTION

Microalgae are ubiquitously occurring photosynthetic microorganisms that can be found in freshwater and marine systems and are known for their rich content of lipids, proteins, polysaccharides, and carotenoids.[1,2] Cultivation is possible for marginal economical costs and on any scale. Microalgae can be grown in fresh water and in brackish or saline water, resulting in a low land and water footprint.[3] *Chlorella vulgaris* is one of

1947-5764/16/$35.00

the most well-characterized microalga and was first isolated by Beijernick in the 1890s.[4] For centuries, these algae have been used in the Far East as alternative medicine and are known as a traditional food source in the Orient.[5] Many proteins and carotenoids have been identified in microalgae,[2,6] in particular, high-value compounds with antitumor and immune-stimulating properties. Polysaccharides such as β-1,3-glucan are linked to antiviral, antitumor, and immune protective activities. This polysaccharide is also known for its positive influence on blood glucose level in human body. Carotenoids in microalgae, such as lutein or zeaxanthin, act as free-radical scavengers and may neutralize radicals such as hydroxyl (OH) radicals, which are related to the development of neurodegenerative and cardiovascular diseases.[7]

A major obstacle for extracting compounds from microalgae is their strong and rigid cell wall, which is composed of complex carbohydrates and glycoproteins with a high physical and chemical strength.[8] Conventional extraction processes are often energy intensive and most suffer further from the use of environmentally harmful solvents and/or long treatment times and high energy demand.[9] Moreover, changes and modifications to extracted compounds are often inevitable due to the use of these chemicals or the need for high temperatures, resulting in a loss of bioactivity or, worse, their destruction. An economically viable technique for down-streaming these processes has yet to be found.

Various physical methods based on fast heating or mechanical damage have already been used for disintegration of plant material; these include microwave exposure, ultrasound (also known as sonication), and homogenization. Amarni and Kadi showed that the extraction of oil from olives with microwaves was highly efficient. The underlying mechanism was explained by an internal heating of the *in situ* water within the biological cell. The heating accelerates cell wall rupture by sudden temperature rise. This allows a fast dissolution of the oil, which is released from broken cells into the solvent.[10] Ultrasound also showed good efficiency for cell wall disruption of plant material due to cavitation phenomena. Wu et al. used ultrasound to enhance the yield of ginseng saponins compared with Soxhlet extraction. Remarkably, this enhancement was achieved with a threefold faster treatment time compared with the classical extraction method.[11] Nevertheless, for microalgae, especially *C. vulgaris*, ultrasound does not seem to be very effective and the combination with other mechanical techniques is thus recommended.[12]

Another method for cell wall rupture, homogenization, exploits the formation of shear forces, when a biological suspension is forced through a narrow tube under high pressure. The classical method, used in microbiology for cell disintegration, is only effective for a minority of different microalgae. Additionally, in large volumes, the method consumes high amounts of energy, which makes it costly.[8] In comparison, ball milling showed that high protein yields can be achieved with moderate expenditures of treatment energy.[13]

In addition to physical methods, chemical procedures are available to disintegrate the cell wall of microalgae. For the enzymatic lysis technique, enzymes such as snailase, lysozyme, or cellulase are added to the biological sample and hydrolyze cell walls with-

out altering compounds. Zheng et al. demonstrated that lipid extraction of *C. vulgaris* could be increased up to 22–24% when lysozyme or cellulase was added. They further suggested that enzymatic lysis can be seen as a gentle and selective method. However, long treatment times of 10 h and continuously stable temperatures are required for sufficient cell wall rupture.[14] Therefore, this method may be gentle, but is also highly time and energy consuming and therefore economically unsustainable.

Nonconventional physical methods such as pulsed electric fields (PEF) or high-voltage electrical discharges have shown to enhance extraction yield from microalgae. In general, these techniques were used together with solvents and, in conjunction, could decrease the use of solvents or treatment time.[15–19]

PEF of sufficient strength and duration cause charging of cell membranes, which eventually can lead to a transient increase of permeability across membranes or a loss of membrane integrity altogether. This process of reversible or irreversible electroporation is used for incorporating large molecules such as DNA or drugs into a cell, but it can be also used to increase the extraction yield of compounds from cells.[20–22]

Plasma may also provide mechanisms that are promising to break the strong and rigid cell wall of microalgae. Depending on method of plasma generation, different processes can be exploited. Generated in water or algal suspensions, corona discharges and spark discharges provide strong electric fields, shockwaves, ultraviolet radiation, and chemical reactive species such as the hydroxyl radical, peroxynitrite, or H_2O_2.[23] In gases, plasmas can be generated in different configurations such as dielectric barrier discharges (DBDs) or plasma jets.[24,25] Depending on the method used, high amounts of reactive oxygen and reactive nitrogen species are produced with air as the working gas. In contact with or immersed in aqueous solutions,short- and long-lived species are also formed in reactions with water.[26–28] The strong oxidizing potential of the created species can be expected to at least partially dissolve the cell walls of microalgae.

Bousetta et al. showed that the extraction of polyphenols from grape seeds was enhanced using pulsed arcs. In their experiments, they compared PEF, streamers, and pulsed arcs applied directly in water. Pulses of 0.8 μs were adjusted to provide an approximate pulse energy of 3–10 J/pulse. In configurations for PEF, field strengths of 20 and 40 kV/cm were applied. Pulse repetition rates varied for the three investigated methods. Pulsed arcs in a point-to-plane configuration (gap distance, 1–3 cm) were applied with a frequency of only 2 Hz because, at higher repetition rates, a strong increase in temperature was observed. In contrast, streamers (point-to-plane gap distance, 3 cm) and PEF (plane-to-plane gap distance, 1 cm) were administered with 20 Hz. It could be shown that, compared with the other methods, pulsed arcs required between 27 and 57 times less energy to extract the polyphenols from the seeds.[16] Similar results were reported by Rajha et al., who investigated the effects of PEF, high-voltage electrical discharges, and ultrasound for the extraction of polyphenol and proteins from vine shoots. PEF of about 13 kV/cm and pulse length of 10 μs, applied at a frequency of 0.5 Hz, were investigated. The same frequency and a voltage of 40 kV were set for high-voltage electrical discharge treatment. The plasma was generated in a needle-to-plate system. A

frequency of 20 kHz was stated for ultrasound treatment. Most efficient was the treatment with high-voltage electrical discharges, resulting in the highest extracted polyphenol content with the lowest energy use.[18]

These results are encouraging for the use of plasmas for the extraction of compounds from microalgae, which has so far not been investigated. The objective of the work presented here was therefore to determine whether plasma sources are a suitable method for this purpose and which plasma source is the most successful at achieving cell disintegration of *C. vulgaris*. Therefore, corona discharges generated in the algae suspension, a plasma jet submerged into the suspension, a DBD applied above the treatment volume, and spark discharges inside the treatment volume were investigated. The different plasma sources and methods provide different direct and indirect reaction mechanisms, including different reaction chemistries and production processes of reactive species. A suitable reference method had to be found to allow a comparison and microwave extraction, ultrasound (sonication), and high pressure homogenization were studied. In addition, PEF were also investigated for the experience from biomass processing. A protein assay was established to determine protein concentration in the supernatant associated with successful cell wall rupture. This assay does not distinguish between proteins from cell wall alone; it also detects proteins from inside the cell. Furthermore, pigments were extracted and their concentration was measured photometrically after cell disruption with the respective chosen method. An increase of these compounds could indicate successful cell disintegration. In addition, scanning electron microscope (SEM) images were taken for each treatment method. Together with the extraction yields, a preliminary evaluation of energy efficiencies was also conducted for the laboratory systems.

II. MATERIALS AND METHODS

A. Cell Culture

C. vulgaris (SAG 211-12, Experimental Phycology and Culture Collection of Algae, University of Goettingen, Germany) was grown in Bold's basal modified medium (Sigma-Aldrich, Germany), containing (in mg/L): boric acid 11.42, calcium chloride dihydrate 25.0, cobalt nitrate * $6H_2O$ 0.49, cupric sulfate * $5H_2O$ 1.57, EDTA (free acid) 50.0, ferrous sulfate * $7H_2O$ 4.98, magnesium sulfate * $7H_2O$ 75.0, manganese chloride * $4H_2O$ 1.44, molybdenum trioxide 0.71, nickel chloride * $6H_2O$ 0.003, potassium hydroxide 31.0, potassium hydroxide 31.0, potassium phosphate monobasic 175.0, potassium phosphate dibasic 75.0, sodium chloride 25.0, sodium nitrate 250.0, sodium selenite 0.002, stannic chloride 0.001, vanadium sulfate * $3H_2O$ 0.0022, and zinc sulfate * $7H_2O$ 8.82.

Cells were cultured according to Schulze et al. photoautotrophically in ventilated, 500 mL Schott flasks, bubbled with air with a flow rate of approximately 1.5 vvm. Temperature was kept at 20 ± 2°C and the culture was illuminated in light:dark cycles (12:12 h) with white fluorescence lamps (50 μmol photons $m^{-2}s^{-1}$).[29]

B. Sample Preparation

For experiments, cells were harvested after inoculation between day 30 and day 45 with an adjusted optical density (OD) of 0.8 at 750 nm (Shimadzu UV-1280 spectrophotometer) for a final volume of 50 mL. Subsequently, the algae suspension was centrifuged at 3000 × *g* (centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Germany) for 10 min, washed with 20 mL of deionized water, and centrifuged at 3000 × *g* for 5 more min. The cells were kept on ice until use.

Dry weight was determined with 20 mL algae suspension (OD 0.8) in a circulating air drying oven (Heraeus Oven UT12, ThermoScientific, Germany). Experiments were repeated in triplicate.

C. Standard Methods

To determine protein yield in the supernatant and for the extraction of pigments, the algae suspensions were treated as described below with standard methods, five plasma sources, and PEF treatment, respectively. After these initial treatments, proteins and pigments were extracted as described under the "Measurement of protein content" and "Pigment extraction" sections.

1. Microwave Extraction

After washing and centrifugation, the supernatant was discarded and the cells were refilled with 3 mL deionized water, transferred into 4 mL brown-glass vials (Agilent Technologies, Germany), and closed. The cap was prepared with a septum, which allowed pressure balance. Subsequently, the vial was placed inside the microwave (Bosch, distributed by Carl Roth, Germany) and extraction was conducted at 600 W for 20 s. Afterward, the suspension was allowed to cool down to ambient temperature, refilled to 50 mL, and centrifuged for further investigations.

2. Ultrasound Extraction (Sonication)

The cell suspension was prepared as described above and filled with deionized water to a final volume of 50 mL. To avoid thermal heating due to cavitation, the extraction was conducted under ice cooling with a treatment time of 20 min and a frequency of 20 kHz (Sonopuls HD 2070 Bandelin, Germany). Subsequently, algae were centrifuged for further investigations.

3. Homogenization

After preparing the cell suspension, the liquid with a volume of 50 mL was transferred into the homogenization apparatus (EmulsiFlex-C5, Avestin, Canada). The pressure was adjusted to 5000 psi and treatment time was 8 min. One minute of treatment time ac-

counts for one cycle; therefore, the algae were pressurized in eight cycles. Afterward, the cells were prepared for further investigations as described above.

D. Plasma and PEF Treatment

1. Corona Discharges

For corona discharge treatment, a coaxial electrode geometry was used that is described in more detail by Banaschik et al.[30,31] Two pure, uncoated, twisted tungsten wires of 0.05 mm diameter (W-005135/13, Goodfellow, Huntingdon, UK) were fixed in the middle of a glass tube as inner electrodes. The twisted wires were chosen for a more durable electrode configuration during the application of discharges. A stainless steel mesh, fixed on the inner wall of the tube, served as theouter electrode. The glass tube had a length of 70 mm and an inner diameter of 34 mm, thus containing a total volume of 64 mL. High-voltage pulses were generated with a 300 ns stacked Blumlein line pulse generator with a repetition rate of 11 Hz. Voltage was measured with a 120 kV/80 MHz high-voltage probe (PMV-5, NorthStar Marana, AZ) and current with a current monitor (Model 5046, Pearson Electronics, Palo Alto, CA). Electrical parameters were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY).

The suspension was moved in a continuous flow system with a peristaltic pump (FH100x, Thermo Scientific, Waltham, MA) to avoid settling of algae in the system. In addition, the suspension was chilled during the whole treatment time to prevent temperature increase. Algae were prepared as described above and, after centrifugation, the pellet was diluted to 140 mL to fill the entire system properly. To generate long streamers, a conductivity of 70 μS/cm was necessary, which was adjusted with sodium chloride. A voltage of 80 kV was applied and treatment time was set to 30 min. After treatment, the solution was centrifuged and the cell pellet was prepared for pigment extraction. Samples from the supernatant were taken for protein measurements.

2. DC-Plasma Jet

Whether algal cell walls are affected by a plasma jet exposure was determined using a DC-plasma jet as described by Kolb et al. and Kredl et al.[32–34] In this setup, plasma is ignited within a microhollow cathode geometry. The inner electrode is made of brass and the outer electrode consists of alumina. Plasma is generated between the inner and outer electrode when a positive voltage of 2 kV is applied to the discharge circuit by a DC high-voltage power supply (PS FX06R50, Glassman High Voltage Inc., High Bridge, NJ, USA). For experiments, the discharge current was adjusted to 30 mA and the treatment time was set to 30 min. Compressed air was used as the operating gas, flowing through the inner electrode with a flow rate of 4 standard litre per minute (slm). A total of 50 mL of algae suspension with OD_{750} = 0.8 was treated. During the treatment, the algal suspension was chilled on ice.

3. Volume DBD (VDBD)

To observe cell wall rupture caused by indirect plasma treatment, a DBD plasma was applied as described by Koban et al.[35] The VDBD consisted of two metal electrodes, a Petri dish (55 × 14.2 mm, VWR International, Germany), and a titanium disc, which was placed between them. The bottom of the Petri dish was used as dielectric for the DBD. The distance between electrode and dish was 15 mm. The entire system was sealed against entry of ambient air and cooled with a Peltier element. Argon was used as the feed gas with a flow rate of 500 standard cubic centimetres per minute (sccm). Total treatment time was set to 10 min with an input voltage of 10 kV and a frequency of 33.8 kHz (AG Series Amplifier Type AG1021, T&C Power Conversion Inc., NY, USA). Forward power was set to 27 W and reflected power to 18 W. Cells were prepared as described above and the algae pellet diluted to 1 mL with deionized water. After treatment, the cell pellet was refilled with distilled water to its start volume of 50 mL for further investigations.

4. Pin-to-Liquid Discharge

As a further plasma source for the generation of reactive species, a pin-to-liquid discharge setup similar to that described by Chen et al. was used for algae treatment.[36] After centrifugation, the algae pellet was diluted to a total volume of 90 mL; otherwise, the gap between the liquid and the needle electrode would have been too large for the given setup and no plasma could have been ignited. The solution was treated with an AC-driven pin-to-liquid electrode configuration placed on top of a beaker and treatment time was set to 3 min. The electrode configuration consisted of two stainless steel electrodes. An AC transformer (F.A.R.T. 5000/75Pe), controlled by a variac (RFT LTS 002), charged the electrodes. One electrode was submerged into the liquid and the other was positioned about 3 mm above the surface of the solution. Due to considerable heating from the sparks, a treatment time longer than 3 min was not feasible. Temperatures more than 25°C were achieved within 3 min and a longer treatment time would have destroyed the algae near the surface. To ensure convection and a uniform distribution of plasma-generated species, the liquid was stirred with a magnetic stirrer (VMS-C4, VWR, Germany). Temperature was controlled with a handheld infrared thermometer (PeakTech 4975, PeakTech Prüf- und Messtechnik GmbH, Germany) pointed at the liquid surface in a 30 s cycle. After the treatment, the liquid was centrifuged and prepared for further measurements.

5. Spark Discharges

For spark discharge treatment, a chamber with a rod-to-rod-configuration was constructed. The cylindrical body of the chamber consisted of polymethyl-methacrylate and an outlet at the bottom and top of the chamber (Fig. 1). Outlets were connected with plastic tubes to a peristaltic pump (Meredos TL, Medorex, Germany). This continuous flow sys-

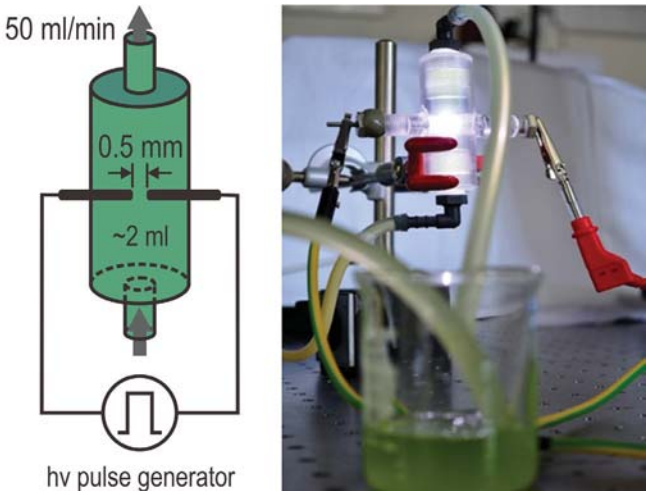

FIG. 1: Spark discharge chamber (left: schematic, right: setup) for the recirculating treatment of microalgae suspensions.

tem ensures homogenous treatment and avoids settling of algae at the bottom of the treatment chamber. The whole system, including tubes and expansion tank, held a total volume of 50 mL with the spark-discharge reactor accommodating approximately 2 mL. The flow rate was adjusted to 50 mL/min to ensure a treatment of the complete suspension in 1 min. Rectangular to the direction of suspension flow, two tungsten electrodes with a diameter of 2.4 mm and gap distance of 0.5 mm were set inside the chamber. Positive high voltage pulses were generated with a Blumlein line generator, delivering high-voltage pulses of 100 ns duration at a spark repetition rate of 4 Hz for an input voltage of about 6–7 kV from a DC high-voltage power supply (PS/EQ060R020-22, Glassman High Voltage Inc., High Bridge, NJ, USA). With the low conductivity of the algal suspension, the overall electrical resistance of the filled treatment chamber was much higher than the impedance of the pulse generator of 400 Ω. By refraining to match pulse generator and reactor impedance, a train of 100 ns high-voltage pulses with decreasing amplitudes was applied between the electrodes, with the first pulse reaching an amplitude of about 80 kV (instead of only a single pulse of only 40 kV amplitude).[37] A typical waveform is displayed in Fig. 2. The repetitively applied high electric fields eventually result in a breakdown, usually between the fourth and eighth applied pulse. The remaining energy, which is stored in the pulse generator, is then dissipated in the instigated spark discharge. The short pulse duration corresponds to a maximum energy of only 1.6 J.

Voltage was measured with a 40 kV/75 MHz high voltage probe (Tektronix P6015A, Tektronix Inc., Beaverton, OR) and current with a Pearson current monitor (Model 5046, Pearson electronics, Palo Alto, CA). Both measurements were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). Experiments were conducted within a treatment time of 30 min and temperature was controlled with

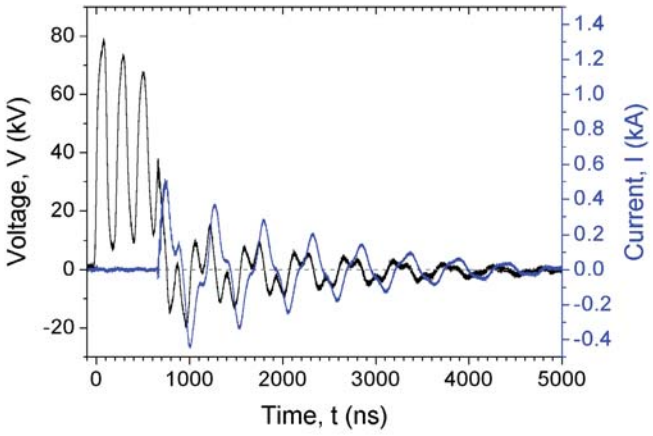

FIG. 2: Current and voltage for the spark discharge treatment. Because the resistance of the treatment chamber filled with microalgae is not matched to the impedance of the Blumlein pulse-forming network, several reflected pulses are applied across the discharge gap before a breakdown occurs and a spark is initiated.

a hand-held infrared thermometer (PeakTech 4975, PeakTech Prüf- und Messtechnik GmbH, Germany) pointed at the expansion reservoir and spark discharge chamber. For each experiment, 50 mL of algae suspension with OD_{750} = 0.8 was prepared as described in the "Sample preparation" section.

6. PEF Treatment

For PEF treatment, the algae suspension was treated in a polytetrafluoroethylene (PTFE) treatment chamber as described by Goettel et al. and Eing et al.[17,38] The same continuous-flow system with a flow rate of 50 mL/min as described for spark discharges was used to avoid settling of algae and to allow air bubbles to exit the chamber. Two stainless steel electrodes with a diameter of 32 mm were paired in parallel and separated by a gap distance of 4 mm in the PTFE housing. The system was connected to a 1 µs pulse-forming network (PFN) that consisted of 16 capacitors (paired in parallel) with 2 nF, a spark gap switch, and an impedance of 480 kΩ. The PFN delivers square pulses with a flat amplitude and a rise time of 0.13 µs into a matched load. One thousand pulses with an amplitude of the first unmatched pulse of 14 kV were administered (resulting in a field strength of 35 kV/cm) at a frequency of 5.5 Hz (power supply Glassman High Voltage Inc., High Bridge, NJ, USA). Voltage was measured with a 40 kV/75 MHz high voltage probe (Tektronix P6015A, Tektronix Inc., Beaverton, OR) and current was measured with a Pearson monitor (Model 5046, Pearson Electronics, Palo Alto, CA). These parameters were recorded with an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). Because the pulse generator impedance and load impedance of the treatment chamber were not the same (matched), a train of pulses with decreasing amplitude

similar to the system described in the "Spark discharges" section were applied without any breakdown. A typical voltage waveform is shown in Fig. 3.

All exposure parameters were adapted from Goettel et al. and Eing et al.[17,38] For all other experiments, 50 mL of algae suspension with OD_{750} = 0.8 was prepared as described above and treated.

All investigated methods with their essential treatment parameters are described in Tables 1 and 2.

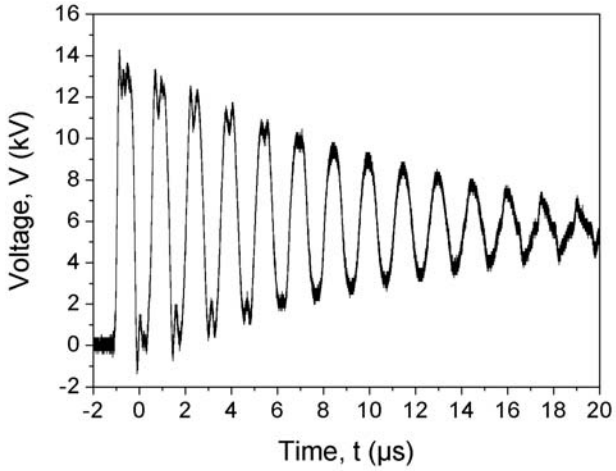

FIG. 3: Typical voltage waveform for the PEF system. The impedance of the Blumlein line pulse-forming network is much lower than the resistance of the PEF treatment chamber. Therefore, a train of 1 µs pulses with decreasing amplitudes is an applied instead of a single pulse.

TABLE 1: Summary of treatment parameters for standard extraction methods

Standard Method	Parameters	Treatment Time
Microwave	600 W	20 s
Ultrasound (sonication)	20 kHz	20 min
Homogenization	5000 PSI	8 min

TABLE 2: Summary of treatment parameters for plasma methods and PEF exposure

Plasma	Setup	Treatment Time
Corona discharges	Coaxial, directly in suspension; recirculation system	30 min
Plasma jet	DC jet; operated with air; submerged in suspension	30 min
VDBD	Above suspension (15mm)	10 min
Pin-to-liquid discharge	From pin to suspensions; distance 3 mm	3 min
Spark discharges	Rod-to-rod discharge (gap distance 0.5 mm) inside suspension; recirculation system	30 min
PEF	1 MS, 35 KV/CM, 5.5 HZ	1000 pulses

E. Measurement of Protein Content

To prove a successful rupture of the cell wall, the protein content in the supernatant was measured with the Bradford assay.[39] An increase of protein content can be used as a marker for efficient disintegration of microalgal cell walls. Therefore, after plasma treatment, the algae suspension was centrifuged at 3000 × *g* for 5 min. Then, 1 mL of supernatant was mixed with 1 mL of Bradford assay reagent (ThermoFisher Scientific, Germany) and measured at 595 nm with a UV/VIS spectrophotometer (Evolution 300, ThermoFischer Scientific, Madison, WI). Bovine standard serum albumin was used for calibration.

F. Pigment Extraction

Pigments such as chlorophylls and carotenoids are located inside chloroplasts, which are protected by the strong cell walls of microalgae. Therefore, an increase in concentration of these substances in the extract can be used as a marker for successful cell wall rupture. Extracted pigments were gained according to Luengo et al.[19,40] After centrifugation at 3000 × *g* for 5 min, the supernatant was discarded and 100 µL of treated or untreated algae (control) pellet suspension was mixed with 96% ethanol (V/V, Carl Roth, Germany) and thoroughly vortexed. Subsequently, the mixture was incubated in the dark for 20 min at room temperature and centrifuged for 2 min at 21 × *g* (Heraeus Pico21, ThermoScientific, Germany). The absorbance of the supernatant was measured at 450, 649, and 664 nm against a 96% ethanol blank. Calculations of total carotenoid, chlorophyll *a*, and chlorophyll *b* were conducted with the following equations:[41]

$$\text{Chlorophyll } a \text{ (Ca) in µg/mL: } (13.36 * A664) - (5.19 * A649)$$

$$\text{Chlorophyll } b \text{ (Cb) in mg/mL: } (27.43 * A649) - (8.12 * A664)$$

$$\text{Total carotenoids in µg/mL: } (1000 * A470 - 2.13 * Ca - 97.64 * Cb)/209$$

G. pH Measurements

Because acidification of the algae suspension may occur due to radical formation during plasma treatment, the pH was measured before and after plasma treatment and, for completeness, also for PEF treatment.[42] The liquid was analyzed with a pH meter (S-20 SevenEasy, Mettler Toledo, Giessen, Germany).

H. SEM

Damage to cell walls was also investigated directly by SEM (PhenomPro, PhenomWorld, Netherlands). For analysis, 10 µL of treated algae suspension was placed on a pin stub mount with a double-sided adhesive patch (PLANO GmbH, Wetzlar, Germany), dried with nitrogen, and transferred into the microscope.

I. Statistical Analysis

Experiments were done in triplicate if not stated otherwise. Means and standard deviations (SDs) of data were calculated. Error bars in all figures correspond to SD.

III. RESULTS

All algae samples had a volume of 50 mL with an OD (measured at 750 nm) of 0.8. Determination of extracted protein and pigment yields was acquired after treatment with standard methods, plasma, and PEF treatment, respectively, by the procedures as described in the "Measurement of protein content" and "Pigment extraction" sections. Experiments were conducted over a longer period of time, so different growth phases of algae affected the results. Pigment concentration in particular can vary for different growth phases. Accounting for differences in cultures and the effect of growth phases, algae cultures were analyzed individually; for each culture, 100 µL of algae suspension was extracted with 1 mL of 96% ethanol and extraction yields compared between microwave treatment results and their corresponding untreated control. Protein content was always determined with 1 mL of the supernatant, which corresponded to a total volume of 50 mL. Dry weight was determined to 0.1167 g/L (SD = 0.0287 g/L).

A. General Effects

In this work, the efficiency of three different standard extraction methods, five different plasma sources, and PEF treatment for rupturing algae cell wall are described. With the chosen model organism, a sufficient cell wall rupture could be achieved with microwave extraction and treatment with a spark discharge plasma source. In comparison, no satisfying rupture could be achieved with homogenization and ultrasound treatment. It could be shown that spark discharge treatment with a frequency of 4 Hz in a contentious flow system was as effective as microwave treatment or even better for pigment extraction. For all other plasma sources and PEF treatments, no effect or a smaller effect compared with microwave was observed.

B. Protein Extraction

The measurement of protein in the supernatant after treatment with different methods was chosen as a marker for successful cell wall rupture. Therefore, a protein assay was used. From the absorption at 595 nm, the relative protein concentration in the supernatant was determined. Results were related to dry weight (0.1167 g/L).

Figure 4 shows the protein content in the supernatant for the extraction by standard methods. With ultrasound, a protein content of 0.4% was achieved. Comparing homogenization and microwave treatment, microwave yielded more than twice the amount (5.9%) compared with homogenization (2.5%).

Protein extraction yield after plasma treatment or PEF treatment is shown in Fig. 5. Corona discharges, plasma jet, VDBD, pin-to-liquid discharge, and PEF achieved less

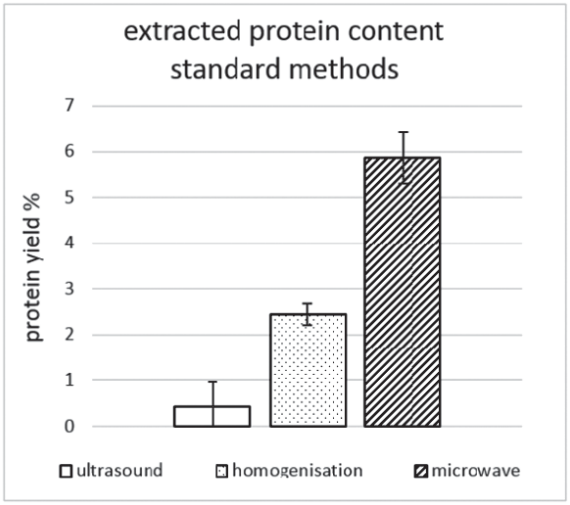

FIG. 4: Protein concentration (%) related to dry weight in supernatant after treatment with ultrasound, homogenization, and microwave. Each bar describes mean values from three independent experiments. Error bars indicate standard deviation (SD).

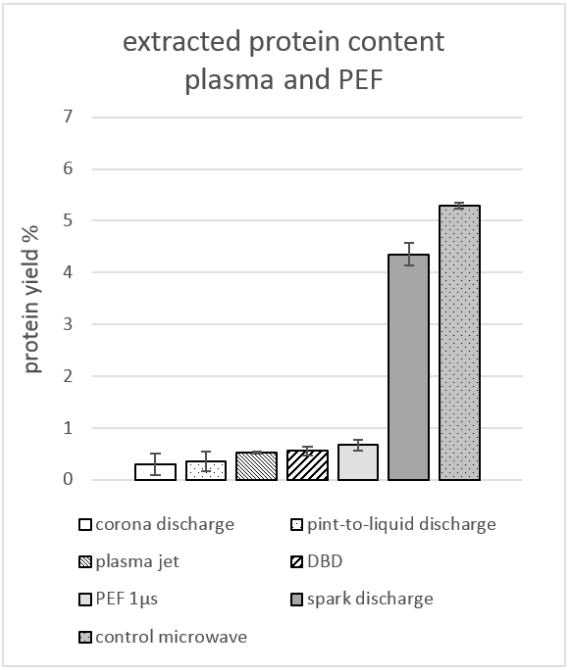

FIG. 5: Protein content (%) related to dry weight, in supernatant after plasma treatment with five different plasma sources and PEF. Each column represents mean values from three or 12 independent experiments (microwave n = 12). Error bars indicate SD.

than 1% protein in amount of the equivalent dry weight, whereas with the microwave standard, more than 5% protein was achieved. However, spark discharges are nearly as effective as microwave treatments. With this plasma source, operated at a spark frequency of 4 Hz, a protein yield of 4.35% was obtained.

C. Pigment Extraction

Pigments are located in the thylakoids inside the chloroplast, which is protected by the cell wall. An increase of chlorophyll *a* and *b* and total carotenoids in the extract therefore indicates successful cell wall rupture. In addition to the comparison with microwave extraction, results were also compared with an untreated control: a 100 µL *C. vulgaris* pellet suspension was extracted with ethanol 96% without any pretreatment. These experiments were conducted to estimate whether plasma had any effect at all due to growth-phase-dependent pigment content inside the algae. To account for variations due to individual cultures and growth phases, results for treatments are described with respect to this untreated control. All results are expressed as pigment concentration in mg/g culture$_{(dw)}$ (dw: dry weight). In figures, chlorophyll *a* is represented by "Chl a," chlorophyll *b* content by "Chl b," total carotenoid content by "TC."

1. Standard Methods

Results for the amount of extracted pigments after treatment with ultrasound, homogenization, or microwave exposure are shown in Fig. 6. As shown for the protein content, microwave extraction is the most effective technique in contrast to ultrasound and homogenization. The chlorophyll *a* content for ultrasound treatment is 1.317 mg/g culture$_{(dw)}$, for homogenization 1.935 mg/g culture$_{(dw)}$. An approximately 4- to 7-fold higher content was obtained with microwave treatment (8.613 mg/g culture$_{(dw)}$). Similar results can be shown for chlorophyll *b* and total carotenoid content. A yield of chlorophyll *b* of 2.680 mg/g culture$_{(dw)}$ and total carotenoid of 3.162 mg/g culture$_{(dw)}$ could be achieved. Only 0.686 mg/g culture$_{(dw)}$ for ultrasound and 0.855 mg/g culture$_{(dw)}$ for homogenization could be measured for chlorophyll *b*. In addition, only a small amount of carotenoids of 0.607 mg/g culture$_{(dw)}$ could be extracted with ultrasound and of 0.925 mg/g culture$_{(dw)}$ with homogenization.

2. Corona Discharges

Corona discharges were ignited directly in the algae suspension in a wire cylinder geometry. The results of pigment extraction are shown in Fig. 7. The amount of extracted pigments is increased compared with the untreated control. Nevertheless, the pigment amount extracted with microwaves is higher. In particular, the total carotenoid content is nearly four times higher (microwave 4.494 mg/g culture$_{(dw)}$, corona discharges 0.946 mg/g culture$_{(dw)}$). However, chlorophyll *b* content was 0.601 mg/g culture$_{(dw)}$: higher for corona discharges than microwave extraction. No positive effect for corona

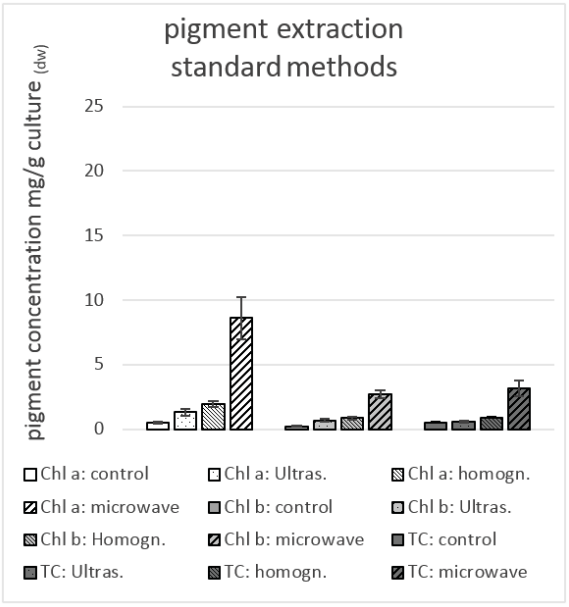

FIG. 6: Pigment concentration (mg/g culture$_{(dw)}$) after ultrasound (Ultras.), homogenization (homogn.), and microwave treatment. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

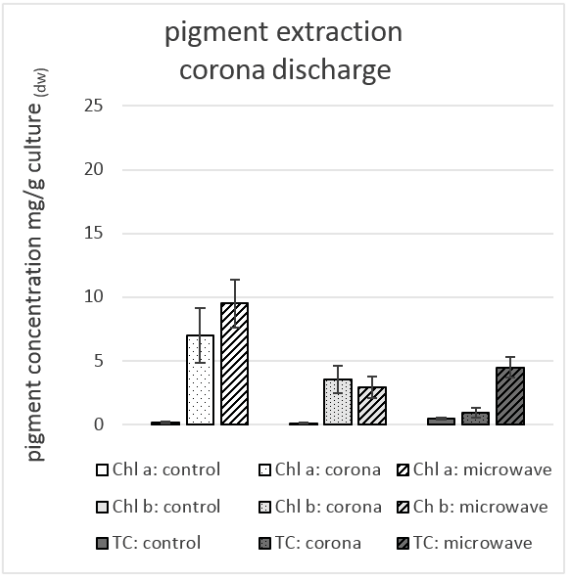

FIG. 7: Pigment concentration (mg/g culture$_{(dw)}$) after corona discharge treatment in a wire cylinder geometry as described in the "Corona discharges" section in the text. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

discharge treatment could be observed for extraction of chlorophyll *a*, either. The content for corona discharge was measured with 7.024 mg/g culture$_{(dw)}$. For microwave treatment an amount of 9.531 mg/g culture$_{(dw)}$ was determined.

3. Plasma Jet

The effect of plasma on pigment extraction generated with an air-driven plasma jet that was immersed in the treatment volume for 30 min was determined. Figure 8 shows the pigment yield after plasma jet treatment compared with microwave extraction and untreated control. Only a minor effect was observed when the algae suspension was treated with the plasma jet. Chlorophylls *a* and *b* extracted with microwave (15.350 mg/g culture$_{(dw)}$ and 4.566 mg/g culture$_{(dw)}$) were considerably higher than for the plasma jet-treated suspensions (0.842 mg/g culture$_{(dw)}$ and 0.210 mg/g culture$_{(dw)}$, respectively). A similar result could be shown for total carotenoid content extracted with microwaves and plasma jet (5.718 mg/g culture$_{(dw)}$ and 1.587 mg/g culture$_{(dw)}$, respectively).

4. Volume-DBDs

Microalgae suspensions were exposed for 10 min to the plasma of a VDBD and its effluents. The results (Fig. 9) show that this kind of plasma has no distinct effect on extracted pigment content compared with microwave extraction. The extracted amounts

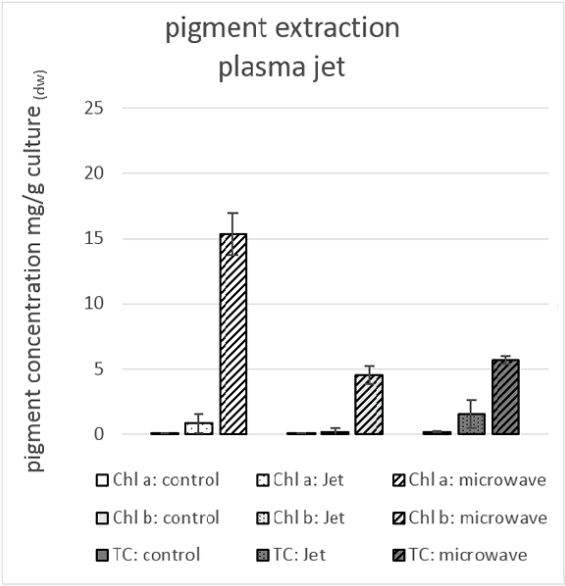

FIG. 8: Pigment concentration (mg/g culture$_{(dw)}$) after treatment with a plasma jet (Jet) as described in the "DC-plasma jet" section of the text. Each bar describes mean values of three independent experiments. Error bars indicate SD.

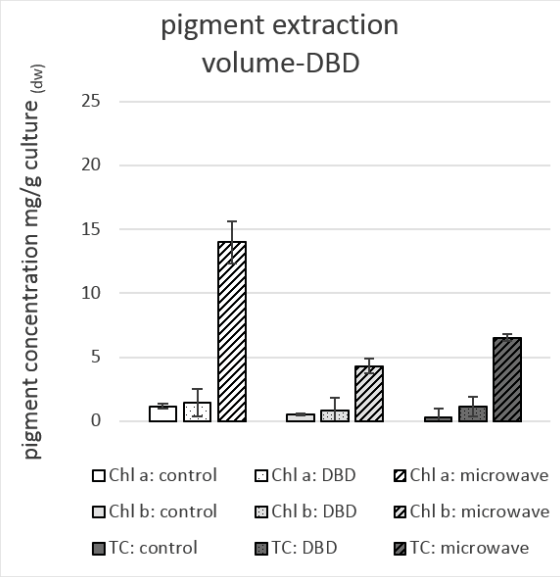

FIG. 9: Pigment concentration (mg/g culture$_{(dw)}$) after VDBD treatment as described in the "VDBD" section of the text. Each bar describes mean values from three and two independent experiments (n = 3, microwave n = 2). Error bars indicate SD.

after VDBD treatment are in a similar range as untreated control. Total carotenoid content after microwave treatment was 6.535 mg/g culture$_{(dw)}$ compared with 1.160 mg/g culture$_{(dw)}$ achieved after plasma treatment.

5. Pin-to-Liquid Discharge

In Fig. 10, the pigment concentration required after pin-to-liquid discharge treatment is shown. Contrary to the low protein content, the pigment concentration after this plasma treatment is increased compared with untreated control. However, the chlorophyll *a* and total carotenoid contents gained with the microwave method are still higher than those achieved with this plasma source. For microwave extraction, the total carotenoid content (3.069 mg/g culture$_{(dw)}$) is three times higher than pin-to-liquid discharge (1.021 mg/g culture$_{(dw)}$). Chlorophyll *a* is also 2 mg/g culture$_{(dw)}$ higher for the microwave treatment (6.836 mg/g culture$_{(dw)}$) than for the plasma treatment (4.809 mg/g culture$_{(dw)}$). Nevertheless, like corona discharges, the chlorophyll *b* content was increased after plasma treatment compared with microwave (3.124 mg/g culture$_{(dw)}$ and 2.17 mg/g culture$_{(dw)}$, respectively).

6. Spark Discharge

A remarkable rise in pigment concentration could be shown for a plasma treatment with spark discharges. The extracted pigment amounts are shown in Fig. 11. Nota-

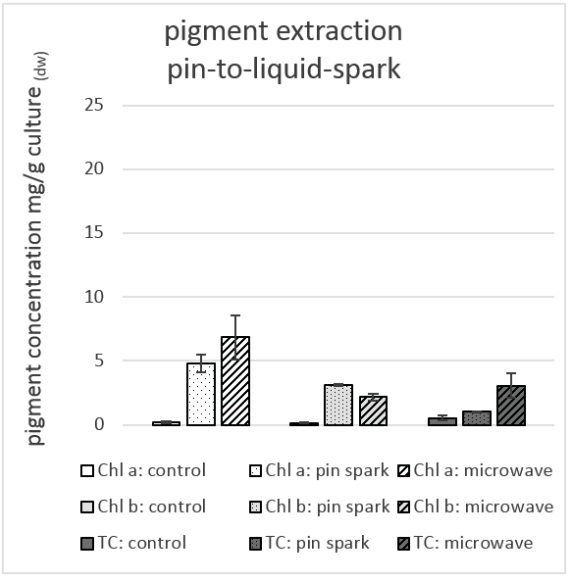

FIG. 10: Pigment concentration (mg/g culture$_{(dw)}$) after pin-to-liquid treatment (pin spark) as described in 2.4.4 of the text compared with microwave extraction and untreated control. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

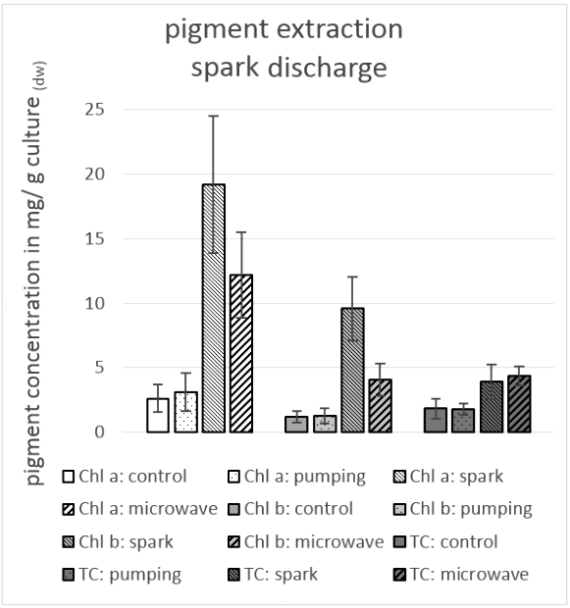

FIG. 11: Pigment concentration (mg/g culture$_{(dw)}$) after spark discharge treatment as described in the "Spark discharges" section of the text compared with microwave extraction. Each bar describes mean values from six or three independent experiments (n = 6, pumping alone n = 3). Error bars indicate SD.

ble, chlorophyll *a* content is increased 1.6-fold compared with microwave extraction (19.204 mg/g culture$_{(dw)}$ and 12.207 mg/g culture$_{(dw)}$, respectively). An increase in chlorophyll *b* can be also be observed for spark discharge treatment (9.583 mg/g culture$_{(dw)}$) compared with 4.058 mg/g culture$_{(dw)}$ for microwave. Furthermore, a comparable yield of total carotenoids after plasma treatment (3.952 mg/g culture$_{(dw)}$) was obtained compared with microwave extraction (4.386 mg/g culture$_{(dw)}$). To determine the influence of the continuous flow system, pigment extraction was conducted after 30 min of circulating the algae suspension through the system without applying any sparks. This did not result in an increased extraction yield compared to control, so algae cells seem to be unaffected by movement (results displayed as "pumping" in the diagram).

7. PEF Treatment

Pigment concentrations after PEF treatment were also determined and are shown in Fig. 12. Chlorophyll *a* and *b* extraction yields (0.280 mg/g culture$_{(dw)}$ and 0.249 mg/g culture$_{(dw)}$, respectively) were nearly the same as achieved for untreated control samples (0.249 mg/g culture$_{(dw)}$ and 0.275 mg/g culture$_{(dw)}$, respectively). A total carotenoid concentration of 0.726 mg/g culture$_{(dw)}$ for PEF-treated samples were obtained compared with the smaller amount for total carotenoid concentration of 0.468 mg/g culture$_{(dw)}$ for the untreated algae suspension. In contrast, after microwave treatment, a chlorophyll *a* content of 9.727 mg/g

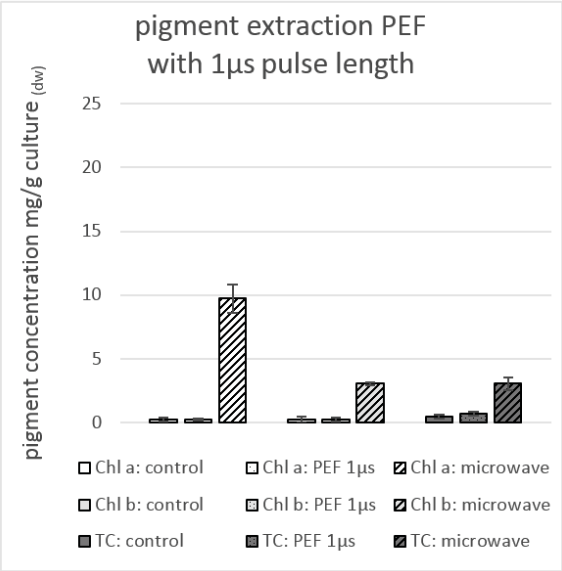

FIG. 12: Pigment concentration (mg/g culture $_{(dw)}$) after PEF treatment as described in the "PEF" section of the text. Each bar describes mean values from three independent experiments (n = 3). Error bars indicate SD.

culture$_{(dw)}$ and a chlorophyll *b* content of 3.051 mg/g culture$_{(dw)}$ were obtained. A total carotenoid concentration of 3.871 mg/g culture$_{(dw)}$ could be measured as well.

D. Changes in pH Values

Plasmas have also been investigated with respect to the generation of reactive species that might have the potential to promote the rupture of cell walls, for example, through oxidative processes. Accordingly, a drop in pH values of the directly and indirectly treated aqueous suspensions can be observed. Individual experiments were therefore accompanied by measurements of pH values. Start values slightly differ depending on the respective microalgae culture. The end values for different plasma treatments and PEF exposures are presented in Table 3. Corona discharges and the plasma jet showed only a very slight effect on pH value: a drop from 6.00 to 5.05 for corona discharges and a small increase from 7.01 to 7.10 for the submerged plasma jet. In contrast, for the DBD and the spark discharge, a drop of 1.41–2.00 could be determined. With the pin-to-liquid discharge, the highest decrease in pH value was measured, indicating a strong reaction chemistry. A difference of 3.65 between the start pH value (6.73) and the end pH value (3.08) was achieved. In comparison, no significant change in pH value was detected for PEF treatment; only a minor decrease from 7.50 to 7.30 was observed.

TABLE 3: pH measurements before and after plasma and PEF treatments

	Corona Discharge	Plasma Jet	VDBD	Spark Discharge	Pin-to-Liquid	PEF
Start	6.00	7.01	6.07	6.77	6.73	7.50
End	5.50	7.10	4.66	4.77	3.08	7.30

E. SEM images

To verify potential cell damage by different methods, SEM images were taken. Images for microwave treatment, spark discharge treatment, and untreated algae are shown in Fig. 13. Usually, *C. vulgaris* has a round shape and a smooth surface, which can be seen in Fig. 13*a*. If an algae suspension is exposed to microwave radiation, a single hole on the cell surface can be observed (Fig. 13*b*). In comparison, the spark discharge treated algae appear to be wizened and some are deflated completely (Fig. 13*c*). In both cases, the cell wall is apparently ruptured; however, different mechanisms seem to be responsible. All other tested methods (i.e., homogenization, ultrasound, corona discharges, plasma jet, VDBD, pin-to-liquid discharges, and PEF) showed no differences between untreated control and treated samples (respective images are therefore not shown).

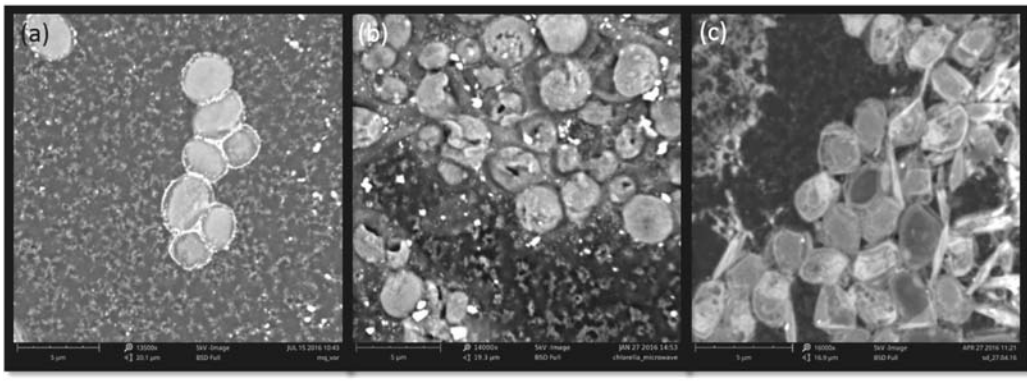

FIG. 13: SEM images of C. vulgaris before any treatment (a), after microwave treatment (b), and after spark discharge treatment (c).

IV. DISCUSSION

The objective of this work was a comparison of selected standard extraction methods, novel plasma methods, and PEF with respect to improving extraction yields of valuable compounds from microalgae. Therefore, five different plasma sources and PEF treatment were chosen and compared with treatments with ultrasound, homogenization, and microwave exposure. Different physical and chemical reaction pathways of plasmas have been exploited to different degrees by the different methods.

For the applied standard methods, the mechanisms of cell wall disruption are understood. When cells are treated with ultrasound waves, cavitation phenomena occur, leading to cell rupture. During this cavitation process, microbubbles are formed, which expand in the rarefaction phase of the sound wave. Subsequently, the bubbles are jolted in the compression phase, resulting in a collapse of these microbubbles. Collapsed microbubbles release a shock wave that propagates throughout the liquid; therefore, a high amount of sonic energy is converted into mechanical energy.[43] A disadvantage of the method is the associated considerable temperature increase due to cavitation. Therefore, chilling of the suspension is necessary to prevent heating and destruction of components.[8,44] We could observe that sonication of *C. vulgaris* has only a minor effect on cell disruption, supposedly due to its remarkable strong, rigid cell wall. No major increase in extracted protein content and pigment concentration could be determined. The effect of sonication and homogenization on cell disintegration for lipid extraction from *C. vulgaris* was investigated previously by Park et al.[12] For conditions similar to our experiments, a frequency of 20 kHz and 20 min treatment time, they could likewise not observe significant cell wall rupture. In addition, they could not find a significant effect for homogenization. According to Park et al., it is likely that cavitation bubbles and induced shockwaves, which occur under sonication processes, fail to disrupt the three thick and strong layers of the cell wall for this type of algae.[12] This assessment was

confirmed by Lee et al., who tested different disruption methods on *C. vulgaris*. Sonication with a frequency of 10 kHz and a treatment time of 5 min had only a minor effect on the disruption of microalgae.[45]

The underlying mechanisms of homogenization of biological cells are strong shear forces. The cell suspension of a certain volume is compressed in a small chamber and pressed through a narrow gap, resulting in cavitation processes. The sudden increase of energy in a small volume results in strong shear stress and, therefore, cell disintegration is possible.[3] In agreement with the results of Park et al., a sufficient cell wall rupture with homogenization could not be achieved in our experiments. Only a low increase of protein content in the supernatant and of pigment concentration was determined. It is likely that, not only the rigid cell walls of *C. vulgaris*, but also its small round shape is responsible for insufficient cell disintegration. Probably, the round shape of these microalgae enables it to resist high pressure changes. However, higher pressures might be more effective.

A strong increase of protein content in the supernatant and pigment concentration was achieved with microwave extraction. When comparing different cell rupture techniques, Lee et al. found that microwave extraction seems to be most suitable to break the cell walls of *C. vulgaris*. They could show that the extracted lipid content was highest (10% w/w) compared with sonication, bead-beating, autoclaving, and osmotic shock.[45] Microwave exposure causes migration of ions and rotation of dipoles. These molecular motions result in a rapid heating of the algae suspensions, particularly of water molecules inside the cells. The temperature increase results in a fast expansion of cells until the wall is ruptured and the cell content is released into the adjacent solvent.[46, 47] This might explain the single hole that was observed on the cell wall surface in the SEM images. Apparently, water inside the cells has been heated rapidly. With the associated increase in pressure, cell and cell wall are eventually ruptured in a weak spot, releasing content through the hole, thus effectively bursting open from the inside. It should be pointed out that the rather high temperatures related to microwave treatment can simultaneously have an adverse effect on sensible compounds such as proteins, chlorophylls, and carotenoids. Accordingly, a loss in activity of these compounds may occur. The harmful temperature effects of microwave exposure in our experiments can be seen in Fig. 14. The color of the pigment extract was yellowish-green compared with the lush green color gained with spark discharges and the light green of untreated control extract.

Standard extraction methods in general are usually based on mechanical damage, fast heating, or chemical reactions such as oxidation. The disadvantages of these methods include insufficient cell wall disintegration, long treatment times, a need for harsh solvents, or thermal degradation of compounds. With the investigated plasma exposures, it might be able to avoid or at least alleviate these shortcomings by exploiting other mechanisms. Plasmas have been shown to provide mechanical forces and oxidizing chemical species such as hydroxyl radicals, H_2O_2, or peroxynitrite.[23,42,48,49] Depending on the specific type of plasma source, different pathways and reaction chemistries are available.[31,32,42,50,51] Therefore, five different plasma sources generating different types of plasmas and thus different

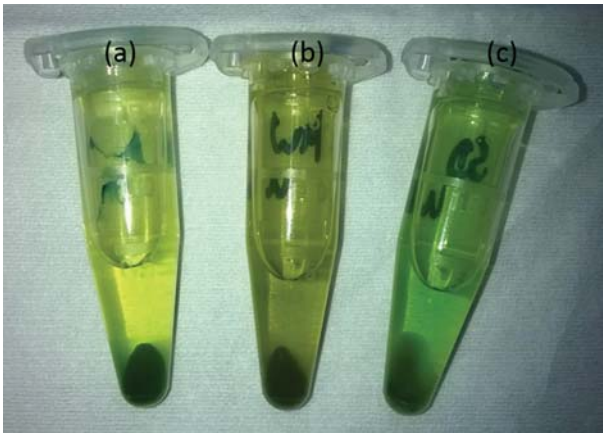

FIG. 14: Color images of extracted pigments for untreated control (a), after microwave exposure (b), and after spark discharge treatment (c). The dark objects at the bottom of the tubes are the centrifuged algae pellets.

physical and chemical characteristics were tested to determine which effect may be the reason for successful cell wall rupture. A common indication for the different processes is changes in pH values. The specific reaction chemistry for all plasma sources has been described thoroughly elsewhere.[31,32,52–55]

A decrease of pH values after a 10 min treatment time was found for VDBD to indicate the generation of chemical reactive species such as reactive nitrogen species, which recombine to nitric acid, leading to liquid acidification. In addition, the generation of H_2O_2 due to reactive oxygen species can be found for such a DBD system.[51,55] In contrast, plasma generated by the investigated jet had no influence on pH values, which stayed constant during the treatment time. However, from previous studies, it is known that in particular peroxynitrites are formed in aqueous solutions.[28] However, for both methods, the exposure of the cells to shear forces seems to be unlikely. Altogether, the experiments show that the chemistry provided by either VDBD or plasma jet are not sufficient to affect the cell wall. This has also been confirmed by SEM images (data not shown).

No drop of pH value was also measured for the application of corona discharges. A detailed study conducted by Banaschik et al. has shown that copious amounts of OH radicals and subsequently H_2O_2 are generated in this system.[31] The provided OH reaction chemistry does not seem to have an effect on the cell wall of *C. vulgaris*. In particular, because the total carotenoid content in the supernatant was lower than with microwave extraction and cell wall rupture was not detected, the generated shockwaves in the system do also not seem to be strong enough for the disintegration of *C. vulgaris*. That chemistry and shockwaves have no effect on cell walls can be seen in the SEM images (data not shown). This (in comparison) higher extraction yield could be attributed to PEF. In a previous work, we could show that a very strong electric field is established in the close vicinity of the wire electrode at the center of the reactor with and without the generation of corona

discharges. A calculated field strength directly around the wire (of 50 μm in diameter) of approximately 2.7 MV/cm was achieved for an applied voltage of 80 kV.[56] Therefore, a cell wall rupture could not be achieved, but a pore formation due to the electric field might occur. This electroporation might further explain the low protein content of the supernatant, but an increase of chlorophyll concentration compared with microwave extraction. Luengo et al. could demonstrate that pulses in the microsecond and millisecond range can increase pigment concentration compared with untreated microalgae. For example, a field strength of 20 kV/cm for a pulse length of 3 μs and a treatment time of 75 μs resulted in an increase of extraction yields for carotenoids and chlorophylls (*a* and *b*) of 42%, 54%, and 195%, respectively, when extraction was conducted after 1 h of preincubation. It was also described that no significant effect could be observed when extraction was conducted immediately after PEF treatment. Luengo et al. suggested that, because pigments are located in thylakoids inside the chloroplast, these compounds first have to cross the chloroplast membrane and afterward the cell membrane.[19,40] The treatment time in this work was set to 30 min, so a retarded chloroplast membrane poration may facilitate compound transport outside the cell.

The possibility of increasing the extraction yield from *C. vulgaris* by electroporation has motivated the experiments with PEF alone. The exposure showed no change in pH value because the generation of oxidizing species was not expected and only physically induced damage is expected. In this work, no effect on the cell wall by the applied PEF could be detected, as can be seen from the low protein content in the supernatant. The results are in agreement with previous observations by Postma et al. In their work, it could be shown that permeabilization of algal cell wall due to PEF treatment was not effective enough to release high quantities of large molecules such as proteins.[57] SEM images showed no influence of PEF on cell wall structure either, which confirms previous findings for the cell walls of bacteria.[21] Moreover, the pigment concentration stayed in the same range as for the untreated control, supporting that a cell wall destruction due to PEF treatment, as was also discussed by Günerken et al., seems unlikely.[3] Taking into account overall volume and continuous flow rate of the setup used in this work, about 58 pulse trains with a pulse length of individual pulses of 1 μs and a field strength of up to 35 kV/cm were applied to each algae cell while circulating through the treatment chamber during the treatment time. For the application of 50 pulses with a pulse length of 3 μs and a maximum field strength of 25 kV/cm, an increased pigment yield was observed by Luengo et al.[19] However, for our treatment conditions, we found no significantly higher concentrations. In addition to the longer pulse durations used by Luengo et al., which should, however, be compensated in part by the application of pulse trains in our case, differences might also be due to differences in experimental procedures. Pigment extraction was conducted immediately after PEF treatment as for plasma experiments. However, as Luengo et al. and also Goettel et al. suggest in their work, 1–2 h between PEF application and extraction might be necessary to achieve efficient yields of compounds from algae.[19,38] However, neither these experiments nor ours suggest a rupturing of cell walls.

The highest change of pH values was measured for the pin-to-liquid discharge, indicating a strong reaction chemistry. Before the plasma was switched on, the algae suspension had a pH value of 6.73, but after treatment, the pH value was 3.08. The formation of high amounts of nitrogen radicals such as peroxynitrites and other nitrogen oxides are the main reason for the drop of pH values.[42] However, the formation of these radical species seem not to be effective in rupturing cell walls either, because only a low protein content was measured after treatment. In addition, the SEM images showed no ruptured cells. Few cells appear dented slightly, but not ruptured. Interestingly, the chlorophyll content in the supernatant similar to that after the microwave treatment was observed. Again, the reason might be the electroporation of the cell membrane underneath the cell wall. Bruggeman et al. determined electric fields of 49.5 kV/cm in a comparable configuration, although operated by a DC voltage.[58] The curvature of our electrodes was even smaller, so the electric fields were higher. At least near the electrodes, an efficient electroporation process could be established, even for the AC voltage operation of the discharge. At an operating frequency of 50 Hz, the respective pulse duration given by half waves and pulse numbers within 3 min of treatment time are expected to be sufficient. A considerable disadvantage of the method, regardless, is the temperature increase in the suspension, which will degrade heat-sensitive pigments and other compounds.

The highest extraction yields with respect to both proteins and carotenoids were obtained for spark discharge treatments. Responsible for the results could be the reaction chemistry, but were more likely the strong and fast shockwaves from the rapid high-voltage breakdown for the short applied high voltage pulses. Before plasma application, the suspension had a pH value of 6.77 and after treatment a pH value of 4. Although the radical chemistry for our system has not been determined yet, a wide range of radicals seem to be generated. Comparable pH values were reported by Sugiarto et al., when the decoloration of dyes by pulsed discharges in water were investigated.[59] The formation of radicals in a plasma system depends on whether the spark or arc discharges are generated directly in water or if the high voltage electrode is placed above the water. Spark discharges in water have shown to produce OH, H, and O radicals and H_2O_2.[53,60,61] Therefore, produced species are similar to the once observed for corona discharges generated in water. However, Sun et al. found that spark discharges produce even higher amounts of OH radicals than streamer discharges due to a higher plasma density, although in a smaller volume.[62] Regardless, the similarities in reaction chemistries of spark discharge treatments and corona discharge treatments, together with the lack of enhanced extraction yields observed for the latter, are a strong indication that oxidation processes are not responsible for the results observed for the spark discharge treatment. In summary, an active influence of radicals generated by plasma on disintegration of cell walls seems unlikely.

Corona discharges and spark discharges are also known for the formation of shockwaves, providing another conceivable mechanism responsible for cell wall rupture. Sugiarto and Sato have investigated the degradation of phenol with streamer and spark discharges and concluded that spark discharges are more effective in particular for a

small treatment volume. In their work, they argue that this is due to a single strong plasma channel in the spark discharge, whereas the energy is dissipated among multiple paths in a corona discharge. Accordingly, a much stronger shockwave is formed during the propagation of the plasma channel. In this plasma channel, radical formation could also be enhanced.[63] Locke et al. describe in their work that streamer corona discharges form relatively weak shock waves compared with spark discharges.[61] Bousetta et al. and Rajha et al. observed that shockwaves are most likely the major mechanism for the structural damage to vine roots and grape seeds that are treated by pulsed arcs. As a result, polyphenol content is higher compared with PEF treatment or streamer discharge exposure.[16,18]

Because spark plasmas are associated with high temperatures, it is necessary to consider possible temperature effects that are associated with the treatment. In this work, a spark repetition rate of 4 Hz was found, a reasonable compromise with respect to cell wall rupture at moderate temperature increase. In this case, temperature could be held at around 25°C without any chilling system. The suspension maintained a lush green color compared with the thermal damage by microwave treatment, which resulted in similar extraction yields (Fig. 14). Decreasing the repetition rate of spark discharges to 2 Hz (for the same treatment time), protein content in the supernatant was half the one achieved with microwave treatment. Conversely, a frequency of 7 Hz resulted in a higher protein yield than microwave exposures, but temperatures rose above 32°C and algae suspension changed its dark green color to greyish-green (data not shown), indicating thermal degradation and thus loss of valuable components.

In general, spark discharges with a suitable frequency seem to be efficient for cell wall rupture of microalgae, yet are a gentle method to extract thermosensitive compounds such as pigments. Whether formed radicals have an influence on the extracted components in any way will have to be investigated in more detail. Further studies also need to focus on electrode geometries and the generation process of shockwaves by high-voltage pulses of different duration and amplitude.

V. CONCLUSION

In this work, the efficiency of three different standard extraction methods, including microwave treatment, five different plasma treatments, and PEF exposure for rupturing the cell walls of *C. vulgaris* are described. It could be shown that microwave extraction was best suitable for comparison with highest extraction yields. However, disadvantages are very high temperatures and a low scalability. Valuable, but heat-sensitive compounds may be destroyed and an economic large-scale implementation is difficult. Plasma sources, providing strong shock waves, were found to be a potential alternative. Plasma sources with only strong reaction chemistries proved to have a very small effect on cell walls, resulting in small extraction yields. Likewise, PEF alone are inefficient. Spark discharges applied with a frequency of 4 Hz achieved extraction yields similar to microwave exposures, but without thermal degradation of compounds. Details of mechanisms, effect on extracted compounds, and energy demand have to be investigated in future studies.

ACKNOWLEDGMENTS

The authors thank Rüdiger Titze from the Leibniz Institute of Plasma Science and Technology, Greifswald, for help with the VDBD and Mandy Schmidt from the University Greifswald for help with algae culture.

REFERENCES

1. Wijffels RH, Barbosa MJ, Eppink MH. Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioproducts and Biorefining. 2010;4:287–295.
2. Pignolet O, Jubeau S, Vaca-Garcia C, Michaud P. Highly valuable microalgae: biochemical and topological aspects. Journal of Industrial Microbiology & Biotechnology. 2013;40:781–796.
3. Günerken E, D'Hondt E, Eppink M, Garcia-Gonzalez L, Elst K, Wijffels R. Cell disruption for microalgae biorefineries. Biotechnology Advances. 2015;33:243–260.
4. Gouveia L, Batista AP, Miranda A, Empis J, Raymundo A. Chlorella vulgaris biomass used as colouring source in traditional butter cookies. Innovative Food Science & Emerging Technologies. 2007;8:433–436.
5. Sousa I, Gouveia L, Batista AP, Raymundo A, Bandarra N. Microalgae in novel food products. In: Konstantinos N, Papadopoulos PP, editors. Food chemistry research developments. New York: Nova Science Publishers; 2008. p. 75–112.
6. Mundt S, Bui HT, Preisitsc M, Kretilow S, Bui HT, Pham HT, Zainuddin E, Le TT, Lukowski G, Jülich WD. Microalgae: A promising source of novel therapeutics. Biotechnology & Bioengineering. 2014;2:1032.
7. Zhang J, Sun Z, Sun P, Chen T, Chen F. Microalgal carotenoids: beneficial effects and potential in human health. Food & Function. 2014;5:413–425.
8. Kim J, Yoo G, Lee H, Lim J, Kim K, Kim CW, Park, Min S, Yang JW. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol Adv. 2013;31:862–876.
9. Grima EM, Fernández FA, Medina AR. Downstream processing of cell-mass and products. In: Grima EM, Fernández FA, Medina AR, editors. Handbook of microalgal culture: biotechnology and applied phycology. New York: Wiley; 2004. p. 267–309.
10. Amarni F, Kadi H. Kinetics study of microwave-assisted solvent extraction of oil from olive cake using hexane: comparison with the conventional extraction. Innovative Food Science & Emerging Technologies. 2010;11:322–327.
11. Wu J, Lin L, Chau F. Ultrasound-assisted extraction of ginseng saponins from ginseng roots and cultured ginseng cells. Ultrasonics Sonochemistry. 2001;8:347–352.
12. Park JY, Lee K, Choi SA, Jeong MJ, Kim B, Lee JS, Oh YK. Sonication-assisted homogenization system for improved lipid extraction from Chlorella vulgaris. Renewable Energy. 2015;79:3–8.
13. Postma P, Miron T, Olivieri G, Barbosa M, Wijffels R, Eppink M. Mild disintegration of the green microalgae Chlorella vulgaris using bead milling. Bioresource Technology. 2015;184:297–304.
14. Zheng H, Yin J, Gao Z, Huang H, Ji X, Dou C. Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Applied Biochemistry and Biotechnology. 2011;164:1215–1224.
15. Boussetta N, Vorobiev E, Deloison V, Pochez F, Falcimaigne-Cordin A, Lanoisellé JL. Valorisation of grape pomace by the extraction of phenolic antioxidants: Application of high voltage electrical discharges. Food Chemistry. 2011;128:364–370.
16. Boussetta N, Lesaint O, Vorobiev E. A study of mechanisms involved during the extraction of polyphenols from grape seeds by pulsed electrical discharges. Innovative Food Science & Emerging Technologies. 2013;19:124–132.
17. Eing C, Goettel M, Straessner R, Gusbeth C, Frey W. Pulsed electric field treatment of microal-

gae: benefits for microalgae biomass processing. IEEE Transactions on Plasma Science. 2013;41: 2901–2907.
18. Rajha HN, Boussetta N, Louka N, Maroun RG, Vorobiev E. A comparative study of physical pretreatments for the extraction of polyphenols and proteins from vine shoots. Food Research International. 2014;65:462–468.
19. Luengo E, Condón-Abanto S, Álvarez I, Raso J. Effect of pulsed electric field treatments on permeabilization and extraction of pigments from Chlorella vulgaris. Journal of Membrane Biology. 2014;247:1269–1277.
20. Schoenbach KH, Peterkin FE, Alden III RW, Beebe SJ. The effect of pulsed electric fields on biological cells: experiments and applications. IEEE Transactions on Plasma Science. 1997;25:284–292.
21. Qin S, Timoshkin IV, Maclean M, Wilson MP, MacGregor SJ, Given MJ, Anderson JG, Wang T. Pulsed electric field treatment of microalgae: inactivation tendencies and energy consumption. IEEE Transactions on Plasma Science, 2014; 42(10):3191–3196.
22. Kotnik T, Frey W, Sack M, Meglič SH, Peterka M, Miklavčič D. Electroporation-based applications in biotechnology. Trends Biotechnol. 2015;33:480–488.
23. Sunka P, Babický V, Clupek M, Lukes P, Simek M, Schmidt J, Cernak M. Generation of chemically active species by electrical discharges in water. Plasma Sources Science and Technology. 1999;8: 258–265.
24. Conrads H, Schmidt M. Plasma generation and plasma sources. Plasma Sources Science and Technology. 2000;9:441–454.
25. Laroussi M, Akan T. Arc-free atmospheric pressure cold plasma jets: a review. Plasma Processes and Polymers. 2007;4:777–788.
26. Malik MA, Ghaffar A, Malik SA. Water purification by electrical discharges. Plasma Sources Science and Technology. 2001;10:82–91.
27. Laroussi M. Low temperature plasma-based sterilization: overview and state-of-the-art. Plasma Processes and Polymers. 2005;2:391–400.
28. Hao X, Mattson AM, Edelblute CM, Malik MA, Heller LC, Kolb JF. Nitric oxide generation with an air operated non-thermal plasma jet and associated microbial inactivation mechanisms. Plasma Processes and Polymers. 2014;11:1044–1056.
29. Schulze C, Wetzel M, Reinhardt J, Schmidt M, Felten L, Mundt S. Screening of microalgae for primary metabolites including β-glucans and the influence of nitrate starvation and irradiance on β-glucan production. Journal of Applied Phycology. 2016;28:2719–2725.
30. Banaschik R, Koch F, Kolb JF, Weltmann KD. Decomposition of pharmaceuticals by pulsed corona discharges in water depending on streamer length. IEEE Transactions on Plasma Science. 2014;42: 2736–2737.
31. Banaschik R, Lukes P, Jablonowski H, Hammer MU, Weltmann KD, Kolb JF. Potential of pulsed corona discharges generated in water for the degradation of persistent pharmaceutical residues. Water Research. 2015;84:127–135.
32. Kolb JF, Mattson AM, Edelblute CM, Hao X, Malik MA, Heller LC. Cold dc-operated air plasma jet for the inactivation of infectious microorganisms. IEEE Transactions on Plasma Science. 2012;40: 3007–3026.
33. Kredl J, Drache S, Quade A, Polak M, Müller S, Peglow S, Hippler R, Kolb JF. DC operated air plasma jet for antimicrobial copper coatings on temperature labile surfaces. IEEE Transactions on Plasma Science. 2014;10:2756–2757.
34. Kredl J, Kolb JF, Schnabel U, Polak M, Weltmann K-D, Fricke K. Deposition of antimicrobial copper-rich coatings on polymers by atmospheric pressure jet plasmas. Materials. 2016;9:274.
35. Koban I, Holtfreter B, Hübner NO, Matthes R, Sietmann R, Kindel E, Weltmann K- D, Welk A, Kramer A, Kocher T. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs in vitro: proof of principle experiment. Journal of Clinical Periodontology. 2011;38:956–965.

36. Chen CW, Lee H-M, Chang MB. Inactivation of aquatic microorganisms by low-frequency AC discharges. IEEE Transactions on Plasma Science. 2008;36:215–219.
37. Kolb JF, Scully N, Schoenbach KH, Dilip P. System and method for applying plasma sparks to tissue. U.S. Patent No.8,709,006. 2014 Apr. 29.
38. Goettel M, Eing C, Gusbeth C, Straessner R, Frey W. Pulsed electric field assisted extraction of intracellular valuables from microalgae. Algal Research. 2013;2:401–408.
39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72:248–254.
40. Luengo E, Martínez JM, Coustets M, Álvarez I, Teissié J, Rols M-P, Raso J. A comparative study on the effects of millisecond-and microsecond-pulsed electric field treatments on the permeabilization and extraction of pigments from Chlorella vulgaris. Journal of Membrane Biology. 2015;248:883–891.
41. Lichtenthaler HK, Buschmann C. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. Current Protocols in Food Analytical Chemistry. 2001;F4.3.1–F4.3.8.
42. Lukes P, Dolezalova E, Sisrova I, Clupek M. Aqueous-phase chemistry and bactericidal effects from an air discharge plasma in contact with water: evidence for the formation of peroxynitrite through a pseudo-second-order post-discharge reaction of H_2O_2 and HNO_2. Plasma Sources Science and Technology. 2014;23:015019.
43. Chisti Y, Moo-Young M. Disruption of microbial cells for intracellular products. Enzyme and Microbial Technology. 1986;8:194–204.
44. Safi C, Zebib B, Merah O, Pontalier P-Y, Vaca-Garcia C. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. Renewable and Sustainable Energy Reviews. 2014;35:265–278.
45. Lee JY, Yoo C, Jun SY, Ahn CY, Oh HM. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technology. 2010;101:S75–SS7.
46. Pare J J R. Microwave extraction of volatile oils. U.S. Patent No. 5,338,557. 1994 Aug. 16.
47. Camel V. Recent extraction techniques for solid matrices: supercritical fluid extraction, pressurized fluid extraction and microwave-assisted extraction: their potential and pitfalls. Analyst. 2001;126:1182–1193.
48. Lukes P, Clupek M, Babicky V, Janda V, Sunka P. Generation of ozone by pulsed corona discharge over water surface in hybrid gas–liquid electrical discharge reactor. J Phys D Appl Phys. 2005;38:409–416.
49. Bruggeman P, Leys C. Non-thermal plasmas in and in contact with liquids. J Phys D Appl Phys. 2009;42:053001.
50. Bruggeman P, Liu J, Degroote J, Kong MG, Vierendeels J, Leys C. Dc excited glow discharges in atmospheric pressure air in pin-to-water electrode systems. J Phys D Appl Phys. 2008;41:215201.
51. Oehmigen K, Hähnel M, Brandenburg R, Wilke C, Weltmann K-D, von Woedtke T. The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids. Plasma Processes and Polymers. 2010;7:250–257.
52. Liu F, Sun P, Bai N, Tian Y, Zhou H, Wei S, Zhou, Y, Zhang, J, Zhu W, Becker K. Inactivation of bacteria in an aqueous environment by a direct-current, cold-atmospheric-pressure air plasma microjet. Plasma Processes and Polymers. 2010;7:231–236.
53. Miron C, Bratescu M, Saito N, Takai O. Time-resolved optical emission spectroscopy in water electrical discharges. Plasma Chemistry and Plasma Processing. 2010;30:619–631.
54. Ikawa S, Kitano K, Hamaguchi S. Effects of pH on bacterial inactivation in aqueous solutions due to low-temperature atmospheric pressure plasma application. Plasma Processes and Polymers. 2010;7:33–42.
55. Oehmigen K, Winter J, Hähnel M, Wilke C, Brandenburg R, Weltmann K-D, von Woedtke, T. Estimation of possible mechanisms of Escherichia coli inactivation by plasma treated sodium chloride solution. Plasma Processes and Polymers. 2011;8:904–913.
56. Banaschik R, Burchhardt G, Zocher K, Hammerschmidt S, Kolb JF, Weltmann K-D. Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing Legionella pneumophila as model organism. Bioelectrochemistry. 2016;112:83–90.

57. Postma P, Pataro G, Capitoli M, Barbosa M, Wijffels RH, Eppink M, Olivieri G, Ferrari G. Selective extraction of intracellular components from the microalga Chlorella vulgaris by combined pulsed electric field–temperature treatment. Bioresource Technology. 2016;203:80–88.
58. Bruggeman P, Graham L, Degroote J, Vierendeels J, Leys C. Water surface deformation in strong electrical fields and its influence on electrical breakdown in a metal pin-water electrode system. J Phys D Appl Phys. 2007;40:4779–4786.
59. Sugiarto AT, Ito S, Ohshima T, Sato M, Skalny JD. Oxidative decoloration of dyes by pulsed discharge plasma in water. Journal of Electrostatics. 2003;58:135–145.
60. Sato M, Ohgiyama T, Clements JS. Formation of chemical species and their effects on microorganisms using a pulsed high-voltage discharge in water. IEEE Transactions on Industry Applications. 1996;32:106–112.
61. Locke B, Sato M, Sunka P, Hoffmann M, Chang J-S. Electrohydraulic discharge and nonthermal plasma for water treatment. Industrial & Engineering Chemistry Research. 2006;45:882–905.
62. Sun B, Sato M, Harano A, Clements J. Non-uniform pulse discharge-induced radical production in distilled water. Journal of Electrostatics. 1998;43:115–126.
63. Sugiarto AT, Sato M. Pulsed plasma processing of organic compounds in aqueous solution. Thin Solid Films. 2001;386:295–299.

Publication P2

Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures

Zocher, K., Lackmann, J. W., Volzke, J., Steil, L., Lalk, M., Weltmann, K. D., Wende, K. & Kolb, J. F. (2019). *Algal Research*, 39, 101416.

Algal Research 39 (2019) 101416

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures

Katja Zocher[a], Jan-Wilm Lackmann[a], Johann Volzke[a], Leif Steil[b], Michael Lalk[c], Klaus-Dieter Weltmann[a], Kristian Wende[a], Juergen F. Kolb[a,*]

[a] Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Straße 2, 17489 Greifswald, Germany
[b] University of Greifswald, Interfaculty Institute for Genetics and Functional Genomics, Felix-Hausdorff-Straße 8, 17475 Greifswald, Germany
[c] University of Greifswald, Institute for Biochemistry, Metabolomics, Felix-Hausdorff-Straße 4, 17487 Greifswald, Germany

ARTICLE INFO

Keywords:
Shotgun proteomics
Plasma
Microwave
Microalgae
Proteins
Reactive species

ABSTRACT

The efficient extraction of the rich amount of primary and secondary metabolites from microalgae has motivated considerable research. Besides biofuel production, microalgae are especially interesting as source for pharmaceutically and nutritionally valuable compounds. Major obstacle for economic down-streaming processes is the strong and rigid cell wall of microalgae, which impedes efficient compound extraction. Standard extraction methods suffer from several disadvantages and hence, alternative techniques are needed. Especially techniques, which do not modify components and are suitable for thermolabile compounds are required. Microwave burst exposures and submerged spark plasmas have been shown to lead to the release of higher protein yields than other methods. For comparison, *Chlorella vulgaris* was chosen as model organism because of its particular strong cell wall. Shotgun proteomics revealed comparable results of both methods, though several protein groups of interest showed differences in label-free quantification. Peptide modifications caused by reactive oxygen and nitrogen species (RONS) were observed after spark plasma treatment. However, spark discharges have been found more suitable for the extraction of heat sensitive compounds, such as photosystem-related proteins or histones.

1. Introduction

Microalgae are photosynthesis conducting microorganisms that are ubiquitously found in nature. Most microalgae can be grown in almost any environment, requiring only sunlight and some elementary nutrients [1,2]. Economically, they are known not only for biofuel production due to their high amount of lipids, but also for pharmaceutical and nutraceutical components, such as proteins, pigments, carbohydrates, vitamins or minerals [3,4]. One of the best-studied microalga is *Chlorella vulgaris*, which has shown, amongst other benefits, antitumor as well as immune protective and antioxidative potential [5,6].

A major issue for the extraction of relevant compounds from microalgae is a sturdy and thick cell wall. Particularly, the cell wall of *Chlorella vulgaris* is composed mainly of hemicellulose and polysaccharides inhibiting the release of intracellular lipids and providing immense physicochemical strength [7]. Although mechanical and chemical standard extraction methods are available, they are not without significant disadvantages and associated complications, such as high energy demand, alteration of extracted substances or the use of environmentally harmful solvents [8–10]. Therefore, alternative methods, that are efficient but without degradation of the extracted compounds, are highly desirable.

In a previous work, we could show that microwave irradiation is the most suitable reference method for extracting compounds from microalgae with respect to protein yield [11]. A similar conclusion was drawn by Lee et al. and Guldhe et al. for lipid extraction. In these studies, lipids for biofuel production were extracted from different microalgae strains, applying different standard extraction methods and microwave treatment was shown to be the most efficient and yet fastest technique [12,13]. However, scale-up of microwave treatments is challenging and more importantly, less suitable for thermolabile compounds, e.g. pigments or proteins. Spark discharge plasmas within algal suspensions that are generated by the application of nanosecond high-voltage pulses, provide an alternative. Total protein extraction yields are similar to microwave exposures and for extracted pigments no thermal degradation has been observed [11]. Spark plasmas have several physical and chemical characteristics, i.e. strong shockwaves and electric fields, UV radiation and the generation of reactive species [14,15],

* Corresponding author.
E-mail address: juergen.kolb@inp-greifswald.de (J.F. Kolb).

https://doi.org/10.1016/j.algal.2019.101416
Received 23 July 2018; Received in revised form 30 November 2018; Accepted 8 January 2019

which might contribute to an efficient extraction. Moreover, the different disintegration mechanisms might favour different substances, e.g. proteins, in the extraction process in comparison with microwave treatments. Furthermore, non-thermal modifications of compounds might occur, especially by reactive species that are produced by the plasma. These questions were addressed by evaluating proteins, which were extracted from *Chlorella vulgaris* by either microwave or spark plasma treatment. Shotgun proteomic analysis was chosen due to its high sensitivity and its ability to detect various chemical modifications caused by reactive species. Although *Chlorella vulgaris* is a well-known microalga, the main obstacle for applying this technique is the lack of a full proteome database for this microorganism [16,17]. Therefore, a database for the order *Chlorellales* was used as reference in this study, which was taken from the Uniprot® databank [18] and hosts all microalgae from the *Chlorellaceae* family.

2. Material and methods

2.1. Culturing and harvesting of Chlorella vulgaris

Chlorella vulgaris was chosen as a model organism for the extraction from microalgae in general. The cells (SAG 211-12, Experimental Phycology and Culture Collection of Algae, University of Goettingen, Germany) were cultured in Bold's basal medium (Sigma Aldrich, Germany).

Cells were cultured photoautotrophically in ventilated 500 ml Schott flasks, bubbled with filtered ambient air and a gas flow rate of about 1.5 vvm. The flasks were illuminated in light:dark cycles (12:12 h) with white fluorescence lamps (50 µmol photons $m^{-2} s^{-1}$; T5 LongLastTM F39W/840/LL", GELightning) and temperature was kept at 20 ± 2 °C [19].

For experiments, algae were harvested between day 30 and day 37 after inoculation with an adjusted density of OD_{750} 0.8 (Shimadzu UV-1280 spectrophotometer) to a final volume of 50 ml. Afterwards, cells were centrifuged at 3000*g* for 10 min (centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Germany), washed with 20 ml deionised water and centrifuged again for 7 min. The cells were kept on ice until use.

2.2. Microwave treatment

Microwave treatment was employed as standard extraction method for the purpose of comparison with plasma treatment. The effectiveness of this extraction technique was already shown in our previous work [11]. After washing and centrifugation of the harvested cells, the supernatant was discarded and the algae cells were transferred into 4 ml brown glass vials (Agilent Technologies, Germany). Subsequently, cells were refilled with 3 ml deionised water and the vial was closed with a cap that contained a septum for pressure balance. The vial was placed inside a standard 2.45 GHz microwave oven (Bosch HMT75M451, Serie 4 microwave, 2.45 GHz, distributed by Carl Roth, Germany) and treated at a setting of 600 W (mean power) for 20 s to the boiling point. After treatment, the vial was allowed to cool down to ambient temperature, refilled to final volume of 50 ml and centrifuged at 3000*g* (centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Germany) for 10 min for further investigations.

2.3. Plasma treatment

Spark discharge plasma was ignited in the gap of 0.5 mm between two tungsten rod electrodes with a diameter of 2 mm, which were submerged in the algae suspension. The electrodes were housed in a cylindrical reaction chamber, made from polymethyl-methacrylate (PMMA) with a volume of about 2 ml. In- and outlet at the top and bottom, respectively, permitted a continuous flow, driven by a peristaltic pump (TL 150, medorex Messtechnik + Bio-Verfahrenstechnik, Germany). Including the expansion tank and tubes, the entire system holds 50 ml. A flow rate of 50 ml/min was set, corresponding to a treatment time of the sample volume in 1 min and a residence time in the chamber of 2.4 s, respectively.

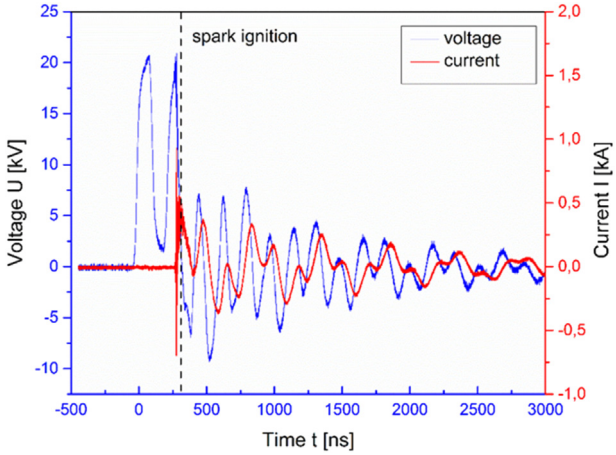

Fig. 1. Typical voltage and current waveforms for a mismatched train of pulses, delivered by a 100-ns Blumlein pulse-forming network, instigating a spark on the second applied pulse.

A Blumlein line pulse-forming network (BPFN) was used for the generation of spark discharges. For a matched load impedance of 50 Ω, a single high-voltage pulse of 100 ns duration is applied to the electrodes. However, due to the low conductivity of the algal suspension (1–2 µS/cm), the impedance of the reaction chamber, filled with the liquid, is much higher than the impedance of the pulse generator. Accordingly, a mismatched train of 100 ns pulses with decreasing amplitudes was applied between the electrodes.

The BPFN was charged with a positive high voltage from a DC power supply (PS/EQ060R020-22, Glassman High Voltage Inc., High Bridge, NJ, USA). Mismatched pulse trains were applied with a repetition rate of 4 Hz. For a pulse amplitude of about 20–25 kV across the electrode gap, a spark discharge was instigated within every train of pulses. A typical waveform is displayed in Fig. 1. The voltage was measured differentially with two high voltage probes (Tektronix P6015A, Tektronix Inc., Beaverton, OR), while the current was determined with a Pearson current monitor (Model 5046, Pearson electronics, Palo Alto, CA) and both were recorded on an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). When a spark is ignited, the voltage rapidly drops and at the same time, the current increases. The onset of the spark discharge is accordingly indicated in Fig. 1 (dotted line).

All experiments were conducted within 30 min with a suspension volume of 50 ml and an OD_{750} of 0.8. Samples were prepared as described in Section 2.1. Temperature was controlled with a hand-held infrared thermometer (PeakTech 4975, PeakTech Prüf- und Messtechnik GmbH, Germany) with measurements taken at the spark reactor and at the expansion reservoir. Under the chosen conditions, temperature stayed always below 25 °C.

2.4. Gel-free proteomic analysis

To determine the nature of extracted algal proteins, gel-free proteomic analysis was conducted. After the respective treatment method, samples were divided, whereat two samples were prepared immediately and two samples were kept on ice for 2 h before further processing. Thereafter, the algae suspensions were centrifuged at 3000*g* (centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Germany; used for all centrifugation steps besides SPE centrifugation) for 10 min and subsequently separated into their supernatant and their remaining pellet. All samples were frozen with liquid nitrogen to prevent

unnecessary protein damage and stored at −80 °C. The frozen supernatant was lyophilised (Christ Alpha 1–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) to reduce chemical consumption. Protein content was determined with DC Protein Assay.

On behalf of better readability, a detailed supply list of all chemicals and consumables is given in Supplemental S1 and will be not mentioned in the following sections.

2.4.1. Purification of proteins in the supernatant

After lyophilisation, the supernatant was lysed, using 1 ml HHT buffer.

The solutions were gently shaken on ice for 30 min and centrifuged at 1000*g*, 4 °C for 5 min. Due to a small protein yield in the supernatant, the whole sample was employed for proteomic analysis. After lysing, 10 µl iodoacetamide (concentration 505 mmol/l) was added to each sample and incubated for 30 min in the dark at room temperature. To precipitate proteins, 4 ml of −20 °C cold acetone were added and samples were stored overnight at the same temperature.

After precipitation, samples were centrifuged at 3220*g* for 10 min and subsequently, washed twice with acetone (−20 °C). To enhance enzymatic digestion, 100 µl of 0.05% Rapigest ®, dissolved in 25 mmol/l ammonium bicarbonate, was added as surfactant and the samples were transferred to 1.5 ml low-binding tubes (safe seal microcentrifuge tubes). Afterwards, protein digestion was conducted with 5 µl trypsin (concentration 0.02 µg/µl) overnight. To remove the surfactant from the samples, 2 µl of 37% hydrochloric acid were added, vortexed thoroughly and incubated at 37 °C for 30 min. The samples were then centrifuged three times at 15,000*g* to obtain phase separation, with the middle phase always transferred into new low-binding tubes (centrifuge 5430R, Eppendorf Vertrieb Deutschland GmbH, Germany). Finally, the samples were kept at 2–8 °C until solid phase extraction.

2.4.2. Purification of proteins in the pellet

To investigate the plasma effects on the proteins, which were kept inside the algal cell, the remaining pellet was also prepared. For cell lysis, the following buffer solutions were utilised:

Buffer A: 50 mmol/l TRIS, pH 8.0, 150 mmol/l NaCl, 1 mmol/l TCEP, Roche® complete mini tablets, EDTA-free, 1 mmol/l PMSF (phenylmethylsulfonylfluoride)
Buffer B: 50 mmol/l TRIS, pH 8.0, 150 mmol/l NaCl, 1 mmol/l TCEP, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)

The fresh pellets were transferred to lysing tubes (lysing matrix C), refilled with 500 µl buffer *A* and homogenised at 4.0 m/s, for 3 cycles of 60 s and two minutes breaks on ice, each (Fastprep 24™ 5^G, MP Biomedicals, LLC, USA). Buffer *A* was used in this case to protect proteins against proteolysis during homogenisation. Storage container and homogenisation tubes were rinsed with buffer solution *B*, 250 µl each. This step was necessary to ensure solvation of proteins that were adhered to tube walls. Subsequently, the homogenate was centrifuged at 16,000*g*; 4 °C for 30 min. A volume equal to 50 µg protein amount was transferred to low-binding tubes and precipitated by acetone (−20 °C), with one volume of protein solution mixed with a 4-fold volume of acetone and incubated overnight at −20 °C.

After precipitation, all samples were treated as described in Section 2.4.1, additionally with adding 1 µl of 100 mmol/l TCEP for 5 min and 1 µl of 505 mmol/l iodoacetamide for 30 more minutes at room temperature before trypsin digestion (concentration 0.02 µg/µl).

2.4.3. Solid phase extraction

For solid phase extraction, a stage tip system with C18 gel-loaded pipette tips was applied. Prior to extraction, the columns were preloaded with Luna® 3 µm C 18 (2) material (Phenomex Inc., CA, USA), suspended in a 6:3 methanol-to-water-mixture. After preloading, columns were washed once with 100 µl acetonitrile/0.1% acetic acid solution and twice with 100 µl water/0.1% acetic acid solution. After each step, the washing solution was removed by centrifugation. Subsequently, samples were loaded onto the columns by centrifugation. Samples were desalted by an additional washing step, using 100 µl water/0.1% acetic acid and the peptides were then eluted from the column using 50 µl of 60% acetonitrile/0.1% acetic acid. The elution solution was pressed through the columns into MS vials, utilising ca. 4 bar of nitrogen. Evaporation of the solvent was conducted with a speed vacuum device (Eppendorf Concentrator plus, Eppendorf Vertrieb Deutschland GmbH, Germany). The dried samples were dissolved in 10 µl of 5% acetonitrile in water/0.1% acetic acid solution and stored at −80 °C until measurement with tandem mass spectrometry.

2.4.4. LC-MS/MS spectrometry

To determine the origin of proteins, their distribution and probable plasma effects, samples were analysed with a nano-LC system (UltiMate 3000), coupled to a QExactive classic mass spectrometer (MS) with a nanoFlex source (all Thermo Scientific Inc., MA; USA), using a steel emitter. To separate peptides, reversed-phased LC was conducted by operating an in-line setup with a trap column (PepMap 100, C18, 75 µm × 15 mm) and a separation column (PepMap 100 RSLC, C18, 75 µm × 150 mm, both Thermo Scientific Inc., MA; USA). The 245 minute-gradient was utilised with solvent *A* (Aqua dest + 0.1% acetic acid) and solvent *B* (acetonitrile + 0.1% acetic acid) as follows: initial trapping step 0–4 min, 2% *B*; 190 min, linear to 35% *B*; 210 min, linear to 50% *B*, keep for 10 min; 221 min, linear to 80% *B*, keep for 10 min; 232 min, linear to 2% *B*, equilibration step to 245 min 2% *B*. The detection of peptides was performed by the MS in DDA-Top10 mode over the complete run, combining MS survey scans with fragmentation (MS/MS) spectra of the peaks of interest. Signals were monitored in the range of 300 to 1650 *m/z* with a spray voltage of 2.6 kV. Resolution was set to 70,000 for MS and 17,500 for MS/MS scans with an AGC target of 1×10^6 (MS) and 2×10^5 (MS/MS), respectively. Fragmentation for MS/MS was achieved by applying a normalized HCD energy of 27.5 eV. Additionally, the exclusion time for MS/MS isolation masses was set to 30 s.

2.4.5. Data analysis

After separation and fragmentation, peptide fractions were compared to a proteome database via Proteome Discoverer™ 1.4 (PD, Thermo Scientific), to identify original proteins in the samples. Although *Chlorella vulgaris* is a well-studied microalga, there is no full proteome database available, yet. The approach in this study was to apply a database from the order *Chlorellales* to identify the fragments according to their original protein. This database was taken from Uniprot® [18]. Statistical settings, such as peptide and protein score were kept rather strict (> 1 and > 10, respectively) with at least two unique peptides found per protein, required for further processing. To exclude ambiguous results, a false discovery rate (FDR) of 0.01 was applied and for modification studies, only modifications with an FDR of 0.05 or better were taken into account.

Experiments were repeated in duplicate and each replicate was injected twice into the nano LC-MS system. Subsequently, corresponding runs were merged to enhance coverage.

Venn diagrams for the results were generated using the VENNY platform [20].

Proteome Discoverer data files were shared with Mendeley Data [dataset] [21] and furthermore deposited to the ProteomeXchange Consortium via the PRIDE [22] partner repository with the dataset identifier PXD011411 [23].

2.5. Hydrogen peroxide and nitrite/nitrate yield

For the comparison of oxidation and nitration patterns with the

results obtained by proteomic analysis, hydrogen peroxide as well as nitrite/nitrate yields were determined with photometric test kits. To avoid disturbances of the absorption from the algae suspension, measurements were conducted with 50 ml distilled water. All other experimental conditions were kept identical to those of protein extraction and experiments were repeated in triplicate.

Hydrogen peroxide is a product of recombined reactive oxygen species and can therefore be detected with titanium (IV) oxysulfate solution. The yellow coloured solution was quantified at 407 nm (Evolution 300, ThermoFischer, Scientific, Madison, WI) and calibration was conducted with hydrogen peroxide solution.

The amount of nitrite and nitrate allows conclusions whether reactive nitrogen species are generated during plasma treatment. Nitrite and nitrate yield were determined with Spectroquant® Test Kits at 525 nm and 340 nm, respectively. Calibration curves were recorded with sodium nitrite and sodium nitrate solutions.

3. Results

A wide range of proteins from different cell compartments was identified with shotgun proteomics after plasma or microwave treatment. Chloroplastic-, cytoplastic-, nucleus-related, or mitochondrial proteins were found. Furthermore, proteomic analysis revealed amplified reactive oxygen-related modifications on peptide level for plasma treated algae, but nitro-modifications caused by reactive nitrogen species were not increased in comparison to microwave treated algae.

3.1. Overall protein content in comparison

Samples were divided as described in Section 2.4 with direct processing or a time lag of 2 h before freezing. An incubation time of 2 h was chosen to assess protein content and quality without significant degradation of proteins. An overview of the number of proteins that was found is given in Table 1. The overall shared amount of both extraction techniques varied minimally for analysis directly after processing and after the two hour time lag for proteins found in the supernatant and in the pellet.

In the supernatant, 1022 proteins could be identified directly after processing with both treatment methods (Table 1a). The unique proteins only found in the plasma treated samples could not all be identified due to an incomplete database. These four proteins were classified as three *uncharacterised proteins* and one *ATP synthase F0 subunit beta* protein. The number of proteins only found after microwave treatment was 65.

Contemplating the amount of proteins found inside the pellets when directly processed, microwave and spark plasma shared an amount of 968 identified proteins. Microwave treatment featured 58 unique proteins, whereat plasma treatment showed 83 unique proteins (Table 1b).

Table 1
Number of proteins in the supernatant (a) and proteins kept inside the pellet (b) after microwave and spark plasma treatment. Number of proteins account for direct processing and two hour incubation time, respectively.

	Method		
	Microwave (unique amount)	Shared amount	Spark plasma (unique amount)
a) Number of proteins in supernatant			
Directly after processing	65	1022	4
2 hour time lag	288	792	3
b) Number of proteins in pellet			
Directly after processing	58	968	83
2 hour time lag	24	986	102

After two hour incubation time, 792 proteins were identified in the supernatant, which were shared by microwave and plasma treatment (Table 1a). The number of proteins that were only found for microwave treatment was higher compared to plasma treatment (288 proteins and three proteins, respectively). The three unique proteins, which were found for plasma treatment (and which were different from the four proteins found in the supernatant), could be identified as *expressed protein*, *50S ribosomal protein L16* from chloroplastic origin and one *photosystem I reaction centre subunit XII* protein. Similarly, also for microwave extraction a high number of uncharacterised proteins was observed.

For proteins found in the pellets after two hour time lag, only a few different proteins could be identified for both treatments in comparison to directly after processing (Table 1b). In total, 968 proteins were identified after 2 h. However, for microwave treatment a sharp drop in the number of identified unique proteins can be observed after 2 h of incubation (24 unique proteins were identified). It seems that a time lag plays a role with respect to the amount of proteins that was observed after microwave exposure. Moreover, this decrease goes along corresponds with the rather high amount of proteins found in the supernatant for microwave treatment after an incubation time of 2 h. A time lag, in contrast, has only a minor effect for plasma treated samples. Here the number of unique proteins increases from 83 to 102.

3.2. Semi-quantitative protein yields

To determine whether protein extraction after plasma treatment showed differences in yield and classes, a semi-quantitative analysis was conducted. The areas under the curves (AUCs) from LC-MS/MS spectrographs for all determined proteins were set into relation with equivalent AUCs for the microwave treated proteins. A ratio higher 2 or smaller 0.5 was assumed as statistically significant, which means a two-fold increase or decrease in protein yield after spark plasma treatment compared to microwave treatment. AUCs were calculated from the three highest peptide peaks (high3) by Proteome Discoverer™. This resulted in a sigmoidal curve for all ratios as shown in Fig. 2. The fold-change axes are displayed in logarithmic scale to account especially for some very high ratios and the limits of 2 and 0.5 are marked with horizontal lines. For example, 278 proteins identified in the supernatant directly after processing were at least twice as abundant after plasma treatment compared to microwave treatment, i.e. ratios higher than 2. Conversely, for 204 proteins their amount decreased after plasma treatment compared with microwave exposure, i.e. ratios smaller than 0.5 (Fig. 2a).

A similar sigmoidal curve resulted from AUC-ratios, which were calculated from supernatant samples with two hour incubation time. In this case, 283 proteins showed a ratio higher than 2, while a ratio smaller than 0.5 was determined for 275 proteins.

The ratios for all proteins, which were detected inside the pellet, are shown in Fig. 2b. In short, directly after processing a ratio higher than 2 was calculated for 174 and smaller than 0.5 for 485 proteins. With a time lag of 2 h, 263 proteins exhibited a ratio higher than 2 and 345 showed a ratio below 0.5.

3.3. Protein yield of selected protein groups

To ascertain changes in yields for selected protein categories, a more detailed analysis of five selected groups was conducted, the ratios of the corresponding AUCs were calculated as described in Section 3.2. The following groups were chosen: antifreeze proteins, chlorophyll *a–b* binding proteins, photosystem-related proteins (e.g. reaction centre or light harvesting complex proteins), histones, and heat shock proteins. These groups represent either rather thermolabile proteins, such as photosystem-related proteins or histones or less sensitive groups, e.g. antifreeze or heat shock proteins. Some of these proteins are already of interest for industrial applications; hence, their extractability was paid

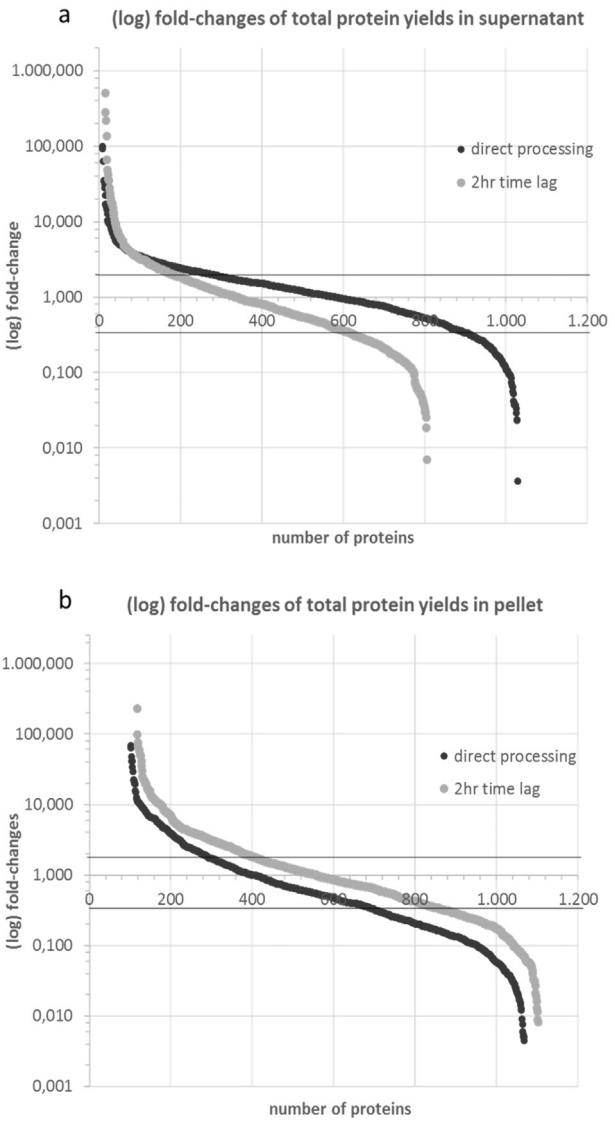

Fig. 2. Fold-changes for proteins identified after plasma treatment in comparison to microwave treatment, according to areas under curves (AUCs) for respective signals of LC-MS/MS spectrographs for (a) the supernatant and (b) pellets after direct processing (black symbols) and after two hour time lag (grey symbols). Horizontal lines display the threshold of ratios of 2 or 0.5.

particular attention. The names of the selected proteins were applied by their submitted protein name and their related accession number on the Uniprot® database, regardless of their annotation score.

Accession numbers, which are displayed in the graphs, were taken from Uniprot® database. Labelling all diagrams with full protein names was not possible in all cases because some proteins are only putative and have therefore no specific name, such as the group of antifreeze proteins.

3.3.1. Antifreeze proteins

Similar to higher plants, *Chlorella* strains are able to induce so-called hardening-induced antifreeze capability. Amongst other genes, antifreeze proteins, such as *hiC12* or *hiC6* are up-regulated in *Chlorella vulgaris* at temperatures below 4 °C [24]. In this work, *hiC12* and two other putative antifreeze proteins were detected in all samples. However, ratios for these proteins were not significant for the supernatant (Fig. 3a). In contrast, for all antifreeze proteins left inside the pellet (Fig. 3b), the fold change was over 3.15 and over 1.50 after plasma treatment. This agreed well with the lack of antifreeze proteins in the

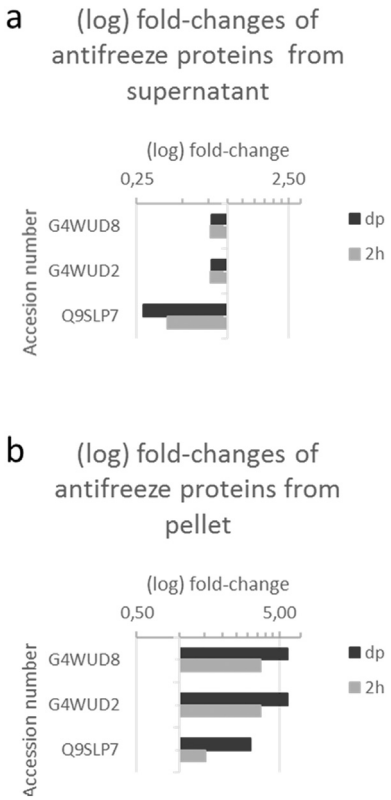

Fig. 3. Fold-changes according to areas under curve (AUCs) for respective signals of LC-MS/MS spectrographs for antifreeze proteins after plasma treatment compared to microwave treatment. Proteins were found in the supernatant (a) and in the pellet (b) directly after processing (dp) and after 2 hour incubation time (2 h). Accession numbers were determined according to the Uniprot® database.

supernatant, which indicates that antifreeze proteins are better extractable with microwave burst heating.

3.3.2. Chlorophyll *a–b* binding proteins

Chlorophyll binding proteins are a good indicator for temperature-sensitive compounds in plant or algal material. Seven proteins found in the supernatant after plasma exposure showed a fold-change of > 2 in comparison with microwave treatment (directly after processing). Only three proteins showed ratios of < 0.5 (Fig. 4a), indicating that these three candidates were better accessible by microwave treatment. After a time lag of 2 h, the number of proteins with a fold-change above 2 decreased down to four. However, the number of proteins with a fold-change of < 0.5 stayed the same.

Inside the pellet samples, 17 chlorophyll *a–b* binding proteins were detected. Eight of them showed a fold-change higher 2 directly after processing and also after a two hour time lag. For another four proteins, observed directly after processing, and three proteins; found after the two hour time lag, only a fold-change of < 0.5 was derived (Fig. 4b).

3.3.3. Photosystem-related proteins

The group of photosystem-related proteins contains all other proteins, which are associated with the photosystems I and II. Reaction centre subunits, assembly proteins, or apoproteins were collected in this group amounting to altogether 20 proteins. As already shown for the group of chlorophyll *a–b* binding proteins, the amount of photosystem-connected proteins after plasma treatment was higher than after microwave exposure. Directly after processing, 12 of the 20 proteins had a fold-change higher than 2 and only one protein, found for microwave treatment, was not observed after plasma exposure (accession number P56311, *photosystem I assembly protein Ycf3*). Notably, this protein was

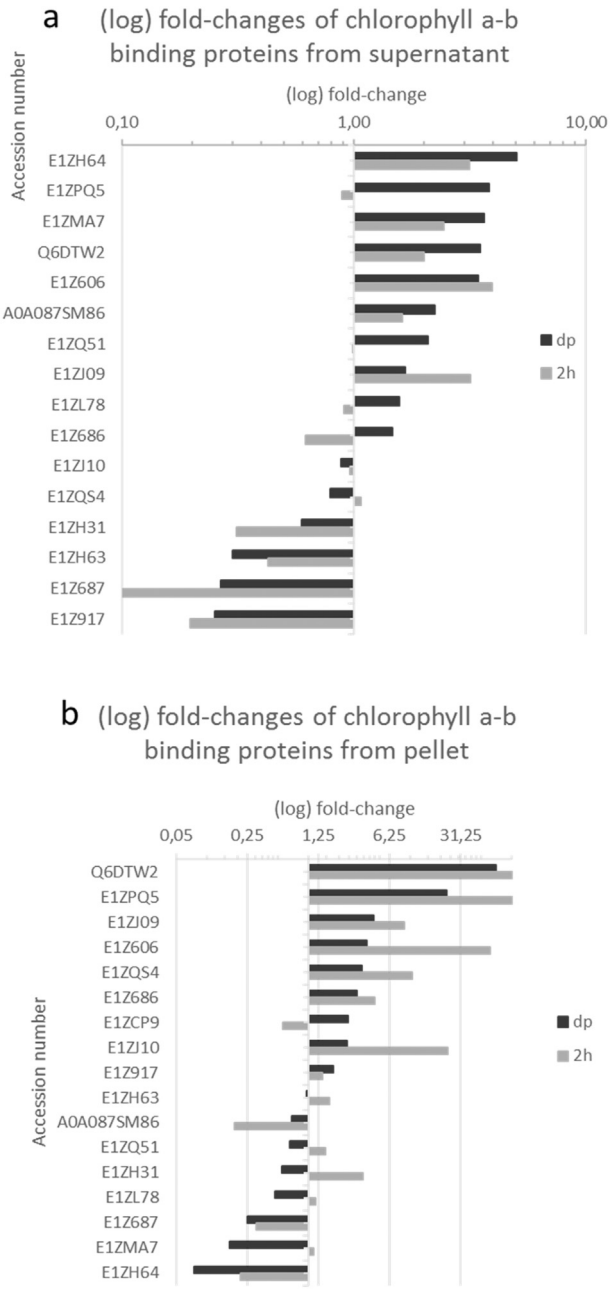

Fig. 4. Fold-changes according to areas under curve (AUCs) for respective signals of LC-MS/MS spectrographs for chlorophyll *a–b* binding proteins in plasma treated samples compared to microwave treatment. Proteins were found in the supernatant (a) and in the pellet (b) directly after processing (dp) and after 2 hour incubation time (2 h). Accession numbers were determined according to Uniprot® database.

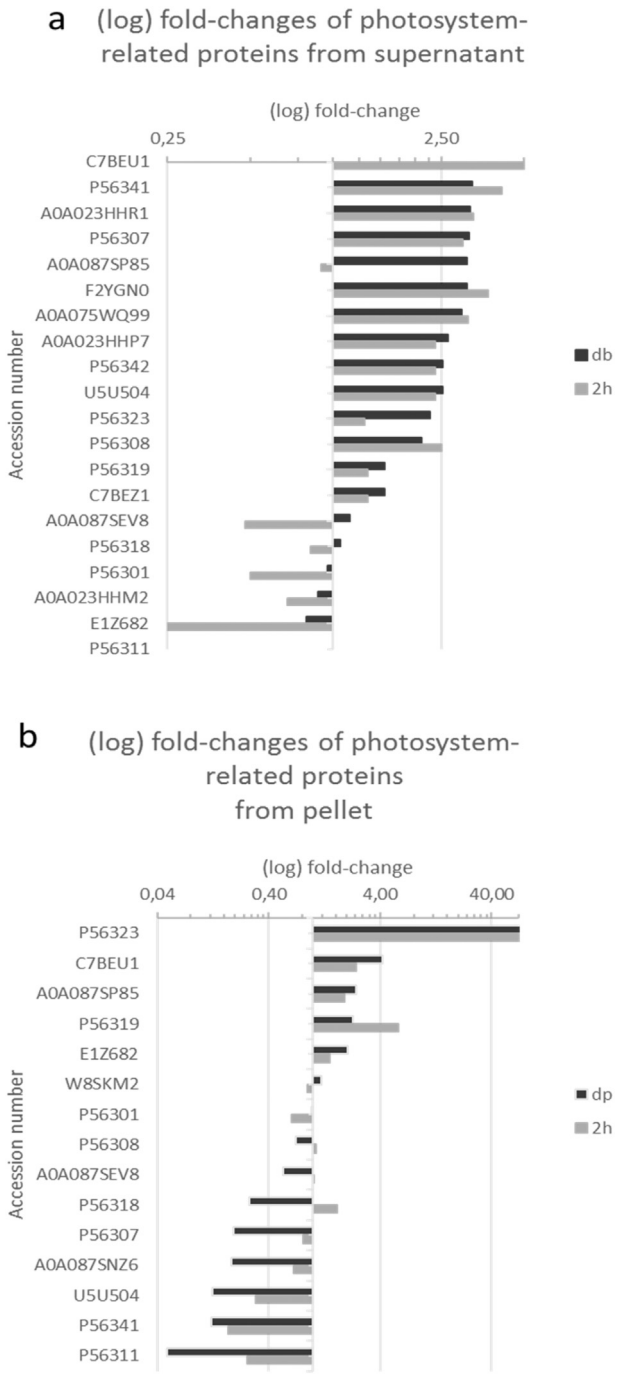

Fig. 5. Fold-changes according to areas under curve (AUCs) for respective signals of LC-MS/MS spectrographs for all photosystem-related proteins after plasma exposure compared to microwave treated samples. Proteins were found in the supernatant (a) and in the pellet (b) directly after processing (dp) and after 2 hour incubation time (2 h). Accession numbers were determined according to the Uniprot® database.

also not found after 2 h. For the 2 hour time lag, the number of proteins with ratios above 2 decreased slightly from 12 to 10, and two proteins showed a fold-change lower than 0.5. However, the protein *photosystem I reaction centre subunit XII* (C7BEU1) was no longer observed after 2 h in the microwave-treated group, but had the highest ratio of 9.76 after plasma treatment (directly after processing). Results are presented in Fig. 5a.

In this group of photosystem-related proteins, 15 proteins were found in the remaining algae pellet (Fig. 5b). Plasma treated samples contained only five of them with a fold change above 2 and six proteins with a change < 0.5 (directly after processing). In the group with the two hour time lag, three proteins showed a fold-change higher than 2 and also three proteins a ratio < 0.5. Notably, the protein *photosystem II reaction centre protein H* (accession number P56323) was not discovered in all microwave samples.

3.3.4. Histone proteins

Histones are part of the algal nuclei and their function is to bind DNA; hence, they play an important role in replication [25]. For this reason, a more detailed analysis whether plasma affects this group was conducted as well (Fig. 6). From all eight histones found in the

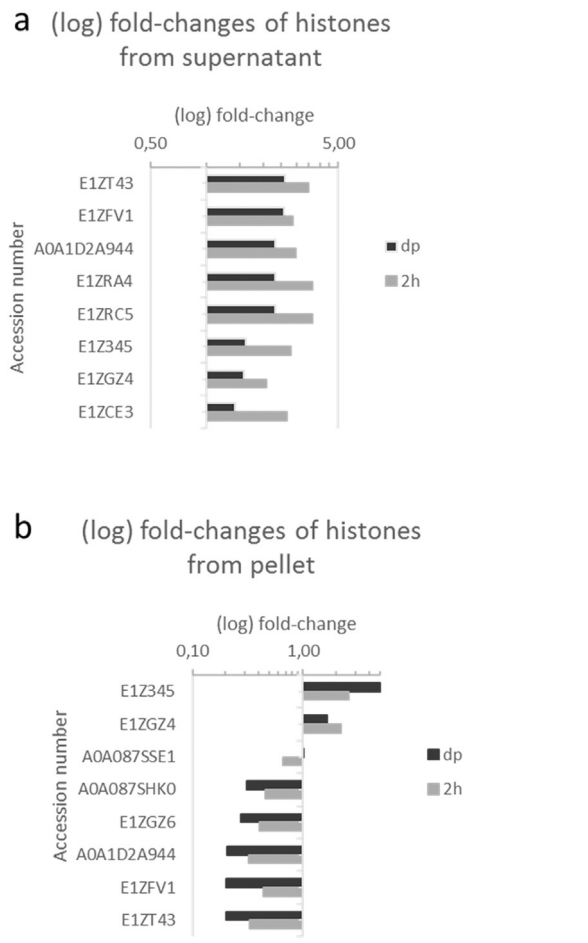

Fig. 6. Fold-changes according to areas under curve (AUCs) for respective signals of LC-MS/MS spectrographs for histones after plasma treatment compared to microwave exposure. Proteins were found in the supernatant (a) and in the pellet (b) directly after processing (dp) and after 2 hour incubation time (2 h). Accession numbers were determined according to the Uniprot® database.

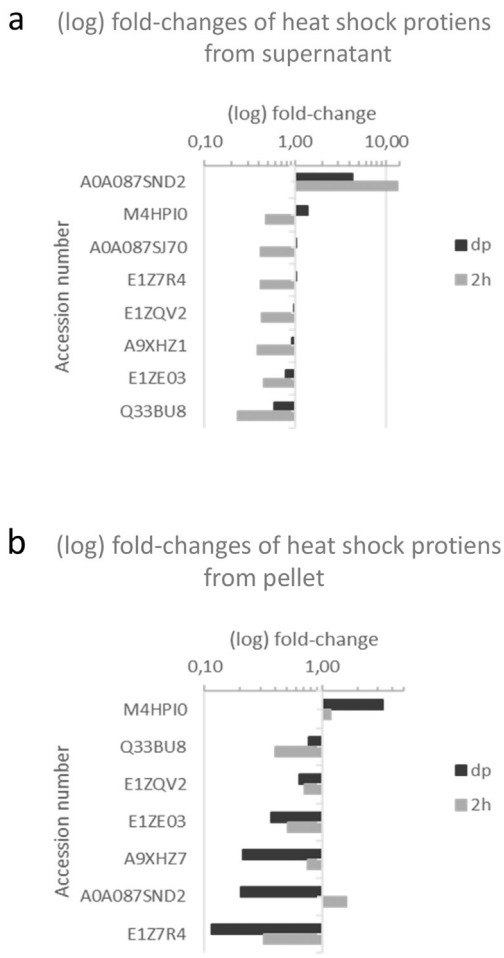

Fig. 7. Fold-changes according to areas under curve (AUCs) for respective signals of LC-MS/MS spectrographs for all heat shock proteins found after plasma treatment compared to microwave treatment. Proteins were found in the supernatant (a) and in the pellet (b) directly after processing (dp) and after 2 hour incubation time (2 h). Accession numbers were determined according to the Uniprot® database.

supernatant, five showed a higher ratio than 2 and none was found with a ratio < 0.5 with no delay in processing/storage. Remarkably, after 2 h all eight histones, found in the supernatant, achieved a higher ratio than 2 for plasma treated samples compared to microwave exposures (Fig. 6a).

In addition, eight proteins were discovered in the remaining algal pellet and in all sample types, five showed a fold-change between 0.46 and 0.20. A higher fold-change increase over 2 could only be observed for a single protein (directly after processing) and for two proteins for a delay of 2 h (Fig. 6b).

3.3.5. Heat shock proteins

Heat shock proteins are often expressed in response to stress factors, e.g. heat, cold, metal ions or UV radiation and belong to a large family of proteins [26–28]. In this work, eight antifreeze proteins were found after treatment, with one heat shock protein (accession number M4HPI0) belonging to the HSP 90 group and all others to the HSP 70 group. In Fig. 7a, the results are shown for the samples from the supernatant. For all plasma-treated samples, measured directly after processing, a fold change of > 2 was calculated for only one protein (fold-change 4.31, *mitochondrial Heat shock 70 kDa protein*, accession number A0A087SND2); the same protein showed a fold-change of 13.61 after 2 h. All other proteins revealed no significant ratios (i.e. between 1.37 and 0.59). Interestingly, after 2 h, these proteins showed a fold-change between 0.47 and 0.23, indicating a possible up-regulation of these heat shock proteins after microwave exposure.

The heat shock protein from the HSP 90 group was also observed in the remaining pellet (Fig. 7b), showing a fold change of 3.27 directly after processing and a change of 1.18 after the two hour time lag. The protein *Heat shock cognate 70 kDa protein* (accession number A0A087SJ70) was not identified in the pellet for any treatment regimen. All other HSP 70 proteins had a calculated fold-change between 0.77 and 0.12 directly after processing. After 2 h, the fold changes ranged between 1.63 and 0.32.

3.4. Oxygen and nitrogen modifications

Physical plasmas generate reactive oxygen and nitrogen species (RONS), which are known to interact with biological compounds [29,30]. To determine whether reactive species produced during spark discharges affect proteins that were extracted from *Chlorella vulgaris*, peptides were screened for chemical modifications. Only peptide alterations with a false discovery rate of 5% (= medium) or less were selected and thus, 7777 peptide sequences could be studied.

3.4.1. Trioxidation

One possible oxidation pattern, caused by ROS, is oxidation of cysteine. While single oxidation events are unstable in positive ionisation mode, triple oxidations (e.g. cysteine sulfonic acid) are highly stable and can easily be detected by MS [31]. Samples showed high amounts

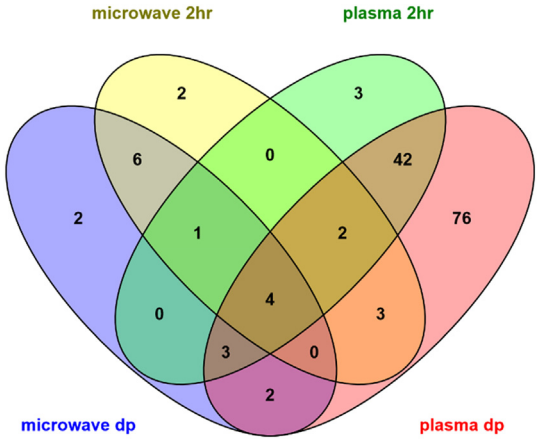

Fig. 8. Number of trioxidised proteins in the supernatant after microwave exposure (blue and yellow ellipses) and plasma treatment (green and red ellipses). Directly after processing, 19 modifications were found for microwave-treated samples (microwave dp) and 132 after spark discharge treatment (plasma dp). With the 2-hour time lag, 19 modifications, different from the ones for direct processing, were detected in microwave samples (microwave 2 h), but 56 remained after spark plasma treatment (plasma 2 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of trioxidation after spark plasma treatment, especially for proteins found in the supernatant. Directly after processing, 19 peptides showed trioxidation for microwave treatment. In contrast, 132 peptides were altered by plasma treatment. After 2 h, the number of trioxidised peptides decreased to 56. Results are presented in Fig. 8. The number of modified proteins (i.e. 19) did not change for the microwave-treated samples; however, the proteins that were found after the two-hour time lag were not entirely the same as were found directly after processing. Here, only 11 proteins are shared directly after processing and for the two-hour time lag in the microwave-exposed samples.

Proteins, which remained inside the pellet, were also affected by oxygen species, although these ratios differ between incubation times. After plasma treatment, 25 (directly after processing) and 43 (after 2 h) modified peptides were found and for microwave-exposed samples, 6 oxygen modifications were found immediately and 14 after 2 h (Fig. 9).

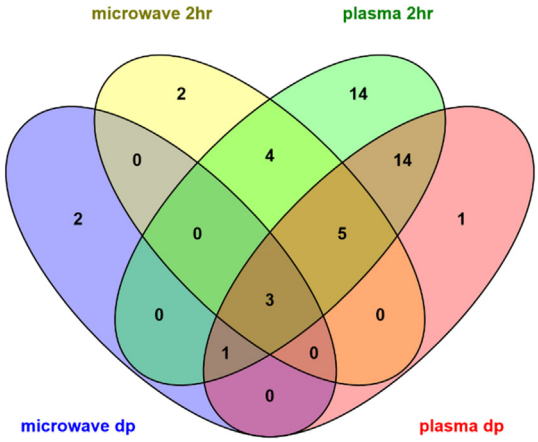

Fig. 9. Number of trioxidised proteins kept inside the pellet after microwave exposure (blue and yellow ellipses) and plasma treatment (green and red ellipses). Directly after processing, 6 modifications were found for microwave treated samples (microwave dp) and 25 after spark discharge treatment (plasma dp). With the 2-hour time lag, 14 modifications were detected in microwave samples (microwave 2 h) and 43 after spark plasma treatment (plasma 2 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

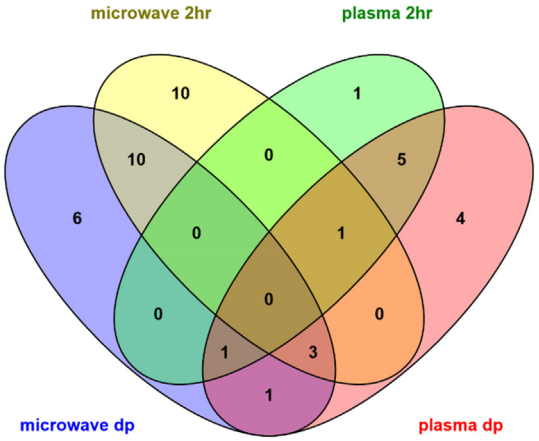

Fig. 10. Number of nitrated proteins found in the supernatant after microwave exposure (blue and yellow ellipses) and plasma treatment (green and red ellipses). Directly after processing, 21 modifications were found for microwave treated samples (microwave dp) and 15 after spark discharge treatment (plasma dp). With the 2-hour time lag, 25 modifications were detected in microwave samples (microwave 2 h) and 8 after spark discharge treatment (plasma 2 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4.2. Nitration

The appearance of the nitration of proteins would be a sign for reactive nitrogen species generated during plasma generation. Whether RNS are generated during spark development, and therefore cause alterations of proteins, was hence investigated.

In Fig. 10 (supernatant) and Fig. 11 (pellet), numbers of nitrated peptides are displayed directly after processing and after two hour incubation time, respectively. Directly after processing, 21 modifications in the supernatant were detected for microwave treatment, whereas 15 modifications could be found after plasma exposure. Within a time lag of 2 h, the number of alterations after microwave extraction increased merely up to 25, but decreased for plasma-treated samples to eight (Fig. 10).

Observing modifications for proteins inside the algae pellets, the

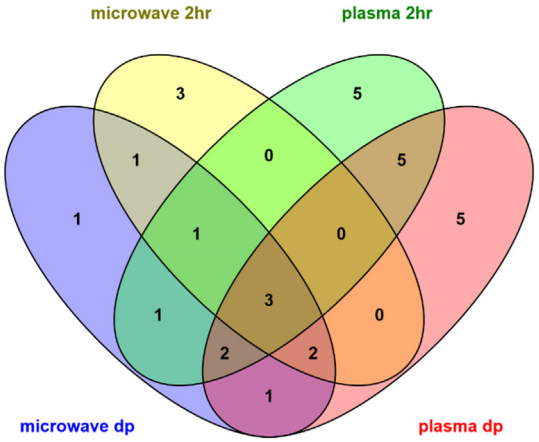

Fig. 11. Number of nitrated proteins kept inside the pellet after microwave exposure (blue and yellow ellipses) and plasma treatment (green and red ellipses). Directly after processing, 12 modifications were found for microwave treated samples (microwave dp) and 18 after spark discharge treatment (plasma dp). With the 2-hour time lag, 12 modifications were also detected in microwave samples (microwave 2 h), as well as 18 after spark discharge treatment (plasma 2 h). It has to be noted that the type of proteins were different, although the number stayed constant directly after processing and for the 2-hour time lag. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Hydrogen peroxide and nitrite/nitrate concentrations after 30 minute spark plasma treatment, conducted for experiments with distilled water as described in Section 2.5. Standard deviations are given in brackets.

Species	Yield in mg/l
H_2O_2	3.00 (± 0.29)
NO_2^-	0.13 (± 0.01)
NO_3^-	Below detection limit

number of modifications stayed constant over time. For microwave treated samples, 12 alteration were detected and 18 for plasma treatment (Fig. 11). Although the number of proteins stayed the same, the modified proteins differed depending on treatment and processing conditions, indicating no prime target for plasma-generated RNS.

3.5. Hydrogen peroxide and nitrite/nitrate content

To characterise the spark plasma regarding its RONS development and verify the results found after proteomic analysis, yields for H_2O_2 and NO_2^-/NO_3^- were quantified with photometric kits.

Hydrogen peroxide concentration after 30 minute treatment was determined with 3.00 mg/l (± 0.29 mg/l). The nitrite yield obtained was 0.13 mg/l (± 0.01 mg/l) and nitrate concentration ranged below the detection limit. Results are shown in Table 2 (standard deviations are given in brackets). These findings correlate with the relative high amount of trioxidised proteins in the supernatant, caused by oxygen species and the only small amount of nitration of proteins in the supernatant and pellet.

4. Discussion

Chlorella vulgaris is considered an interesting model organism for the extraction from algal biomass in particular for its sturdy and robust cell wall [32]. Spark discharge plasmas have been proven an effective method to disintegrate cell walls of this microalga. Yields for proteins and other valuable substances are comparable with treatment by microwave heat bursts and both extraction methods deliver higher amounts than other procedures that were tested, including pulsed electric fields. However, microwave treatments are more likely to have a detrimental effect on temperature-sensitive compounds [11]. Since plasmas are known for the generation of reactive oxygen and nitrogen species, associated modifications of extracted compounds might be possible. Therefore, in this study, origin of extracted proteins and possible differences in the distribution pattern between microwave and plasma treatment in particular, were examined.

4.1. Protein yield

Lacking of a full proteome database for *Chlorella vulgaris*, it was necessary to compare the peptides against a more general database for the order *Chlorellales*, which is one of two divisions of green algae. A similar approach was conducted by Guarnieri et al. [16], who examined biosynthetic pathways of *Chlorella vulgaris* were examined via de novo transcriptomic and proteomic analyses. After an in silico six-frame translations of the annotated transcriptome, the product ion data were then searched against a *Chlorophyta* database, which means using all available sequenced microalgae. Utilising transcriptomic sequences to identify proteins from *Chlorella vulgaris* is often used; for instance, Gao et al. [33] applied this approach successfully for the evaluation of sample extraction methods for proteomic analysis. Nevertheless, transcriptomics is not always a suitable substitute for the lack of a proteomic database. For example, transcriptomics platforms are not set up to systematically include changes of splice variants, but proteomics typically observes these proteins, which are encoded by those variants. Furthermore protein turnover or post-translational modifications have a considerable influence on the nature and level of protein expression, and are not sufficiently detected by transcriptomics [34]. Shortcomings in the obtained results based on transcriptome databases have therefore to be kept in mind. Another option is to search against the full protein database, as was for example conducted by Wang et al. [35] for the also non-sequenced green algae *Haematococcus pluvialis* or by Campos et al. [36] for the evaluation of extracellular proteins from *Chlorella vulgaris*. In those studies, detected peptide fragments were compared against the complete SWISS-PROT® database. Advantage of this approach is that BLAST methods are highly suitable for high-throughput analysis because of their simplicity, but are less appropriate for precise in-depth annotation [17].

Major advantage of applying the database for the order *Chlorellales* is that it allows an overview of all extracted proteins. It is therefore possible to draw conclusions on the origin of proteins. This practice can obtain more precise results than applying the database of the phycological division or a comparison with the complete SWISSPROT® database. However, most of these proteins were labelled as uncharacterised proteins and thus, a more stringent differentiation was so far not possible. Nevertheless, the approach is sufficient for the comparison of the two extraction techniques. The search against the *Chlorellales* database gave already a revealing insight into distribution patterns. For industrial applications, the generalised family-database can be refined with regard to specific proteins of interest. In contrast, in-depth pathway analysis would require a more sophisticated database, e.g. by fully sequencing the organism of interest.

Regarding the overall protein amount, displayed in Table 1 a, 1094 proteins were found in total (i.e. for microwave and plasma treatment together) in the supernatant within the given statistical parameters, as described in the material & method section. Overall protein numbers of 1022 (directly after processing) and 792 (after a two-hour time lag) were found for both extraction techniques. This result confirms that the two methods are able to extract similar numbers of proteins. The ascertained decrease in the total number of proteins that were determined might be caused by inactivation during the storage time of 2 h on ice, which was chosen to observe whether a time lag affects proteins in any way. Due to the time lag of 2 h, proteases and peptidases of the cells may be able to degrade proteins more extensively and this might explain the decrease in the eventually obtained numbers of proteins. Additionally, precipitation of proteins due to heat or light exposure is also a conceivable mechnanism for further modifications. The rather low change in protein number within the algal pellet might be due to a residual protective function of the remaining cell wall, even when cells are comprehensively damaged (Table 1b).

Whether a time lag after spark plasma treatment has a positive effect on extracted yields can be answered by observing the sigmoidal curves from ratios of all yield AUCs in Fig. 2a and b. The ratios of the protein yields show no major difference between samples after directly processing and those with the two hour incubation time. Hence, a need for a break before further processing seems not necessary. Contrary, an interruption before further analysis seems to harm proteins, extends processing times and thus, raises costs for industrial application.

4.2. Temperature effects and temperature-related proteins

The increased number of proteins in the supernatant after microwave extraction could be a result of higher bulk temperatures. It seems conceivable that proteins are strongly modified accordingly, resulting in a higher amount of unique respective proteins as they were detected by mass spectrometry (see Table 1). It is also possible that microwave exposure alters protein structure, which might increase the number of different detected proteins after 2 h due to precipitation and hence, better accessibility for trypsin digestion. De Pomerai et al. [37,38] have shown already that microwave irradiation favours the aggregation of

bovine serum albumin in vitro. In these studies, it was also observed that modest heat-shock responses are induced by microwave exposure in *Caenorhabditis elegans* and microwave irradiation activates the expression of small heat shock proteins as part of a mild stress response. Those findings might also explain the observed up-regulation of heat shock proteins after 2 h in the supernatant for the microwave-exposure of *Chlorella vulgaris*. Compared to microwave treatment, the AUC-ratios for plasma treated samples were below 0.47, which indicates an increase of heat shock proteins after microwave treatment. Interestingly, although the development of a spark plasma channel is associated with transient temperature increases of at least hundred degrees, only one mitochondrial heat shock 70 kDA protein (accession number A0A087SND2) was detected at elevated levels after spark discharge treatment. Temperature increase by plasma might be too short and too localised to have sustained effects on the bulk of the treated volume. This overall temperature is controlled by pulse duration and spark repetition frequency. For the chosen value of 4 Hz, temperatures of the algal suspension stayed below 25 °C. Conversely, microwave exposures with the same protein yield are associated with higher temperatures in the bulk, i.e. the boiling point of the solvent water.

In opposition to heat shock proteins, antifreeze proteins modify the growth of ice inside cells, which results in the stabilisation of ice crystals over a defined temperature range and inhibit the recrystallization of ice [39]. They are found in many organisms, such as fish, fungi, plants, or algae. In this work, three antifreeze proteins were detected; however, only one could be fully identified as *hiC12*. The two others were categorised as “predicted” in the Uniprot® database. Due to a high amount of putative uncharacterised proteins that were found, it is possible that more antifreeze proteins are encoded by *Chlorella vulgaris*. For example, Li et al. [24] have likewise found *hiC6*, *hsp70* and *rpl10a* in different *Chlorella vulgaris* strains. Nevertheless, due to AUC-ratios between 0.78 and 0.28, spark discharge treatment seems to have a comparable effect as microwave treatment.

Antifreeze proteins are of great interest for instance in the food industry to improve the quality of frozen foods by inhibiting recrystallization. Venketesh and Dayananda [40] further recommend the use of antifreeze proteins for cryosurgery, cryopreservation or for transgenic technologies. They also state that the application strongly depends on costs since large quantities of highly pure proteins are needed. Extracting those proteins with the help of microwave exposure or spark plasma might be a promising application.

Chloroplasts, which contain two photosystems, are the most thermolabile organelles in plant cells and photosynthesis-related proteins are rather heat sensitive compounds, accordingly. The photosystem II (PSII) is considered the primary target of damage-associated components of the photosynthesis system [41]. The light harvesting chlorophyll *a–b* protein complex is associated with PSII (also known as light harvesting complex II, LHCII) and is the major collector of energy in higher plants and green algae [42]. When temperature increases, first the reaction centres of PSII are blocked, followed by dissociation of the light harvesting complexes from the core of PSII [43]. A decrease after microwave treatment of chlorophyll *a–b* binding proteins and all other photosystem-related proteins is most likely the result of heat damage due to microwave exposure, as was found in our study (ratios shown in Figs. 4 and 5). Especially, if reaction centres are blocked first and the LHC complexes follow, it might explain why a *photosystem I reaction centre subunit XII* protein was found in the plasma supernatant after 2 h, but not in the microwave samples because reaction centres are destroyed first by heat. It should be noted that an even higher amount of photosystem-related proteins was found in the supernatant of plasma-treated samples compared to chlorophyll *a–b* binding proteins. This is further proof that spark discharge plasmas are gentle enough for the extraction of heat sensitive compounds.

Photosystem-related proteins are interesting for different industrial applications. For instance, for the production of molecular hydrogen as energy carrier for environmentally friendly and clean energy sources. Accordingly, PSII proteins were successfully integrated for electron transfer into a bio-electrochemical device, which is considered as crucial step towards a semiartificial hydrogen production [44,45]. Thus, a reliable but mild extraction of photosystem-related proteins is of considerable interest.

Chlorella vulgaris has one nuclei, which was affected by both microwave and plasma treatment. Histones are part of the nucleic material, with core histones H3 and H4 are known to be highly conserved, H2B to be more variable than H2A and the linker histone H1 to be the least conserved histone [46]. Eight histones were detected in all samples. Two histones were identified as H3 and H4, four as histones 2B and two as 2A; the histone H1 was not found. The KEGG Genes Database [47] identified within those five histone groups 24 histones that are fully sequenced for *Auxenochlorella protothecoides* and 13 sequenced for *Ostreococcus tauri*, which are both green algae. It is conceivable that the number of *Chlorella vulgaris* histones is similar to that of these two algae. Notably, almost all eight detected histones found in the supernatant show a fold-change above 2 after spark discharge treatment, Two of them found in the pellet showed likewise a fold-change higher than 2. Considering heat development during microwave exposure, the low histone yield in these samples might be explained by heat-induced damage. Saidi et al. [48] describe in their review that histones undergo several modification, e.g. acetylation, methylation, ubiquitination, phosphorylation, or glycosylation when exposed to heat. A complete destruction of histones due to sudden heat exposure might be another reason for the absence of histones in microwave-treated samples. Thus, microwave extraction might not be suitable for these temperature sensitive proteins. However, the extraction of histones and their investigation in regulation of gene expression is a crucial part in biotechnology, e.g. for genetic engineering and stable expression of transgenes [49].

4.3. Chemical modifications by reactive species

Proteomic analysis can also be applied to determine whether reactive species generated during spark propagation modify the chemical composition of proteins. In general, oxidation and nitration play an important role and can lead to different effects on proteins. For instance, loss of function, modification of sidechains, up- or down-regulation or even activation of silent proteins have been observed in several studies [31,50–52]. Several oxidation states and nitration of amino acid sidechains were determined in this study. Chemical modifications after spark plasma exposure were analysed qualitatively with respect to threefold oxidation (trioxidation) and nitration because the respective parental reactive species are expected for plasma generation. Chemical modifications due to microwave exposure are currently unknown in this field and were therefore not investigated. A more detailed examination of microwave exposure-related modifications would be certainly beneficial.

Concerning trioxidation after plasma treatment, a rather high number of threefold-oxidised peptides was found in the supernatant directly after processing (see also Fig. 8), namely 132. In contrast, after 2 h the amount decreased to 56 peptides. However, the total amount of proteins also decreased by about 30%. Therefore, the corresponding ratio of trioxidised proteins seemed to be constant over time. Radicals, generated by plasma, recombine to more stable compounds, such as H_2O_2. Subsequently, these can be decomposed again, e.g. into O_2 and H_2O [53]. Generation, recombination and degradation processes will eventually establish a dynamic equilibrium, depending on operating parameters. The high amount of threefold oxidation presumably due to long-lived H_2O_2 was confirmed by the photometrical-determined yield of 3.00 mg/l after 30 minute treatment time, as shown in Table 2. Conceivable associated protein modifications may influence their function. Changes might not always be detrimental, but can possibly lead to new protein activities of industrial interest, e.g. the enhancement of extracted products. Potential targets for oxidation were not

investigated in detail, but Smith et al. [54] have studied the oxidation of amino acids after plasma treatment. They could show that for amino acids after plasma treatment with a floating DBD, primary targets are any sulphur or nitrogen functionalities. Moreover, they suggest for increased plasma treatment times that fragmentation occurs along the carbon backbone of the amino acids, which may be most likely due to highly oxidising and acidic conditions. The occurrence of hydroxyl radicals enables hydrogen separation and exposes the carbon for further reactions. It has to be noted that these conclusions were drawn for DBD plasma sources, were acidic conditions are common due to reactive nitrogen species, which are responsible for drop in pH values [55]. Spark discharges administered in this study have shown to decrease pH values only slightly [11]. Chemical modifications, similar to the ones reported by Smith et al. were also found by Lackmann et al. [56] and Takai et al. [57], when applying a plasma jet to amino acids and protein.

Although trioxidation was found to a high degree for proteins in the supernatant, the number of oxidised proteins inside the remaining algae pellets is much lower, which can be observed in Fig. 9. Hence, it seems likely that proteins are protected inside the cell. For instance, microalgae are rich in carotenoids, which play an important role as antioxidants. With their ability to scavenge reactive oxygen species, already during photosynthesis, carotenoids contribute strongly to the total antioxidant activity of microalgae [58]. It is therefore conceivable that carotenoids from *Chlorella vulgaris* may quench hydroxyl radicals or their more stable reactants that are generated by spark discharges and thus, prevent the oxidation of proteins inside the cell. Because microalgae undergo photosynthesis, other protective compounds, such as superoxide dismutase (SOD) and catalase may also scavenge oxidising agents. In short, electrons reduce O_2 during photosystem I passage into superoxide radicals. These radicals are then converted into H_2O_2 by SOD and afterwards H_2O_2 is degraded by catalase [59]. An example that SOD can scavenge ROS was given by Gebicki et al. [60]. They have shown that active SOD present during γ-irradiation decreased the yields of protein peroxide that is otherwise generated by reactive oxygen species. In their work, protein solutions (BSA and lysozyme 10 mg/ml) in 20 mmol/l phosphate buffer, pH 7.4, were irradiated with γ-rays with a dose of 1000 Gy. The γ-irradiation was specifically chosen to generate ROS. On the one hand, it may therefore be possible that SOD and catalase inside the algal cell react with ROS, which are directly are indirectly generated by plasma, and hence, protect the surrounding proteins from trioxidation. On the other hand, it is likely that these enzymes are the preferential target of ROS and other proteins are protected indirectly. In addition, Takai et al. [57] reported that especially sulphur-containing and aromatic amino acids were decreased during treatment with a plasma jet, corresponding to a protective bystander effect for other proteins, e.g. with various ring-systems. A more detailed and dedicated study is necessary to confirm these possibilities and is recommended for future research.

In contrast, and as shown in Figs. 10 and 11, no substantial nitration after spark discharge treatment compared to microwave treatment was found neither in the supernatant nor for proteins kept inside the pellet. Thus, formation of reactive nitrogen species (RNS) in applicable amounts during spark propagation seemed improbable. The very small yields of NO_2^- (0.13 mg/l) and NO_3^- (below detection limit, see also Table 2), confirm the findings of the proteomic analysis. It needs to be mentioned that photometrical analysis for the quantification of H_2O_2 and NO_2^-/NO_3^- are sensitive to interferences with other reactive species that are present in the liquid. For a more detailed study, other quantification methods, such as HPLC or EPR measurements are recommended. Nevertheless, for an estimation of stable species and confirmation of findings from the proteomic analysis, photometrical detection is a sufficient method.

4.4. Origin of proteins

All detected proteins are from different compartments inside the cell, according to the proteomic processing protocol. To extract and precipitate proteins, the remaining cell walls or membranes were digested or disrupted, respectively. Hence, cell wall proteins were no longer available for MS detection. Furthermore, plant and algae cell wall proteins are generally glycosylated [61] and present therefore several difficulties. Usually, these proteins are embedded in an insoluble polysaccharide matrix, and interact with other cell wall components. Depending on whether the proteins are weakly or strongly bound, the extraction is often conducted with salts, chelating substances, or by gel-electrophoresis [62]. However, plasma might also have an effect on cell wall proteins. Smith et al. [54] suggest that amino acids of cell walls may in fact be the primary target for plasma treatments.

The semi-quantification method administered in this work was able to determine differences for proteins extraction between microwave exposures and spark discharge treatment. Furthermore, differences in the distribution of selected protein groups could be elucidated. For temperature-sensitive compounds, spark discharge treatment has proven to be gentle and yet effective enough to compromise the cell walls of microalgae. The results indicate also that with the respective extraction method, a preselection of respective proteins of interest might be possible and may help to improve down-streaming processes.

5. Conclusion

Objective of this work was to determine what kinds of proteins are extractable with microwave and plasma treatment, and further, if there are any differences between selected protein groups. Spark discharge plasmas and microwave exposures have shown to achieve comparable results, i.e. in particular extraction yields. Moreover, for thermolabile compounds it could be shown that spark plasmas are gentler and thus, can be recommended for the extraction of these substances. Possible chemical modifications of extracted proteins due to reactive species generated during plasma propagation should be topic of further studies. However, searching peptide fragments against the *Chlorellales* database already provided instructive information with respect to applications. A complete annotation of *Chlorella vulgaris* is, nevertheless, necessary for a deeper understanding of protein origins and possible plasma effects.

The plasma approach itself can be further improved, e.g. by shortening treatment times while increasing spark repetition rates. This is especially applicable towards a more comprehensive detachment of chlorophyll *a–b* binding proteins and histones from cell compartiments. In addition, different pulse durations might enhance extraction quantitatively and qualitatively.

Contributions

KZ designed and performed experiments, analysed data, wrote the paper; JL analysed data, wrote paper, critically revised article for intellectual content; JV designed and performed experiments; LS created database, critically revised article for intellectual content; KW conception and design of experiments, critically revised article for intellectual content; ML, KDW final approval of the article; JK wrote paper, critically revised article for intellectual content; all the authors read and approved the manuscript.

Acknowledgement

Many thanks to Heiko Krause for his kind support on the bioinformatics part. Dres. Henrike Brust and Nicola Wannicke from the Leibniz Institute of Plasma Science and Technology, Greifswald, for their cordial advice on plant physiology.

This work was partially funded by the Federal Ministry of Education

and Research (grant number 03Z22DN12 to K.W.).

The authors have no conflicts of interests, financial or otherwise, to declare. No conflicts, informed consent, human or animal rights applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101416.

References

[1] A. Richmond, Handbook of Microalgal Culture: Biotechnology and Applied Phycology, John Wiley & Sons, 2008.
[2] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, Renew. Sust. Energ. Rev. 14 (1) (2010) 217–232.
[3] M. Olaizola, Commercial development of microalgal biotechnology: from the test tube to the marketplace, Biomol. Eng. 20 (4–6) (2003) 459–466.
[4] I. Sousa, et al., Microalgae in novel food products, Food Chem. Res. Dev. (2008) 75–112.
[5] A. Converti, et al., Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production, Chem. Eng. Process. Process Intensif. 48 (6) (2009) 1146–1151.
[6] S. Mundt, B.H., M. Preisitsc, S. Kretilow, H.T.N. Bui, H.T. Pham, E. Zainuddin, T.T. Le, G. Lukowski, W.D. Jülich, Microalgae – a promising source of novel therapeutics, JSM Biotechnol. Bioeng. 2 (1) (2014) 1.
[7] H. Zheng, et al., Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves, Appl. Biochem. Biotechnol. 164 (7) (2011) 1215–1224.
[8] R.E. Teixeira, Energy-efficient extraction of fuel and chemical feedstocks from algae, Green Chem. 14 (2) (2012) 419–427.
[9] R. Halim, M.K. Danquah, P.A. Webley, Extraction of oil from microalgae for biodiesel production: a review, Biotechnol. Adv. 30 (3) (2012) 709–732.
[10] G. Yoo, et al., An effective, cost-efficient extraction method of biomass from wet microalgae with a functional polymeric membrane, Green Chem. 16 (1) (2014) 312–319.
[11] K. Zocher, et al., Comparison of extraction of valuable compounds from microalgae by atmospheric pressure plasmas and pulsed electric fields, Plasma Med. 6 (3–4) (2016) 273–302.
[12] J.-Y. Lee, et al., Comparison of several methods for effective lipid extraction from microalgae, Bioresour. Technol. 101 (1) (2010) S75–S77.
[13] A. Guldhe, et al., Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production, Fuel 128 (2014) 46–52.
[14] P. Šunka, Pulse electrical discharges in water and their applications, Phys. Plasmas 8 (5) (2001) 2587–2594.
[15] B. Sun, S. Kunitomo, C. Igarashi, Characteristics of ultraviolet light and radicals formed by pulsed discharge in water, J. Phys. D. Appl. Phys. 39 (17) (2006) 3814.
[16] M.T. Guarnieri, et al., Examination of triacylglycerol biosynthetic pathways via de novo transcriptomic and proteomic analyses in an unsequenced microalga, PLoS One 6 (10) (2011) e25851.
[17] M.J. Reijnders, et al., Green genes: bioinformatics and systems-biology innovations drive algal biotechnology, Trends Biotechnol. 32 (12) (2014) 617–626.
[18] UniProt Consortium, UniProt: the universal protein knowledgebase, Nucleic Acids Res. (2016) ([cited 2017 March]; Available from: https://www.uniprot.org/).
[19] C. Schulze, et al., Screening of microalgae for primary metabolites including β-glucans and the influence of nitrate starvation and irradiance on β-glucan production, J. Appl. Phycol. (2016) 1–7.
[20] J.C. Oliveros, Venny. An interactive tool for comparing lists with Venn's diagrams, Available from: http://bioinfogp.cnb.csic.es/tools/venny/index.html, (2007–2015) [cited 2018 March 26th].
[21] J.-W.L. Katja Zocher, J. Volzke, L. Steil, M. Lalk, K.-D. Weltmann, K. Wende, J.F. Kolb, Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures Mendeley Data, 1 (2018), https://doi.org/10.17632/rfr63zkxz7.1.
[22] J.A. Vizcaíno, et al., 2016 update of the PRIDE database and its related tools, Nucleic Acids Res. 44 (D1) (2015) D447–D456.
[23] J.-W.L. Katja Zocher, J. Volzke, L. Steil, M. Lalk, K.-D. Weltmann, K. Wende, J.F. Kolb, Comparison of Protein Extraction Using Microwave and Spark Discharges in Chlorella vulgaris, ProteomeXchange 1 (2018).
[24] H. Li, et al., Enhanced expression of antifreeze protein genes drives the development of freeze tolerance in an Antarctica isolate of Chlorella vulgaris, Prog. Nat. Sci. 19 (9) (2009) 1059–1062.
[25] P. Rizzo, Basic chromosomal proteins in lower eukaryotes: relevance to the evolution and function of histones, J. Mol. Evol. 8 (1) (1976) 79–94.
[26] S. Lindquist, E. Craig, The heat-shock proteins, Annu. Rev. Genet. 22 (1) (1988) 631–677.
[27] E. Vierling, The roles of heat shock proteins in plants, Annu. Rev. Plant Biol. 42 (1) (1991) 579–620.
[28] W.R. Swindell, M. Huebner, A.P. Weber, Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways, BMC Genomics 8 (1) (2007) 125.
[29] J. Lackmann, et al., A dielectric barrier discharge terminally inactivates RNase A by oxidizing sulfur-containing amino acids and breaking structural disulfide bonds, J. Phys. D. Appl. Phys. 48 (49) (2015) 494003.
[30] X. Lu, et al., Reactive species in non-equilibrium atmospheric-pressure plasmas: generation, transport, and biological effects, Phys. Rep. 630 (2016) 1–84.
[31] J.-W. Lackmann, et al., Chemical fingerprints of cold physical plasmas–an experimental and computational study using cysteine as tracer compound, Sci. Rep. 8 (1) (2018) 7736.
[32] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of Chlorella vulgaris and other microalgae for biofuels production, Planta 237 (1) (2013) 239–253.
[33] Y. Gao, et al., Evaluation of sample extraction methods for proteomics analysis of green algae Chlorella vulgaris, Electrophoresis 37 (10) (2016) 1270–1276.
[34] P.S. Hegde, I.R. White, C. Debouck, Interplay of transcriptomics and proteomics, Curr. Opin. Biotechnol. 14 (6) (2003) 647–651.
[35] S.B. Wang, et al., Cell wall proteomics of the green alga Haematococcus pluvialis (Chlorophyceae), Proteomics 4 (3) (2004) 692–708.
[36] A. Campos, et al., Effects on growth, antioxidant enzyme activity and levels of extracellular proteins in the green alga Chlorella vulgaris exposed to crude cyanobacterial extracts and pure microcystin and cylindrospermopsin, Ecotoxicol. Environ. Saf. 94 (2013) 45–53.
[37] D.I. de Pomerai, et al., Growth and maturation of the nematode Caenorhabditis elegans following exposure to weak microwave fields, Enzym. Microb. Technol. 30 (1) (2002) 73–79.
[38] D.I. de Pomerai, et al., Microwave radiation can alter protein conformation without bulk heating, FEBS Lett. 543 (1–3) (2003) 93–97.
[39] M. Griffith, K.V. Ewart, Antifreeze proteins and their potential use in frozen foods, Biotechnol. Adv. 13 (3) (1995) 375–402.
[40] S. Venketesh, C. Dayananda, Properties, potentials, and prospects of antifreeze proteins, Crit. Rev. Biotechnol. 28 (1) (2008) 57–82.
[41] K. Georgieva, Some mechanisms of damage and acclimation of the photosynthetic apparatus due to high temperature, Bulg. J. Plant Physiol. 25 (3–4) (1999) 89–99.
[42] R. Bassi, F. Rigoni, G.M. Giacometti, Chlorophyll binding proteins with antenna function in higher plants and green algae, Photochem. Photobiol. 52 (6) (1990) 1187–1206.
[43] P.A. Armond, U. Schreiber, O. Björkman, Photosynthetic acclimation to temperature in the desert shrub, Larrea divaricata: II. Light-harvesting efficiency and electron transport, Plant Physiol. 61 (3) (1978) 411–415.
[44] A. Badura, et al., Light-driven water splitting for (bio-)hydrogen production: photosystem 2 as the central part of a bioelectrochemical device, Photochem. Photobiol. 82 (5) (2006) 1385–1390.
[45] B. Esper, A. Badura, M. Rögner, Photosynthesis as a power supply for (bio-)hydrogen production, Trends Plant Sci. 11 (11) (2006) 543–549.
[46] M. Chaboute, et al., Histones and histone genes in higher plants: structure and genomic organization, Biochimie 75 (7) (1993) 523–531.
[47] Kanehisa Laboratories, KEGG Genome Database. [Database] 2018 July 5th, Available from: http://www.genome.jp/kegg/genome.html, (2018) [cited 2018 July, 11th].
[48] Y. Saidi, A. Finka, P. Goloubinoff, Heat perception and signalling in plants: a tortuous path to thermotolerance, New Phytol. 190 (3) (2011) 556–565.
[49] Oyler, G. and J. Rosenberg, Enhanced gene expression in algae. 2014, Google Patents.
[50] P. Klatt, S. Lamas, Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress, Eur. J. Biochem. 267 (16) (2000) 4928–4944.
[51] M. Ilbert, et al., The redox-switch domain of Hsp33 functions as dual stress sensor, Nat. Struct. Mol. Biol. 14 (6) (2007) 556.
[52] S. Bekeschus, et al., Hydrogen peroxide: a central player in physical plasma-induced oxidative stress in human blood cells, Free Radic. Res. 48 (5) (2014) 542–549.
[53] R. Banaschik, et al., Fenton chemistry promoted by sub-microsecond pulsed corona plasmas for organic micropollutant degradation in water, Electrochim. Acta 245 (2017) 539–548.
[54] J.B. Smith, I. Adams, H.-F. Ji, Biomolecule response to nonthermal plasma, 7 (4) (2017) 427–443.
[55] K. Oehmigen, et al., Estimation of possible mechanisms of Escherichia coli inactivation by plasma treated sodium chloride solution, Plasma Process. Polym. 8 (10) (2011) 904–913.
[56] J.-W. Lackmann, et al., Photons and particles emitted from cold atmospheric-pressure plasma inactivate bacteria and biomolecules independently and synergistically, J. R. Soc. Interface 10 (89) (2013) 20130591.
[57] E. Takai, et al., Chemical modification of amino acids by atmospheric-pressure cold plasma in aqueous solution, J. Phys. D. Appl. Phys. 47 (28) (2014) 285403.
[58] K. Goiris, et al., Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (6) (2012) 1477–1486.
[59] J.G. Scandalios, L. Guan, A.N. Polidoros, Catalases in plants: gene structure, properties, regulation, and expression, Cold Spring Harb. Monogr. Ser. 34 (1997) 343–406.
[60] S. Gebicki, J.M. Gebicki, Formation of peroxides in amino acids and proteins exposed to oxygen free radicals, Biochem. J. 289 (3) (1993) 743.
[61] G.I. Cassab, Plant cell wall proteins, Annu. Rev. Plant Biol. 49 (1) (1998) 281–309.
[62] E. Jamet, et al., Cell wall proteins: a new insight through proteomics, Trends Plant Sci. 11 (1) (2006) 33–39.

Publication P3

Mechanism of Microalgae Disintegration by Spark Discharge Treatment for Compound Extraction

Zocher, K., Rataj, R, Steuer, A., Weltmann, K-D and Kolb, J.F., *Journal of Applied Physics: D*, 2019 (submitted).

Your submission to J. Phys. D: Appl. Phys.: JPhysD-122186

Journal of Physics D: Applied Physics 16.09.2019 12:54

An: katja.zocher, raphael.rataj, anna.steuer, weltmann, juergen.kolb

Bitte Antwort an jphysd

Dear Ms Zocher,

Re: "Mechanism of Microalgae Disintegration by Spark Discharge Treatment for Compound Extraction" by Zocher, Katja; Rataj, Raphael; Steuer, Anna; Weltmann, Klaus-Dieter; Kolb, Juergen
Article reference: JPhysD-122186

Thank you for submitting your Paper, which will be considered for publication in Journal of Physics D: Applied Physics. The reference number for your article is JPhysD-122186. Please quote this number in all future correspondence regarding this manuscript.

As the submitting author, you can follow the progress of your article by checking your Author Centre after logging in to https://mc04.manuscriptcentral.com/jphysd-iop Once you are signed in you will be able to track the progress of your article, read the referee reports and send us your electronic files.

This journal makes manuscripts available to readers on the journal website within 24 hours of acceptance. Please be aware that if you did not tick the relevant opt-out box on the submission form, the accepted version of your manuscript will be visible on the journal's website before it is proof-read and formatted to our house style.

If you are planning any press activity for your article, or are currently engaging in an IP or patent application, you may wish to opt-out of making your accepted manuscript immediately available online. If you do not wish to make the accepted version of your manuscript immediately visible to readers, and have not ticked the opt-out box during submission, please let us know as soon as possible.

Please do not hesitate to contact us if we can be of assistance to you.

Yours sincerely

On behalf of the IOP peer-review team:
Editor-in-Chief - Joan-Ramon Morante
Publisher - Tom Miller
Editor - Sarah Whitehouse
Associate Editors - Polly Russell, Louise Nugent, Tay Sparks, Ian Stokes
Editorial Assistants - Beth Hammond, Emma Wright and Qamar Scott
Production Editors - James Cottle, Kimberley Graves and George Evans
Marketing Executive - Zoe Anderson

Want to find out what is happening to your submission right now? Track your article here:
https://publishingsupport.iopscience.iop.org/track-my-article/?utm_source=Track%20my%20article&utm_medium=Email

jphysd@ioppublishing.org
Twitter: https://twitter.com/JPhysD

IOP Publishing
Temple Circus, Temple Way, Bristol
BS1 6HG, UK

www.iopscience.org/jphysd

IOP Publishing Journal Title
Journal **XX** (XXXX) XXXXXX https://doi.org/XXXX/XXXX

Mechanism of Microalgae Disintegration by Spark Discharge Treatment for Compound Extraction

Katja Zocher, Raphael Rataj, Anna Steuer, Klaus-Dieter Weltmann and Juergen F. Kolb

Leibniz Institute for Plasma Science and Technology (INP), Felix-Hausdorff-Strasse 2, 17489 Greifswald, Germany

E-mail: juergen.kolb@inp-greifswald.de

Received xxxxxx
Accepted for publication xxxxxx
Published xxxxxx

Abstract

Microalgae possess a cell wall with remarkable physical and chemical strength, which proves to be the bottleneck for extraction techniques. With the application of spark discharges instigated by 100 ns high voltage pulses, a novel method was established that was found effective and yet, gentle for the extraction of sensible, especially heat-sensitive, compounds. The spark, which was ignited directly in the submerged algae suspension, is characterised by several physical and chemical processes that might promote a successful disintegration of the cells. Among other characteristics, the conceivably most destructive mechanism, i.e. strong shockwaves, was examined with Schlieren diagnostics to evaluate associated pressures. The results were compared with the tensile strength, determined by atomic force microscopy, of Chlorella vulgaris, which was chosen as model organism. A shockwave pressure of 500 MPa in the close vicinity of the discharge by far exceeded the elasticity modulus of the algae with 13.95 MPa, confirming the potential of mechanically breaking the cell wall. At the same time, bulk temperatures could be maintained close to room temperature by adjusting the operating parameters for the spark-application, hence, encouraging especially the extraction of thermally unstable intracellular substances.

Keywords: spark discharges, shockwave, microalgae, extraction, tensile strength

1. Introduction

The role of microalgae for biotechnologies has been considerably increasing during the last decades [1]. The major benefit of these photosynthesis performing, aquatic microorganisms is their ability to grow under harsh conditions [2]. A cultivation is simple at low economic costs because only water, light and nutrients are necessary for sufficient growth. The metabolism of valuable compounds in high amounts inside the organism, such as lipids, carbohydrates proteins or pigments have shown to be of high interest for biofuel production or as pharmaceutical or nutraceutical feedstock [3]. Major obstacle for their retrieval is the chemically complex and structurally robust cell wall of microalgae [4], which makes the extraction of compounds an involved and expensive process.

Common extraction techniques can be categorised either as mechanical methods, e.g. sonication, homogenisation, or microwave radiation, or methods based on chemicals, such as acids, bases, surfactants, organic solvents, and enzymes. All these methods have drawbacks, for instance heat development, structural degradation of compounds, high-energy demand, or long treatment times, altogether adversely affecting the value-added chain. Therefore, an industrial need for alternative methods has to be met by novel approaches.

In previous studies, promising results have been achieved for the application of spark discharges, which have shown to be an effective and yet, gentle alternative extraction method. The spark discharges were ignited directly in the algal suspension. As model organism, Chlorella vulgaris was used, which is a well-known green microalga with a remarkable sturdy cell wall that gives the alga outstanding chemical and mechanical robustness. The three layers of cell wall consist, e.g. of cellulose, hemicellulose, and glycoproteins [5] [6]. In comparison with classical methods, similar or even better extraction yields were achieved after spark discharge exposure, without degrading the extractives. It was also found that for proteins, which were analysed with proteomics, the type of extracted protein could be directed. For instance, heat sensitive proteins, such as photosystem-related proteins or histones were found in higher amounts for spark discharges than after the treatment with the classical method. Higher yields were also found for pigments, especially chlorophyll a and b extracts, than with the reference method, which kept their lush green colour. The procedure, based on a direct exposition of the algae to the plasma effects, made the addition of any chemicals during the spark discharge treatment unnecessary, which constitutes an important ecological advantage of the method.

Spark discharges in general are characterised by various physicochemical properties. The generation of reactive species, strong electric fields, UV-light emissions, high temperatures and overpressure shockwaves [7] [8] are provoked by the plasma channel and could contribute to the observed effects on the cell wall. Other plasma sources that were studied in our previous work, such as dielectric barrier discharges (DBDs), pin-to-liquid discharges above the suspension and corona discharges or plasma jets submerged in the suspension had only a minor effect on the cell wall. This indicates that unique features of the spark discharges, i.e. shockwaves, seem to exert strong mechanical forces, affecting the microalgal cell walls. Reactive species provided by plasma had no apparent effect, although DBDs and plasma jets are known for the formation of high amounts of radicals. Besides radical formation, especially corona discharges are also associated with electric fields and shockwaves. However, no respective effect on the cell wall was observed. The shockwaves from the corona discharge seem to be less pronounced and distinct and experiments with pulsed electric field exposures of similar strength did likewise not result in extraction yields comparable to the spark discharges [9]. Another distinct characteristic of spark discharges are high temperatures in the discharge channel. However, these do not necessarily seem to affect the extracts as the preservation in particular of pigments shows. As long as the repetition frequency for the sparks is adjusted accordingly, bulk temperatures can be kept low and a degradation of extracted compounds can be avoided. In this work, the repetition rate was therefore set to 4 Hz, so that the temperature of the suspension never exceeded 25 °C.

For a more detailed evaluation of the shockwave hypothesis, shockwave pressures and expansions for the spark discharges were determined by Schlieren diagnostics. The findings were compared to the tensile strength of Chlorella vulgaris, which was ascertained by atomic force microscopy on viable cells.

2. Material and Methods

2.1 Algae cultivation and harvesting

Chlorella vulgaris (SAG 221-12, Experimental Phycology and Culture Collection of Algae, University of Goettingen, Germany) was cultured photoautotrophically in ventilated Schott flasks (volume 500 ml), aerated with ambient air at a flow rate of ca. 1.5 vvm. The cells were grown in Bold's Basal

modified medium (Sigma Aldrich, Taufkirchen, Germany) with the temperature kept at 20±2 °C. Illumination was conducted with white fluorescent lamps (T5 LongLastTM F39W/840/LL", GELightning) with 50 µmol photons $m^{-2}s^{-1}$ for light:dark cycles of 12:12 hours [10] [9].

Algae were harvested between day 30 and 42 after inoculation when an adjusted optical density (OD) of 0.8 at 750 nm (Shimadzu UV-1280 spectrophotometer) was attained in a final volume of 50 ml. After harvesting, the suspension was centrifuged at 3000 g for 10 minutes (centrifuge 5810R, Eppendorf Vertrieb Deutschland GmbH, Germany), washed with 20-ml-deionised water and centrifuged again for seven more minutes at 3000 g. Subsequently, cells were resuspended with deionised water in a final volume of 50 ml. The cell suspensions were kept on ice for further use.

2.2 Electrical setup

Spark discharges were ignited inside a polymethyl-methacrylate (PMMA) housing, filled with the algae suspension. The dimensions of the housing were 7x8x1 cm, holding a volume of approx. 1 ml. The top and bottom of the housing were connected with plastic tubes to allow for recirculation of the suspension through the device. Front- and backside of the housing were fitted with glass windows for observation and Schlieren diagnostics. Discharges were ignited between two tungsten rods with 2 mm in diameter and a gap distance of 0.5 mm. To prevent algae from settling, the suspension was moved in a continuous flow by a peristaltic pump (TL 150, medorex Messtechnik + Bio-Verfahrenstechnik, Germany). The treatment system of discharge chamber, expansion tank and tubes held a total volume of 50 ml. A flow rate of 50 ml/ min was set to ensure a complete treatment of the entire suspension volume in one minute. For an unambiguous analysis of microscopic images, respective exposures were conducted for an extended treatment time of 30 minutes. A schematic of the experimental setup is shown in Figure 1.

Positive pulses of 100-ns width were generated by a Blumlein line pulse-forming network (BPFN) and applied with a repetition rate of 4 Hz. In this case, the temperature of the suspension did not exceed 25 °C. For cell disintegration, the pulses were applied for a mismatched load, resulting in a train of pulses with decreasing amplitudes until a spark was instigated, usually for the third to fifth pulse in the train. The required pulse amplitude was in the range from 20-35 kV. The BPFN was charged by a high-voltage DC power supply (PS/EQ060R020-22, Glassman High Voltage Inc., High Bridge, NJ, USA) and the circuit closed by a self-breakdown spark gap switch, pressurized with air. Voltage and current across the electrode gap were monitored with a passive high voltage probe (Tektronix P6015A, Tektronix Inc., Beaverton, OR) and Rogowski coil (Model 5046, Pearson electronics, Palo Alto, CA), respectively. Both parameters were recorded on an oscilloscope (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY). A typical waveform is shown in Figure 2. In this graph, voltage (black line), current (red line) and a reference pulse without spark ignition (grey line) are displayed. A spark was associated with an abrupt drop in voltage and concurrent increase in current. Without discharge, the current is negligible on the displayed scale.

In general, algae were prepared and treated as described in section 2.1, but for the observation of shockwaves, distilled water and a needle-to-needle configuration (tungsten, curvature radius=164 µm) was used. This was necessary because even low algae densities disturbed the imaging processes due to absorption and an evaluation of shockwaves was not possible. Moreover, in the rod-to-rod-system, reflections of shockwaves between the metal rods prevented the localisation of the plasma channel and a correct calculation of shockwave pressures. For Schlieren imaging, the system was also matched with an impedance of 50 Ω, connected in parallel to the electrodes to allow for precise synchronisation of the diagnostics. For the unmatched system with varying spark ignitions, a triggering and thus, synchronisation would not have been possible.

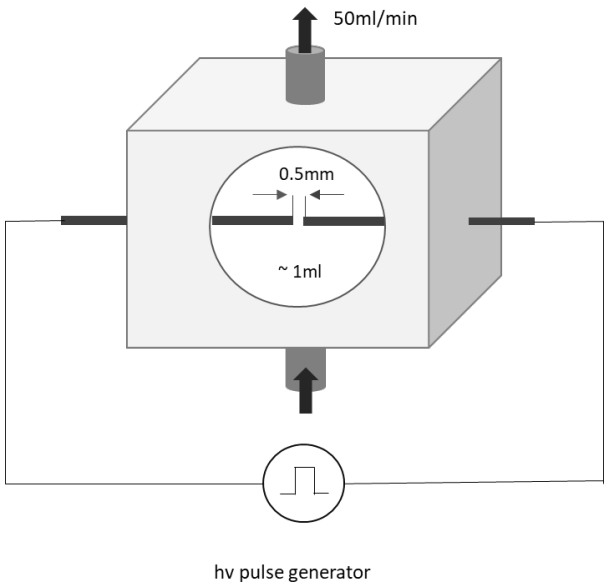

Figure 1 Schematic image of the reaction chamber, as described in section 2.2, for the treatment of the algae suspension in a continuous flow system. The system was operated with a 100 ns Blumlein line pulse-forming network (BPFN).

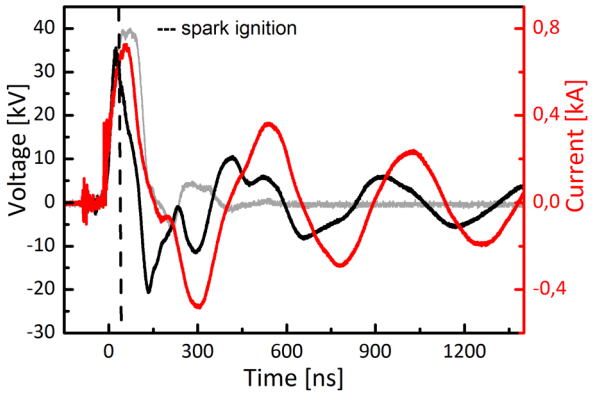

Figure 2 Voltage (black line) and current (red line) for a 100-ns pulse, applied with a Blumlein line pulse-forming network. A spark was ignited when (indicated by the dashed line) the voltage dropped and the current increased simultaneously. For comparison, a high voltage pulse that did not result in the generation of a spark is also shown (grey line).

2.3 Microscopic images

Images of treated and untreated cells were recorded on an inverted microscope (Axio Observer D1 Carl Zeiss, Berlin, Germany). An oil-immersion objective (EC Plan-Neofluar with 100 x/ 1.30 oil magnification, Phase 3/ M27, Carl Zeiss, Berlin, Germany) was chosen together with an immersion oil (Type F immersion liquid, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). A drop of the respective cell suspension was placed onto a glass slide under a cover slip. This procedure was necessary due to a small cells size, and therefore a high magnification of 100X was needed. Fixation would have been detrimental for the study of the cell surface, especially for the untreated living cells.

2.4 Atomic force microscopy (AFM)

2.4.1 Equipment

Elasticity measurements of *Chlorella vulgaris* were conducted with an atomic force microscope (AFM, Nano-wizard 3 BioScience, JPK Instruments, Berlin, Germany and Observer Z1, Carl Zeiss, Berlin, Germany). Force curves were collected with a triangular silicon nitride cantilever of 225 µm in length, a width of 20 µm, at a resonant frequency of 15 kHz. The tip of the cantilever had a radius of 20 nm and a nominal spring constant of 0.03 N/m (MLCT, Bruker, Camarillo, California). The spring constant was determined before each experiment with the thermal noise method.

2.4.2 Fixation of viable cells

To investigate living untreated microalgae without damaging them by drying and shrinking, AFM-measurements had to be carried out in the liquid medium. Fixing the cells at least in place was nevertheless obligatory. Due their spherical shape and rigidity, they tended to move away from the cantilever under pressure, otherwise.

The fixation was achieved by adsorption. Therefore, petri dishes (TC-dish 35 cell+, Sarstedt AG&Co, Nümbrecht, Germany) were conditioned according to A.K. Lee et al. [11]. Petri dishes were cleansed thoroughly with isopropanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and subsequently air-dried. Afterwards, 3 ml poly-L-lysine solution (0.1 % (w/v) in H2O solution, MW 150,000-300,000, Sigma Aldrich, Germany) was pipetted into the dishes, incubated at room temperature for 24 hours and stored. The poly-L-lysine solution was discarded before AFM-analysis and the petri dishes were gently dried by nitrogen gas. A volume of 100 µl of the algae suspension was diluted with 1 ml-deionised water and incubated for one hour at room temperature. Prior to the measurements, the dishes were carefully filled with additional 1 ml of deionised water. Calibration of the laser system was conducted with incubated petri dishes and 2 ml of deionised water.

2.4.3 Imaging

Immobilised cells were first located with the optical microscope and positioned directly under the cantilever. Cells were scanned in contact mode and quantitative imaging™ (QI™, Nano-wizard 3 BioScience, JPK Instruments, Berlin, Germany) to obtain force curves, which were recorded with a 64px x 64px resolution. The maximum applied force was 4 nN at a z-length of 3 µm and a frequency of 50 kHz. Ten independent measurements on each of the altogether 27 different microalgae were recorded for a statistical analysis of the force curves.

2.4.4 Data analysis

For image processing, the JPK data processing (JPK Instrument, Berlin, Germany) software was used. The Poisson ratio of 0.5 was chosen according to Chopinet et al. [12] and calculations were conducted by applying a Hertz model fit for biological samples.

2.5. Schlieren diagnostics

2.5.1 Schlieren imaging

A setup for Schlieren imaging was adopted according to Schmidt-Bleker et al. [13]. Light from a green laser (532 nm) was sent first through two collecting lenses (focal lengths of 30 and 200 mm), to expand the illuminated area to about 80 mm². After passing the region of interest, the light was focussed by an optical filter (532 ± 0.6 nm, FWHM = 3 ± 0.6 nm) and a collecting lens (focal length of 200 mm) onto the edge of a razor blade. The transmitted light was collected with a lens (focal length of 300 mm; all lenses and filters were provided by Thorlabs GmbH, Dachau, Germany) onto a framing camera (Specialised Imaging Ltd, Pitstone, UK). The combination of a green laser together with the filter was selected, since the plasma barely emits light at this wavelength. The camera had a recording time of 10 ns and was able to detect in four images (*i = 0, 1, 2, 3)* for the same event the development of individual shockwaves for subsequent 100-ns time steps. This allowed for a precise determination of propagation velocities and consequently pressure profiles.

2.5.2 Shockwave velocity and pressure

Radius and point of origin (centre) of a spherical expanding shockwave were determined from the first image (*i = 0*). The shock wave radius, r_i, according to each recording and the average propagation velocity, $u_{s,i}$, for the time Δt between subsequent images, could then be derived (Eq. 1):

$$u_{s,i} = \frac{r_i - r_{i-1}}{\Delta t}, \quad i = 1, 2, 3 \qquad (1)$$

Eventually, the shockwave pressure, P, could be calculated from this information from the Hugoniot equation (Eq. 2) [14]:

$$P = \rho_0 u_s \frac{(u_s - A)}{B} \qquad (2)$$

For the density of water, ρ_0, a value of 1 g/cm^3 was assumed and the constant A equals the velocity of sound in water of 1.45 km/s. The parameter B is a correction factor, which was experimentally determined by Nagayama et al. with a value of 1.99 [15].

3. Results

Microscopic images revealed deformed cells after spark discharge exposure and confirmed the assumption of physical damage to cells and cell walls. From the AFM analysis, the tensile strength of *Chlorella vulgaris* was obtained, revealing consistently higher strength than previously reported generally for microalgae in literature [16]. However, the shockwave pressure was found to be sufficiently high to overcome the cells' tensile strength, which in return defines the stress needed to disrupt the cell walls. Accordingly, in addition to *Chlorella vulgaris*, successful cell wall rupture by spark exposures was also found for *Nannochloropsis occulata* and *Cyanidium caldarium* when comparing the protein content in the supernatant with a standard extraction method. However, their even smaller size (< 5 µm) prevented a meaningful microscopic examination and, hence, results are not shown here.

3.1 Microscopic images

The possible effect on the integrity of the algal cell wall was visualised microscopically. Before treatment, cells exhibit an evenly round shape and a smooth surface as can be seen in Figure 3 (panel *a*). After spark exposure, cells appeared shrunk and the surface seemed uneven (panel *b*). Moreover, cell compartments seemed to detach from the organelles as a result of the damaged cell walls, consolidating the response to an external force. This is consistent with a proteomic analysis presented previously, which found that components, e.g. proteins, already disperse into the solution during the plasma treatment. In addition, compounds from inner compartments (chloroplasts, nuclei etc.) were also found in the liquid, which further proofed a subcellular plasma effect [17]. The observed damages that is shown in Figure 3 (panel *b*), demonstrates the result of this comprehensive collapse of the cells.

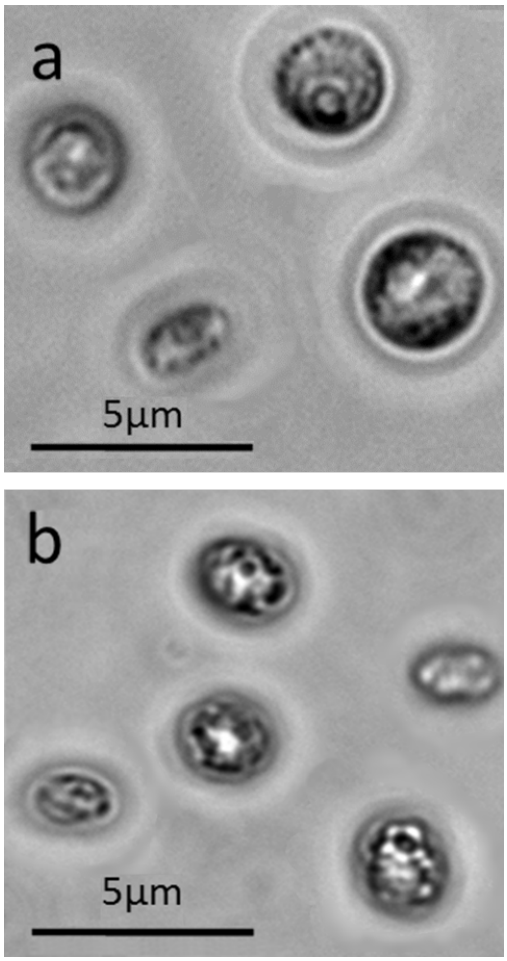

Figure 3 Microscopic images of Chlorella vulgaris cells before (panel a) and after spark discharge treatment (panel b). Before treatment, cells appeared round and smooth, but after treatment, damage to the surface and to subcellular compartments became visible.

3.2 Elasticity module measurements

To determine the elasticity module, and hence, tensile strength of algae, atomic force microscopy was employed. After the cantilever had scanned region of interest, 2D images and 3D profiles of the cells were obtained as shown in Figure 4, panel *a* and *b*, respectively. Bright parts in the middle of the cell indicate the areas for which the elasticity was determined. Every cell was scanned across its entire

size; however, elasticity modules derived for the cell edges were disregarded due to the strong contribution of the underlying surface

From 27 scanned cells, an average elasticity modulus of 13.95 MPa with a standard deviation of 4.5 MPa was derived. However, cells vary in size and shape and hence, values for the tensile strength differ due to that diversity. Interestingly, actual values seem to be independent in particular from cell size, i.e. smaller cells are not harder than larger cells, but might be specie-dependent in accordance to the respective cell wall composition. An AFM-analysis was not attempted for cells that were treated by spark discharges since the respective values of the elasticity modules for the impaired cells would not have any meaning.

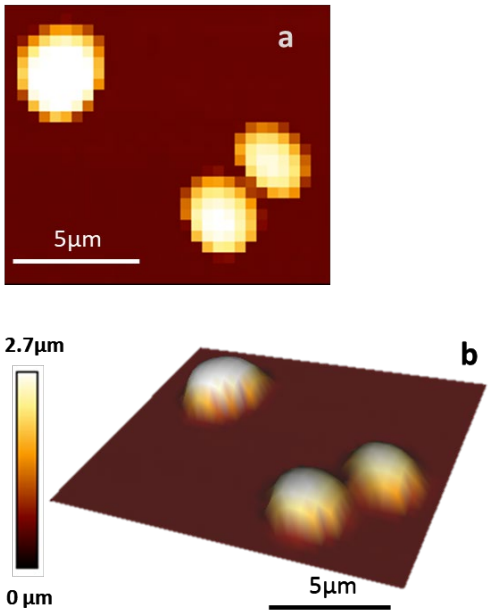

Figure 4 AFM images of untreated algae cells. Panel a displays the 2D image with the height specified by the indicated colour-scale. Panel b shows the corresponding 3D profile of the cells.

3.3 Shockwave pressure

Shockwave pressures were calculated by fitting the radii r_0, r_1, r_2, and r_3 of the shockwave fronts in the recorded images as described in section 2.5.2. As already mentioned in section 2.2, Schlieren imaging was only possible in distilled water due to the strong absorption of light in algae suspensions, even for low cell densities. Figure 5 shows the time-resolved propagation of the shockwave front from 1,100 ns to 1,400 ns in consecutive images of the same discharge event. Arrows indicate the radii that were determined for the respective expansion of the shockwave front. The average propagation velocity of the front was calculated with equation (1) from the radii of subsequent images. With the obtained velocities, the pressure was then derived from the Hugoniot equation (2) as described in section 2.5.2. The pseudo-colouring in the images is based on a linear scale for the spatial resolved light intensities for each of the four individual sensors of the framing camera display the localisation of the shockwave.

Figure 6 summarises the results for the shockwaves generated by altogether 100 independent discharge events and the derived pressures for the propagation of the respective shock fronts for different times after spark initiation, i.e. across the distance that was accessible to observation and reactor walls, respectively. The graph shows, accordingly, how the pressure decreased with distance from the discharge origin. The large errors bars were due to the quadratic dependency between pressures and velocities (c.f. Eq. 2). The shockwave radius could, constricted by the resolution of the camera, also only be determined down to five pixels, corresponding to a spatial resolution of 20 µm. In addition, within the camera opening time for individual images of 100 ns, the shockwave expanded only by another 180 µm between two frames (assuming a velocity of 1.8 km/s). Altogether, this resulted in errors of about 10 % for individual pressure values. The pressure in close vicinity of the discharge, i.e. at 0.6 mm distance from the centre between the electrodes, reached values of about 500 MPa ±42 MPa. At a distance of 2.4 mm, the pressure had dropped to 140 MPa ±10 MPa. For comparison, the range of values that was determined for the tensile strength of *Chlorella vulgaris* is also indicated in the graph as dash-dotted line along the horizontal axis. Notable, shockwave pressures exceeded the tensile strength of the microalgae by far. The pressures would still be sufficient to break the cells, even taking into account the variations that are described by the error bars.

For a further evaluation of the pressure-wave, especially at larger distances, the decrease of the pressure with distance was fitted to a generic $1/r^2$-dependence (dashed red line in Figure 6) [18]. This suggests that the average shockwave pressure at a distance of 20 mm would still be in the order of 20 MPa. At a distance less than 20 mm, the pressure would eventually become comparable to the tensile strength of the algae and presumably, more and more cells would stay intact, accordingly.

.

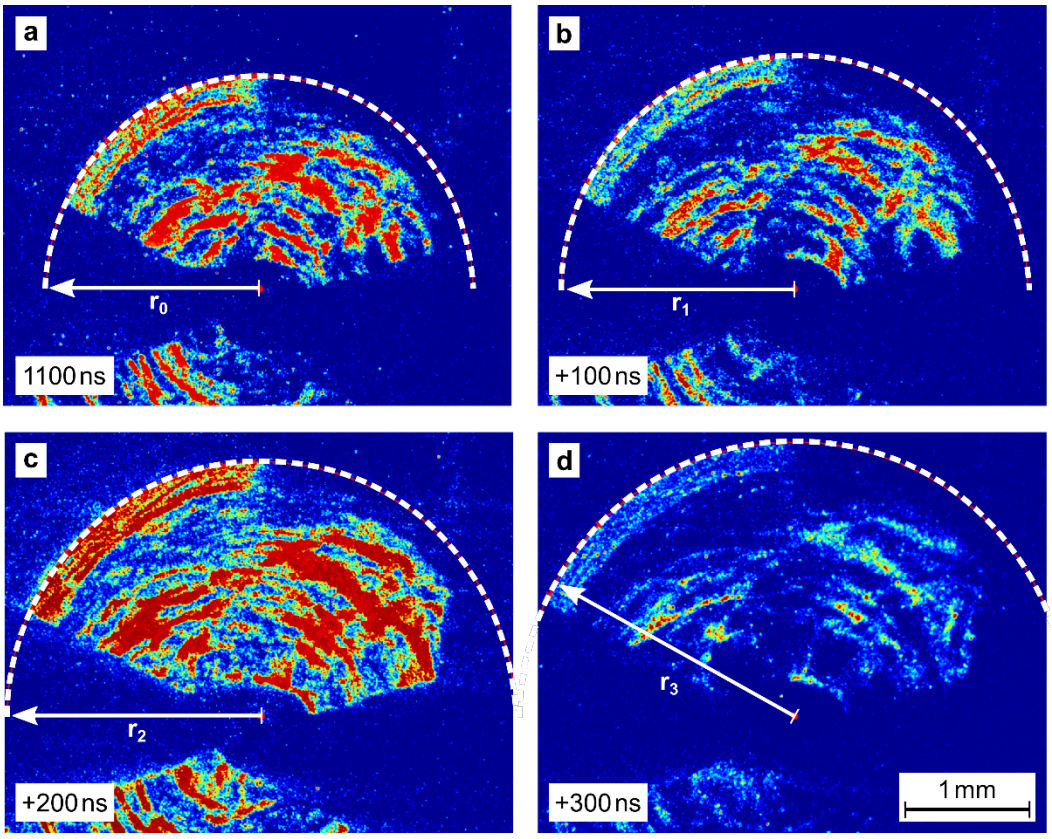

Figure 5 Time-resolved radial propagation of a shockwave front in a needle-to-needle configuration, visualized in consecutive images for subsequent time steps of 100 ns (panel a-d).The different radii of the front are indicated by arrows starting from the centre between the needle electrodes. (Images were reformatted to the same size for presentation) The pseudo-colours in the images describe the recorded intensities on a linear scale. The propagation speed and eventually the pressure in the front were determined from the radii and the time steps between images.

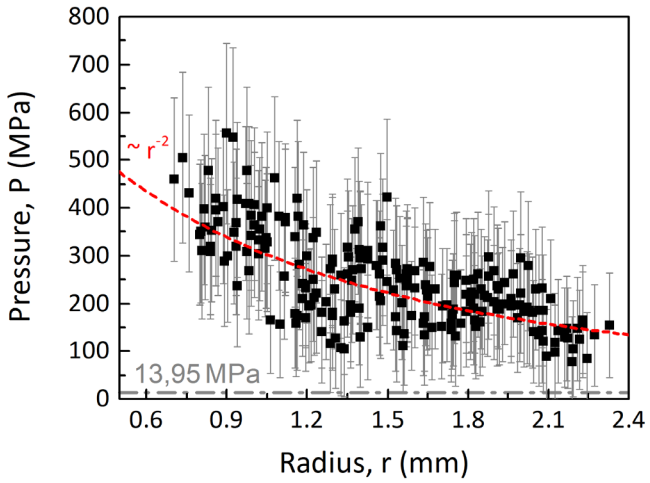

Figure 6 Shockwave pressure in relation to the distance from the centre of the electrode gap of the needle-to-needle discharge configuration. The fit (dashed red line) illustrates a decrease in pressure, P, which is inverse squared proportional to the distance, r, from the centre of the discharge. The grey dash-dotted line along the horizontal axis indicates the pressure range, in which algae might withstand the shockwave pressure.

4. Discussion

The tensile strength of Chlorella vulgaris and the pressure of shockwaves, generated during spark discharges, were determined with AFM and Schlieren diagnostics, respectively. The comparison proofed that the applied plasma-generated shockwave pressure is sufficient to rupture the cell wall of Chlorella vulgaris, as it was already suggested in our previous work [9] [17].

Spark discharges in liquids are fast established but short-lived plasma channels between two electrodes that develop upon the application of a sufficiently high voltage [19]. The process is associated with a sudden drop of the applied voltage and a concurrent rapid increase in current. The respective voltage-current characteristic for the investigated configuration is depicted in Figure 2, where the spark initiation is indicated by a vertical dotted line. The on average dissipated energy in the discharge was calculated from current and voltage measurements with 2 J/ pulse, when the electrode gap was matched by an additional resistance in parallel to the impedance of the BPFN. (In this case, only a single pulse is applied and initiating the electrical breakdown in the gap.) For unmatched pulses, as they were applied for the cell rupture experiments, even lower energies of 0.2 J/pulse were determined for the sparks. In this case, the resistance across the gap, higher than the impedance of the high voltage pulse generator, results in a train of pulses with amplitudes that are sufficient for a breakdown of the gap already with a lower charging voltage. Accordingly, the pressures in the shock fronts might actually be lower than was observed for the matched case. However, the observed damage to the algae confirms that they were still sufficient to compromise cell walls An adequate effect on cells over the treatment time of 30 minutes with possibly weaker shockwaves is still assured. Eventually, these considerations have to be investigated in more detail for a further development of the method, where the expansion of the shockwaves and respective energy needs have to be put in relation with desired extraction yields and efficiencies for the design of larger systems.

The electrical energy that is provided by a high voltage pulse is generally dissipated in a spark plasma channel in aqueous solutions by UV light emission, radiated electromagnetic fields, the generation of reactive species, temperature increase, and the formation of shockwaves [20] [21]. A quantitative analysis of the energy partitioning into each phenomenon is difficult and strongly depends on the specifications of the investigated system and especially on the parameters of the applied high voltage pulses [22]. For microsecond pulses, it was suggested that the energy in the electromagnetic radiations can be about 30 % of the input energy [23] and light emission may account for less than 5 % [24] [25]. In comparison, Robinson et al. calculated an energy share of 36 % for UV light emissions in spark discharges that are developing under water for microsecond pulses [26]. The remaining electrical energy is assumed to be converted primarily into mechanical work [22], i.e. shockwaves and increasing temperatures. However, the energy partition for nanosecond pulses may be different from discharges instigated with microsecond pulses. Especially, together with the pulse duration also much shorter time of nanosecond high-voltage pulses, and corresponding much shorter time lags preceding breakdown initiation, might change the distribution. Since this interesting topic is beyond the scope of the presented study, it is recommended for future research efforts, which will in return help to further to explore the potential of spark discharges that are instigated with short high-voltage pulses. Regardless, the possible different mechanisms that are provided by a spark discharge submerged in aqueous suspensions and their possible contributions towards the observed cell wall damage will be discussed in the following paragraphs.

As mentioned above, in particular for the generation of spark discharges with microsecond pulses, strong emissions

in the visible and UV spectrum of light have been observed [27]. Sun et al. [28] studied the effects of UV-light, shockwaves, and electric fields on the inactivation of *E.coli* bacteria in distilled water, accordingly, by applying spark discharges in a needle-to-plate system with similar electrical parameters as in this work. They found that for peak voltages of 16 kV, 18 kV, and 20 kV, the effect of UV-light on inactivation ratios were constant of around 28 % with respect to controls. Thus, no convincing evidence was found that UV-light exposures are responsible for the inactivation. Notable, results were independent from the distance of the bacteria to the plasma channel. *E.coli* is a gram-negative bacterium, which means the cell wall is thin and sensitive for environmental effects. In comparison, *Chlorella vulgaris* is known for its sturdy cell wall, i.e. a damage on the cell wall due to UV-light is therefore not conceivable and it seems highly unlikely that UV-light is contributing to the observed cell wall damages (c.f. Figure 3b).

Electromagnetic radiation, but especially high electric fields that are associated with the high-voltage pulses that are applied, might also play a role in the disintegration of cells. Considering the electrode gap distance of 0.5 mm and an applied voltage of 30 kV, the corresponding electric field of 600 kV/cm between the electrodes would be in principle sufficient to result in the electroporation of cell membranes [29] [30]. With increasing distance from the electrodes, the electric fields are readily decreasing with at least a $1/r^2$-dependence. In our previous work, we could show that neither pulsed electric fields that were applied with a pulse duration of 1 µs and field strengths of 35 kV/cm nor electric fields of 2.7 MV/ cm that were conveyed by a corona discharge, which was generated with 100-ns high voltage pulses, resulted in a significant release of compounds from *Chlorella vulgaris*. Besides, no observable damage to their cell walls was found [9]. Conversely, these pulsed electric field exposures are known to be adequate for the electroporation of mammalian cells or bacteria [31] [32] [33]. Electroporation is known as a consequence of the charging of cell membranes. Contrarily, a charging of the already porous cell wall seems unlikely. Hence, electroporation of the algae cell walls due to high field strength around the electrodes can be considered negligible.

Plasmas are also known for the generation of reactive nitrogen and oxygen species (RONS), which might disintegrate molecular bonds and, accordingly, decompose cell wall constituents. Especially dielectric barrier discharges (DBDs) are known for their potent RONS chemistry [34]. Smith et al. observed damages on bacterial cell walls when applying plasma from a floating DBD and found that the most likely reason for that damage was the collapse of amino acids (as part of the cell wall) caused by radicals formed by the plasma [35]. Contrarily, Laroussi et al. observed differences between the inactivation of gram-negative and gram-positive bacteria with a resistive barrier discharge [36]. The group found that the plasma lysed gram-negative bacteria, but no disruption was observed for gram-positive bacteria. The latter are known for a rigid coat, comparable to the cell wall of microalgae. It was suggested that radical formation and radiation processes by the plasma are not effective against these more robust envelopes. These findings were confirmed in our studies, which showed that neither a plasma jet nor a DBD had a relevant effect on microalgal cell walls, although both generate usually high amounts of RONS, especially NO_2^- and NO_3^-. Altogether, RONS formed by spark discharges were unlikely responsible or significantly contributing to the rupture of cell walls of *Chlorella vulgaris*. However, their influence on extracted compounds is still unknown and needs to be investigated further.

A pertinent characteristic of a spark discharge is the high temperature of several thousands of Kelvins inside the plasma channel [37]. Accordingly, the observed cell damage might conceivably be caused by thermal degradation. However, high temperatures are only found at the core of the plasma initially. Once formed, the channel starts to expand and the temperature decreases due to the expansion by radiation and conduction processes. Locke and Thagard have calculated the temperature distribution for a plasma channel in water that was formed by a discharge of 1 Joule. At the centre of the discharge channel 5,000 K were estimated, whereas towards the plasma-bulk-liquid-boundary temperatures had dropped to only 300 K [38]. The temperatures for the investigated spark discharges in the algal suspension were probably in the same range. Obviously, only cells inside the plasma channel or in its very close vicinity are prone to the associated thermal degradation. This would not explain the observed extraction yields, which require a more comprehensive effect on the majority of the algae. Furthermore, extracted compounds would certainly be damaged for these rather high temperatures. Thermal energies were readily equilibrated already within the dimensions of the reaction chamber and bulk temperatures of the suspension close to room

temperature were maintained, depending on operating parameters. Assuming that for an energy per pulse of 0.2 J, applied at a repetition rate of 4 Hz for 30 min, the temperature increase of 50 ml of the suspension would still only be about 7 K. Any losses were hereby in fact neglected, while in the actual experiments the medium was even continuously cooled in the flow system. Based on this evaluation and in addition to the observed significant extraction yields, especially for heat sensitive compounds, the role of thermal processes for the degradation of algae was omitted. Conversely, an increase in suspension temperatures and thermal damage to the extracted compounds could be observed, when sparks were applied with repetition rates of 7 Hz and higher (data not shown). The optimisation of repetition rates in favour of faster processing times and with respect to other system and operating parameters, e.g. size of reaction chambers and flow rates, will be a question for an upscaling of the method.

Arguably, one of the most prominent characteristics of spark discharges is the generation of strong shockwaves [7], with pressures that could be in the order of several thousand Megapascal [39]. Accordingly, the disintegration of cells and cell walls, as shown in Figure 3b, which was also confirmed by REM images that were acquired in a previous study [9], by shockwaves were the most likely reason for the observed extraction, both quantitatively as well as qualitatively. The initial pressure of the shockwave front could not be determined. However, a pressure of 500 MPa was measured at a distance of 0.6 mm from the discharge centre. At a distance of 2.4 mm, the pressure decreased to around 140 MPa (Figure 6), which is still higher than the tensile strength of the alga. The fit model indicates that cell wall disintegration is still possible at a distance of 20 mm. The shockwave strength depends, amongst other parameters, on the energy, which is put into the system. Bousetta and Vorobiev identified the energy input (or the number of pulses) as the main parameter to optimise the extraction yield of various plant material [40]. According to Liu et al., 18.7 % of the provided electrical energy is dissipated by shockwaves for sparks that were generated with microsecond pulses [22]. Altogether, an increase in energy input results in higher shockwave pressure, but it should not exceed the threshold of economic viability. It may therefore be reasonable to improve the system by increasing the energy partitioned into the process, e.g. by shaping the applied high voltage pulses (pulse durations and rise times), and to optimise the distribution of shockwaves in the medium. This could be achieved by reflectors or more electrodes, which could be aligned in a row or radially.

Although the study was conducted primarily on one particular algae, i.e. *Chlorella vulgaris* and the principle confirmed for only two additional species (*Nannochloropsis occulata* and *Cyanidium caldarium*), results and the method are instructive and applicable also towards other microalgae or biomass in general. The possibility for the respective physio-mechanical disintegration depends only on the tensile strength of the target cells with respect to the shockwave pressures that can be generated. For the comparison of shockwave pressures with the robustness of the microalgal cell walls, AFM measurements were performed to determine their elasticity modulus and thus, conclusions could be drawn on their tensile strength. The tensile strength of microalgae in general is given in the literature with 9.5MPa [16]. Abbassi et al. have found that a pressure of 10 MPa is enough to rupture 98 % of cells from the microalga *Nannochloropsis occulata*, which is close to the calculated tensile strength of 13.95 MPa in this study and values stated by Lee et al [41]. Lee et al. [11] also studied indentation forces for another green microalga, *Tetraselmis suecica*, in more detail, but did not find identical force curves on various parts of one single cell. The results were explained by ultrastructures inside the cells, which could be the chloroplast, nucleus, starch or lipid bodies. Due to this heterogeneity, local force curves might differ and cell surfaces cannot be completely smooth as well. Notable, elasticity modules, determined in our experiments, had no correlation with the size of the algae and might therefore rather represent characteristics of the cell wall. Hence, the tensile strength credibly depends on the cell wall composition, e.g. amount of cellulose or hemicellulose, or proteins and the number of cell wall layers. Therefore, the elasticity moduli for other microalgae species might vary in comparison to *Chlorella vulgaris*. On average, an elasticity modulus of 13.95 MPa was found in our experiments, but also a not negligible variation of the elastic moduli, i.e. values between 8.06 and 22.31 MPa, was observed for different cells. However, overall the tensile strengths of microalgae in general seems to be much lower than the shockwave pressures that could be generated. Accordingly, the method offers an interesting alternative extraction method, at least for microalgae, without the shortcomings of the processes that are currently in use.

5. Conclusion

Scope of this work was to determine the mechanism of cell wall rupture of *Chlorella vulgaris* by spark discharges. From a comparison of the tensile strength of the microalgae with

shockwave pressures, it was found that from all plasma characteristics only shockwaves could be held responsible for the cell disintegration. The promising results encourage the investigation of the method on a larger industrial scale. An inherent advantage of the process, in comparison to currently used extraction procedures, is that no solvents are needed, which are in some cases toxic or require at least additional purification steps. Furthermore, regarding the optimisation of the downstreaming process, spark discharges can be applied directly to the algae suspension at the time pint of algae harvest, i.e. no pre-processing, such as concentration or initial fermentation is necessary. The method can of course be further optimised by a dedicated design of reaction chambers that distribute the shockwaves in a larger treatment volume, and by adjusting operating and discharge parameters. By increasing repetition rates, shorter treatment times and higher throughputs can be achieved; however, at the same time temperature increases need to be managed. Concurrently of fundamental interest is the question, if the dissipation of the provided electrical energy by shockwaves can be improved by electrode designs and discharge configurations or the application of high-voltage pulses with a more specific shape, i.e. by investigating different pulse durations and rise times.

6. References

1. Pulz, O. and W. Gross, *Valuable products from biotechnology of microalgae.* Applied microbiology and biotechnology, 2004. **65**(6): p. 635-648.
2. Mata, T.M., A.A. Martins, and N.S. Caetano, *Microalgae for biodiesel production and other applications: a review.* Renewable and sustainable energy reviews, 2010. **14**(1): p. 217-232.
3. Olaizola, M., *Commercial development of microalgal biotechnology: from the test tube to the marketplace.* Biomolecular engineering, 2003. **20**(4-6): p. 459-466.
4. Gerken, H.G., B. Donohoe, and E.P. Knoshaug, *Enzymatic cell wall degradation of Chlorella vulgaris and other microalgae for biofuels production.* Planta, 2013. **237**(1): p. 239-253.
5. Zheng, H., et al., *Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves.* Applied biochemistry and biotechnology, 2011. **164**(7): p. 1215-1224.
6. Kim, J., et al., *Methods of downstream processing for the production of biodiesel from microalgae.* Biotechnology advances, 2013. **31**(6): p. 862-876.
7. Locke, B., et al., *Electrohydraulic discharge and nonthermal plasma for water treatment.* Industrial & engineering chemistry research, 2006. **45**(3): p. 882-905.
8. Lukes, P., et al., *Ultraviolet radiation from the pulsed corona discharge in water.* Plasma Sources Science and Technology, 2008. **17**(2): p. 024012.
9. Zocher, K., et al., *Comparison of Extraction of Valuable Compounds from Microalgae by Atmospheric Pressure Plasmas and Pulsed Electric Fields.* Plasma Medicine, 2016. **6**(3-4): p. 273-302.
10. Schulze, C., et al., *Screening of microalgae for primary metabolites including β-glucans and the influence of nitrate starvation and irradiance on β-glucan production.* Journal of Applied Phycology, 2016: p. 1-7.
11. Lee, A.K., D.M. Lewis, and P.J. Ashman, *Force and energy requirement for microalgal cell disruption: an atomic force microscope evaluation.* Bioresource technology, 2013. **128**: p. 199-206.
12. Chopinet, L., et al., *Imaging living cells surface and quantifying its properties at high resolution using AFM in QI™ mode.* Micron, 2013. **48**: p. 26-33.
13. Schmidt-Bleker, A., S. Reuter, and K. Weltmann, *Quantitative schlieren diagnostics for the determination of ambient species density, gas temperature and calorimetric power of cold atmospheric plasma jets.* Journal of Physics D: Applied Physics, 2015. **48**(17): p. 175202.
14. Katsuki, S., et al., *Shock waves due to pulsed streamer discharges in water.* Japanese journal of applied physics, 2006. **45**(1R): p. 239.
15. Nagayama, K., et al., *Shock Hugoniot compression curve for water up to 1 GPa by using a compressed gas gun.* Journal of applied physics, 2002. **91**(1): p. 476-482.
16. Lee, A.K., D.M. Lewis, and P.J. Ashman, *Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements.* Biomass and bioenergy, 2012. **46**: p. 89-101.
17. Zocher, K., et al., *Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures.* Algal Research, 2019. **39**: p. 101416.
18. Vogel, A., S. Busch, and U. Parlitz, *Shock wave emission and cavitation bubble generation by picosecond and nanosecond optical breakdown in water.* The Journal of the Acoustical Society of America, 1996. **100**(1): p. 148-165.

19. Bruggeman, P. and C. Leys, *Non-thermal plasmas in and in contact with liquids.* Journal of Physics D: Applied Physics, 2009. **42**(5): p. 053001.
20. Sato, M., T. Ohgiyama, and J.S. Clements, *Formation of chemical species and their effects on microorganisms using a pulsed high-voltage discharge in water.* IEEE Transactions on Industry applications, 1996. **32**(1): p. 106-112.
21. Sunka, P., et al., *Generation of chemically active species by electrical discharges in water.* Plasma Sources Science and Technology, 1999. **8**(2): p. 258.
22. Liu, S.-W., et al., *Influence of plasma channel impedance model on electrohydraulic shockwave simulation.* Physics of Plasmas, 2019. **26**(2): p. 023522.
23. Roberts, R.M., et al., *The energy partition of underwater sparks.* The Journal of the Acoustical Society of America, 1996. **99**(6): p. 3465-3475.
24. Martin, E.A., *Experimental Investigation of a High-Energy Density, High-Pressure Arc Plasma.* Journal of applied physics, 1960. **31**(2): p. 255-267.
25. Grinenko, A., et al., *Efficiency of the shock wave generation caused by underwater electrical wire explosion.* Journal of applied physics, 2006. **100**(11): p. 113509.
26. Robinson, J.W., M. Ham, and A.N. Balaster, *Ultraviolet radiation from electrical discharges in water.* Journal of Applied Physics, 1973. **44**(1): p. 72-75.
27. Jiang, B., et al., *Review on electrical discharge plasma technology for wastewater remediation.* Chemical Engineering Journal, 2014. **236**: p. 348-368.
28. Sun, B., et al., *Effects of shock waves, ultraviolet light, and electric fields from pulsed discharges in water on inactivation of Escherichia coli.* Bioelectrochemistry, 2018. **120**: p. 112-119.
29. Tsong, T.Y., *Electroporation of cell membranes.* Biophysical journal, 1991. **60**(2): p. 297.
30. Pagan, R. and P. Mañas, *Fundamental aspects of microbial membrane electroporation*, in *Pulsed electric fields technology for the food industry*. 2006, Springer. p. 73-94.
31. García, D., et al., *Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated.* International journal of food microbiology, 2007. **113**(2): p. 219-227.
32. Steuer, A., et al., *Transient suppression of gap junctional intercellular communication after exposure to 100-nanosecond pulsed electric fields.* Bioelectrochemistry, 2016. **112**: p. 33-46.
33. Banaschik, R., et al., *Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing Legionella pneumophila as model organism.* Bioelectrochemistry, 2016.
34. Oehmigen, K., et al., *Estimation of possible mechanisms of Escherichia coli inactivation by plasma treated sodium chloride solution.* Plasma Processes and Polymers, 2011. **8**(10): p. 904-913.
35. Smith, J.B., I. Adams, and H.-F. Ji, *Biomolecule Response to Nonthermal Plasma.* 2017. **7**(4): p. 427-443.
36. Laroussi, M., D. Mendis, and M. Rosenberg, *Plasma interaction with microbes.* New Journal of Physics, 2003. **5**(1): p. 41.
37. Fridman, A., Y. Yang, and Y.I. Cho, *Plasma discharge in liquid: water treatment and applications*. 2012: CRC press.
38. Locke, B.R. and S.M. Thagard, *Analysis and review of chemical reactions and transport processes in pulsed electrical discharge plasma formed directly in liquid water.* Plasma Chemistry and Plasma Processing, 2012. **32**(5): p. 875-917.
39. Sun, B., M. Sato, and J. Clements, *Oxidative processes occurring when pulsed high voltage discharges degrade phenol in aqueous solution.* Environmental science & technology, 2000. **34**(3): p. 509-513.
40. Boussetta, N. and E. Vorobiev, *Extraction of valuable biocompounds assisted by high voltage electrical discharges: A review.* Comptes Rendus Chimie, 2014. **17**(3): p. 197-203.
41. Abbassi, A., M. Ali, and I.A. Watson, *Temperature dependency of cell wall destruction of microalgae with liquid nitrogen pretreatment and hydraulic pressing.* Algal research, 2014. **5**: p. 190-194.

Publication P4

Comparison of Pulsed Corona Plasma and Pulsed Electric Fields for the Decontamination of Water Containing *Legionella Pneumophila* as Model Organism

Banaschik, R., Burchhardt, G., Zocher, K., Hammerschmidt, S., Kolb, J.F. & Weltmann, K.-D. *Bioelectrochemistry*, 112 (2016) 83-90.

Bioelectrochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism

Robert Banaschik [a], Gerhard Burchhardt [b], Katja Zocher [a], Sven Hammerschmidt [b,*], Juergen F. Kolb [a,*], Klaus-Dieter Weltmann [a]

[a] Leibniz Institute for Plasma Science and Technology e.V. (INP Greifswald), Felix-Hausdorff-Straße 2, 17489 Greifswald, Germany
[b] Department Genetics of Microorganisms, Interfaculty Institute for Genetics and Functional Genomics, Ernst Moritz Arndt University of Greifswald, Friedrich-Ludwig-Jahn-Straße 15a, 17489 Greifswald, Germany

ARTICLE INFO

Article history:
Received 1 December 2015
Received in revised form 27 February 2016
Accepted 20 May 2016
Available online xxxx

Keywords:
Non-thermal plasma
Pulsed electric fields
Legionella pneumophila
Decontamination
Bacterial killing

ABSTRACT

Pulsed corona plasma and pulsed electric fields were assessed for their capacity to kill *Legionella pneumophila* in water. Electrical parameters such as in particular dissipated energy were equal for both treatments. This was accomplished by changing the polarity of the applied high voltage pulses in a coaxial electrode geometry resulting in the generation of corona plasma or an electric field. For corona plasma, generated by high voltage pulses with peak voltages of +80 kV, *Legionella* were completely killed, corresponding to a log-reduction of 5.4 (CFU/ml) after a treatment time of 12.5 min. For the application of pulsed electric fields from peak voltages of −80 kV a survival of log 2.54 (CFU/ml) was still detectable after this treatment time. Scanning electron microscopy images of *L. pneumophila* showed rupture of cells after plasma treatment. In contrast, the morphology of bacteria seems to be intact after application of pulsed electric fields. The more efficient killing for the same energy input observed for pulsed corona plasma is likely due to induced chemical processes and the generation of reactive species as indicated by the evolution of hydrogen peroxide. This suggests that the higher efficacy and efficiency of pulsed corona plasma is primarily associated with the combined effect of the applied electric fields and the promoted reaction chemistry.

1. Introduction

Legionella pneumophila are Gram-negative bacteria that were first described in 1979 after an outbreak of pneumonia among members of the American Legion. The elongated non-spore forming aerobic microorganisms with a length of 2–5 μm proliferate in amoeba but can also replicate within alveolar macrophages. Although 15 serogroups of *Legionella pneumophila* are confirmed, serogroup 1 (sg1) is most frequently associated with severe infections [1]. Legionellosis can traditionally be distinguished in two clinical pictures. One is described as Legionnaires' disease (named after the first observed outbreak) causing severe pneumonia. The other is the so called Pontiac fever whose etiopathology is rather moderate, flue-like and most of all self-limiting. The difference to Legionnaires' disease is the lack of pneumonic symptoms [2,3].

In modern societies *Legionella* often persist in water tanks, cooling systems or air conditioning systems causing a severe respiratory disease when contaminated water or aerosol is inhaled by human beings. Several countries reported an increase in cases of legionellosis [4,5]. The Department of Epidemiology (Atlanta, USA) investigated data provided by the Center of Disease Control (CDC) for the years 1990 to 2005. They recognized an increase of 70% from 1310 cases in 2002 to 2223 cases in 2003. Two years later the rate of new infections increased to 12,000 in 2005.

Additional efforts are needed to develop highly efficient disinfection systems to reduce Legionella species in water containing environments [6].

To eradicate *Legionella* several physical and chemical disinfection methods have been described including thermal treatment (superheat-and-flush or instantaneous heating-system), copper/silver ionization, UV-light or hyperchlorination. However, these disinfection methods have limitations [7]. Thermal treatment has disadvantages such as high costs and duration because only temperatures above 60 °C for extended times lead to an almost complete killing of *L. pneumophila*. UV-light is only recommended in combination with superheat-and-flush to provide comprehensive protection. Additionally, prefiltration is necessary to prevent accumulation of chalk residues on the quartz sleeves housing the UV source, which otherwise would

* Corresponding authors.
E-mail addresses: sven.hammerschmidt@uni-greifswald.de (S. Hammerschmidt), juergen.kolb@inp-greifswald.de (J.F. Kolb).

http://dx.doi.org/10.1016/j.bioelechem.2016.05.006
1567-5394/

decrease UV light emission. Chlorine is highly corrosive and can cause severe plumbing damage. A coating of the pipe system is necessary. This, however, cannot eliminate leakage completely. Furthermore, it was demonstrated that *Legionella* is rarely sensitive to chlorine [7,8]. Thus, more advanced disinfection methods are necessary and motivate the development of new treatment techniques such as pulsed electric fields (PEF) and pulsed corona plasma, respectively. Both of them have already proved to be effective, bio-compatible and environmental friendly [9].

Potential applications of PEF treatment are food processing, medical treatment or water treatment [10–14]. If parameters like pulse polarity, conductivity and electrode shape are adjusted correctly, alternatively non-thermal, i.e. corona plasma, can be formed. It has been demonstrated that non-thermal plasma generated directly in water does have a variety of physical and chemical effects known to be effective for pollutant degradation, bacterial killing, including the killing of spores [9,15–20]. Beside the occurrence of strong electric fields, ultraviolet radiations, shockwaves and probably most importantly chemical reactive species such as hydroxyl radicals and hydrogen peroxide (H_2O_2) are generated by the plasma [21]. Nevertheless, processes responsible for bacterial killing with pulsed corona plasma are not fully understood. Especially the role of the electric field in comparison to effects mediated by the plasma itself is unclear. Therefore it is still ambiguous which of the methods is more efficient and causes a higher log-reduction of colony forming units (CFU), requires less time and/or less energy.

Differences between pulsed corona plasma and PEF were already compared previously although in different experimental setups. Slightly higher decontamination efficiency was found for plasma when *Escherichia coli* was used as model organism. Corona plasma was generated in a wire to plate geometry, applying pulses of 600 ns at a repetition rate of 0.1 Hz and with peak voltages of 120 kV. The results were correlated to PEF-treatments conducted in a plate-to-plate setup for an applied homogenous pulsed electric field of 80 kV/cm with pulse durations of 60 ns, 300 ns and 2 μs. In this setting shorter pulses in sub-microsecond range appeared to be more effective than longer pulses [22,23].

Comparative studies were also performed using *Pseudomonas fluorescens* as a model microorganism. Plasma was formed in a needle to plate system when applying pulses of 20 kV with a duration of 6 μs. Air or nitrogen could be bubbled through the needle to enhance energy efficiency. When plasma was applied directly to water, it was found to be more energy efficient than PEF-treatment, which was conducted in plate-to-plate geometry for a homogeneous field of 66 kV/cm and a pulse length of 150 μs [24].

Although the plasma was not generated directly in water a further study showed that the combination of plasma and PEF treatment had synergistic killing effects dependent in which order the methods were applied. Using a plasma jet close to the liquid surface first and afterwards PEF treatment led to an almost complete killing of *Staphylococcus aureus*. Pulsed electric fields were applied with a plate to plate configuration using peak voltage of 3 kV, pulse duration of 100 μs and a repetition rate of 1 Hz [9].

However, all these studies were facing the problem that plasma source and PEF source were not directly comparable due to two different experimental setups for either the application of plasma or the electric field.

In this study two different methods were compared for their effect on the viability of pathogenic *Legionella* in water. An experimental setup was established, which allowed the generation of plasma and pulsed electric fields, respectively.

All experiments were performed with the same experimental setup and an equal peak voltage of about 80 kV. Almost the same amount of energy in either the plasma or PEF treatment was delivered per discharge or pulsed field. This was accomplished by changing the polarity of the applied short high voltage pulses, which resulted either in the generation of corona plasma or an electric field only. This allowed a direct comparison on the effectiveness and differences in killing mechanisms for both methods.

2. Materials and methods

2.1. Electrical setup

A coaxial electrode geometry was used for plasma and PEF treatment, respectively. For a more robust electrode design two twisted tungsten wires (W-005135/13, Goodfellow, Huntingdon, England) with a diameter of 0.05 mm each (pure, uncoated) were aligned in the middle of a glass tube and served as high voltage electrode. The glass tube had a length of 67 mm and a diameter of 34.5 mm. Ground electrode was a metal mesh of stainless steel that was fixed on the inner wall of the class tube. High voltage electrode was replaced after each experiment to establish the most comparable conditions for all experiments. The assembled reactor was holding a volume of 68 ml. Positive or negative high voltage pulses could be applied to the center electrode by a 6-stage Marx-bank with a repetition rate of 20 Hz. The setup was described previously in more detail [19,25]. An inherent advantage of this setup is the possibility to create either a pulsed corona plasma using positive polarity high voltage pulses (Fig. 1) or just a pulsed electrical field using negative polarity high voltage pulses.

During application of positive high voltage pulses in a wire to cylinder or needle to plate system, a strong electric field is located close to the surface of the high voltage electrode (wire). Although the field weakens over distance, electron avalanches result in streamer propagating to the outer electrode (metal mesh) forming a plasma. Negative polarity uses to be less attractive for electron avalanches. If streamers formed at all, they are significantly shorter than with a positive polarity [26,27]. The described mechanism can be employed to develop an experimental setup (pulse width, reactor chamber, conductivity) in which only a positive discharge is formed, even when negative pulses with the same peak voltage were used.

Pulses applied by the Marx-bank are characterized by short rise times of about 20 ns, a peak voltage of 80 kV and an exponential decay resulting in pulse lengths (FWHM) of about 140 ns for positive (plasma) and approximately 240 ns for negative (PEF) polarity (Fig. 2).

Although pulse length for PEF treatment is increasing, the calculated pulse energy is almost similar for both polarities. This can be explained by current flows that compensate for differences in applied voltages. Beyond 400 ns energy dissipated in pulses applied for plasma and PEF

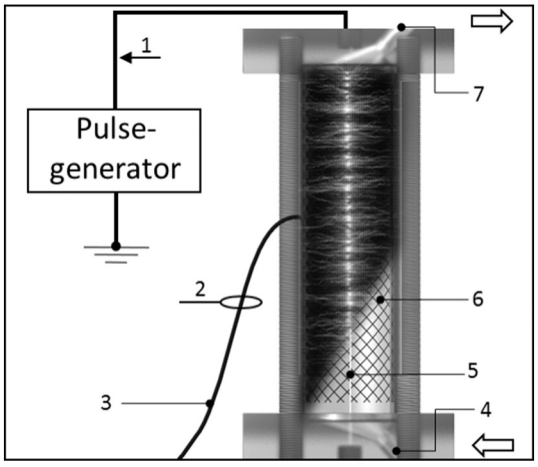

Fig. 1. Pulsed corona plasma in coaxial geometry with increased exposure time. (1) voltage measurement, (2) current measurement, (3) ground connection, (4) bottom connector to peristaltic pump, (5) tungsten high voltage electrode, (6) ground electrode (stainless steel mesh), (7) upper connector to peristaltic pump. Arrows indicate the flow direction of *Legionella* suspension. Positive or negative high voltage pulses were applied to the center electrode from a 6-stage Marx-bank with a repetition rate of 20 Hz. Conductivity of treated suspension was adjusted to 60 μS/cm. A flow rate of 140 ml/min was maintained by a peristaltic pump, which was placed before the setup with a pushing flow from the bottom to the top as indicated by arrows.

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

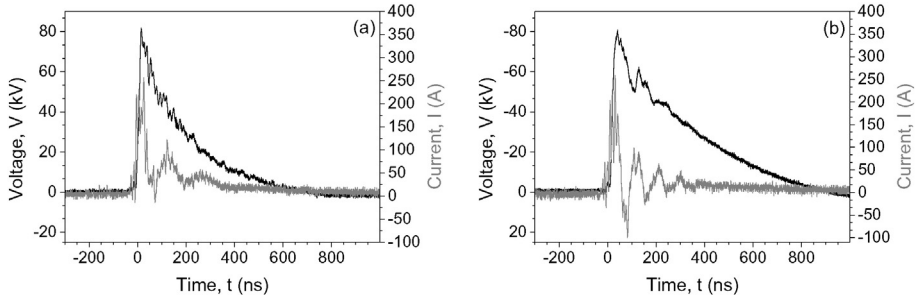

Fig. 2. Current and voltage characteristics for a single positive (a) and negative (b) high voltage pulse that was applied to generate a corona plasma or an electric field. Voltage waveform is close to critically damped with peak voltage of 80 kV, pulse duration of about 140 and 240 ns (FWHM). The current pulses show a damped oscillation with peak currents of 130 A for positive and 120 A for negative polarity. Calculated energy of both pulses was 1.1 J.

treatment is rather low. Therefore a slightly longer exponential decay observed for PEF treatment led only to a small increase of energy consumption. Energy delivered with each pulse (1.1 J) was calculated by integrating the product for the duration of the voltage and current signals. Voltage was measured with a 120-kV/80-MHz high voltage probe (PVM-5, NorthStar Marana, AZ). Current was measured with a Pearson current monitor (Model 5046, Pearson Electronics, Palo Alto, CA). Current monitor was terminated with 50 Ω at the oscilloscope to monitor fast rise times of the current pulses. Voltage and current were recorded for analysis (Wave Surfer 64MXs-B, LeCroy, Chestnut Ridge, NY).

A peristaltic pump (FH100x, Thermo Scientific, Waltham, MA) was used to move the bacterial suspension with a flow rate of 140 ml/min through the setup. Total treated volume including tubes and expansion tank was 140 ml. Samples for each experiment were taken after 7500, 15,000 and 30,000 consecutive discharges. This corresponded to a treatment time of 6.25 min, 12.5 min and 25 min. Taking the volume of the discharge chamber, total volume and flow rate into account, theoretical exposure time to plasma or PEF for a single cell during 1 min of treatment/pumping was 600 discharges or high voltage pulses. To exclude thermal effects due to heating, suspension was cooled with ice cold water to keep the temperature below 28 °C.

2.2. Cultivation of Legionella pneumophila

Cultivation *of L. pneumophila* Philadelphia 1 JR32 [28] was performed in BCYE medium supplemented with 0.04% L-cysteine and 0.025% $Fe^{III}(NO_3)$. In general *L. pneumophila* was inoculated on BCYE agar medium and grown at 34 °C for 2 days. Grown bacteria were transferred to 30 ml BYE medium starting with an $OD_{600\ nm}$ of 0.1 and incubated in a shaking water bath at 34 °C until they reach an $OD_{600\ nm}$ of 0.35. Bacteria were harvested at room temperature at 3270 g for 10 min. Bacterial sediment was resuspended in sterile 20 ml distilled water and centrifuged again to remove residual culture fluid. Approximately 10 ml of grown *Legionella* were transferred to 130 ml sterile distilled water. Conductivity of suspension was adjusted to 60 μS/cm by adding 0.1 M NaCl and pH-value was determined. The reaction chamber was completely filled up with this bacterial suspension and connected to a reservoir by pipes, which were connected to the peristaltic pump (Fig. 1). At specific time intervals 4–6 ml samples were taken to determine $OD_{600\ nm}$, pH-value, conductivity, H_2O_2 concentration. After each time point serial dilutions of treated bacterial suspensions were prepared and 0.1 ml of each sample was plated on BCYE agar plates followed by an incubation step at 34 °C. After three days *Legionella* colonies appeared and were counted after 5 days of incubation.

2.2.1. Determination of hydrogen peroxide concentration

Plasma effects often correlate with the formation of highly reactive species. Of particular interest is the hydroxyl radical with a strong oxidation potential of 2.8 eV. Although hydroxyl radicals cannot be measured easily, detection of relatively high amounts of hydrogen peroxide can prove production by plasma in water, because a major pathway for hydrogen peroxide is the hydroxyl radical recombination in the plasma channels (Eq. (1)) [19,29].

$$\cdot OH + \cdot OH \rightarrow H_2O_2 \quad (1)$$

Furthermore, H_2O_2 is known for the mediation of cytotoxic effects and can be used as disinfectant itself. Hence, additive effects of hydrogen peroxide to bacterial killing should be taken into account and the H_2O_2 concentration has to be quantified.

For determination of H_2O_2 a 1 ml sample was centrifuged at 3500*g* for 6 min, the supernatant was transferred into a new tube and 0.5 ml Titanium(IV)oxysulfate sulfuric acid was added [30]. Color development was determined at $OD_{407\ nm}$. The concentration of H_2O_2 was estimated with a calibration curve and a range between 0 and 6 mM H_2O_2.

2.2.2. Sample preparation for scanning electron microscopy

Applying strong electric fields to cell membranes often induces the formation of pores, thereby causing leakage and possibly cell death [22]. Furthermore, in streamer breakdown processes the formation of shockwaves can be expected as an integral part [31]. Thus, effects on bacterial morphology seem to be conceivable.

To visualize possible effects treated and untreated bacterial suspensions were investigated by scanning electron microscopy. Microscopic images were generated from samples after plasma and PEF treatment, respectively. A sample of 4 ml was centrifuged at 3500*g* for 6 min and resuspended in 1 ml distilled water supplemented with 1% para-formaldehyde. Bacteria were loaded on a 0.2 μm pore size polycarbonate filter and stored in fixation solution (5 mM Hepes, 50 mM NaN_3, 1% glutaraldehyde, 4% para-formaldehyde, pH 7.4) until use for the scanning electron microscopy. Further preparation of samples was as described previously [32]. Finally, *Legionella* loaded filters were examined in a scanning electron microscope EVO LS10 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

2.3. Statistical analysis

Bacteria for control and bacteria exposed to plasma and/or PEF were derived from the same culture and all experiments were conducted at least in triplicate. In graphs, each bar represents the mean of three independent experiments with standard deviation. Student's *t*-test was chosen for statistical analysis with significance for a p-value <0.05. Tests were carried out in Microsoft Excel 2013 and Prism 6.01.

3. Results

3.1. Plasma and PEF treatment

The experimental setup enabled to study the impact of pulsed corona plasma or pulsed electric fields on bacterial suspensions of *L. pneumophila* in water. Bacterial suspension was always adjusted to a

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

conductivity between 60 and 62 μS, pH-value of 6.0 and a temperature range between 25 and 27 °C. A flow rate of 140 ml/min was maintained using a peristaltic pump. For the same treatment, i.e. without the application of either plasma or PEF, the suspension was circulated 25 min through the discharge chamber.

Bacterial concentration after 25 min decreased by <10% ($p = 0.03$) when compared to control. Thus, mechanical stress caused by the continuous flow had only a minor effect on the survival of *Legionella* (Fig. S1).

The *Legionella* suspension was exposed to pulsed corona plasma (Fig. 3). Treatment of *Legionella* suspensions with pulsed corona plasma (a) or PEF (b). 7500, 15,000 and 30,000 consecutive discharges or pulsed electric fields for a peak voltage of 80 kV and repetition rate of 20 Hz were applied. This is equal to a treatment time of 6.25 min, 12.5 min and 25 min (Fig. 3a). Plasma treatment for 6.25 min corresponding to 7500 consecutive discharges resulted in a loss of viable *Legionella* by 33% (log 1.79). Longer treatment with plasma for 12.5 min resulted in a complete bacterial killing and no *Legionella* colonies were monitored on agar plates. Consequently also no viable bacteria were detected after 25 min.

By using the same experimental setup for PEF treatment it was possible to determine survival of *Legionella* without plasma generation (Fig. 3b). Experimental parameters for the application of high voltage pulses, such as peak voltage and conductivity were kept constant but polarity of the power source was changed. Negative applied voltage pulses therefore only generated an electrical field along the wire without igniting a corona plasma. Similar to plasma treatment a CFU log reduction of approximately 32% (log 1.9) was achieved after 6.25 min of treatment. However, compared to plasma treatment, doubling the treatment time did not completely kill *Legionella* and CFU/ml decreased only for approximately log 2 for each sampling point. Thus, compared to plasma treatment almost log 1 of *Legionella* survived this experiment after 25 min treatment.

Antimicrobial effects of plasma treatment were accompanied by an increased formation of hydrogen peroxide reaching a maximum of about 2 mM H_2O_2 after 25 min. In comparison, PEF treatment resulted in H_2O_2 concentrations in the range of 0.13 mM after 25 min (Fig. 4). Formation of hydrogen peroxide for plasma and PEF treatment after 7500, 15,000 and 30,000 consecutive discharges with a repetition rate of 20 Hz. This equals a treatment time of 6.25 min, 12.5 min and 25 min. High voltage pulses of 80 kV were applied (Fig. 4).

In most organisms hydrogen peroxide is produced during cell-metabolism, however, due to its oxidative potential it is regarded as cytotoxic. To assess the effect of H_2O_2 *L. pneumophila* was incubated with 1 mM H_2O_2. This concentration was chosen because it appears after 12.5 min of plasma treatment, where no viable bacteria were observed anymore. Samples were taken at 4 different time points, similar to

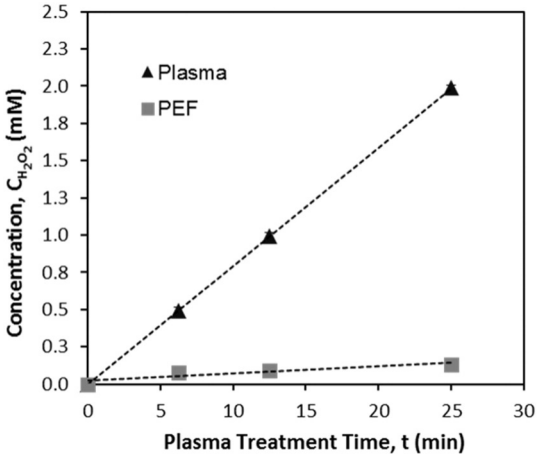

Fig. 4. Formation of hydrogen peroxide for plasma and PEF treatment after 7500, 15,000 and 30,000 consecutive discharges with a repetition rate of 20 Hz. This equals a treatment time of 6.25 min, 12.5 min and 25 min. High voltage pulses of 80 kV were applied. Each data point represents the mean of 3 independent experiments with standard deviation of 0.02 mM H_2O_2 for plasma and 0.04 mM H_2O_2 for PEF treatment. (Hence, error bars are actually smaller than the symbols in the graph.)

experiments conducted with plasma or PEF. After 12.5 min the CFU/ml decreased by about 23% (log 1.47). After 25 min incubation with 1 mM hydrogen peroxide less than log 1.9 CFU/ml of *Legionella* were killed (Fig. 5).

3.2. Scanning electron microscopy

The bacterial morphology after plasma and PEF treatment was illustrated by scanning electron microscopy (Fig. 6). During both treatments a bacterial damage and an alteration of *Legionella* appearance was observed when compared to untreated bacteria. After *L. pneumophila* was exposed to PEF no obvious difference in cell shape was observed. For increasing treatment times, i.e. increasing number of pulses applied, the number of ruptured cells observed post-exposure to PEF was substantially lower when compared to treatments with plasma generating pulses. Occasionally bacteria appeared deflated and surrounded by small vesicles. Obvious damage of the unit membrane led to lysis and release of the cytoplasmic content. Findings for PEF treatment can also be applied to plasma treatment, however, cell damage was generally more severe. In addition it seemed that more bacteria were completely disrupted after plasma treatment and an increase of cell debris was visible. Longer treatment of 25 min encouraged the formation of plaque-like structures. No cell damage was observed when *L. pneumophila* was exposed to 1 mM hydrogen peroxide (Fig. S2).

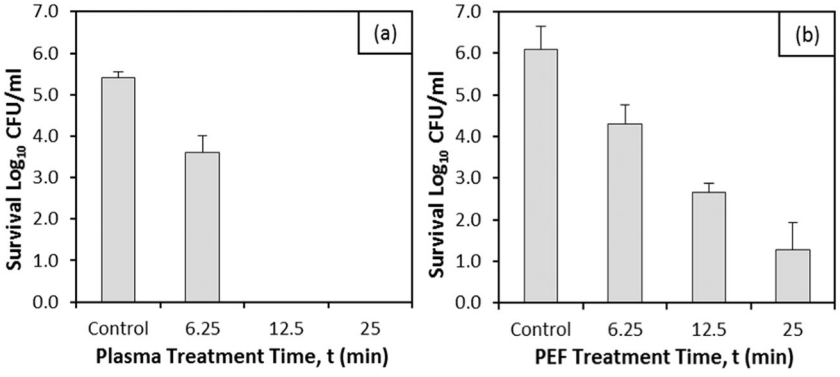

Fig. 3. Treatment of *Legionella* suspensions with pulsed corona plasma (a) or PEF (b). 7500, 15,000 and 30,000 consecutive discharges or pulsed electric fields for a peak voltage of 80 kV and repetition rate of 20 Hz were applied. This is equal to a treatment time of 6.25 min, 12.5 min and 25 min. Each bar represents the mean of three independent experiments with standard deviation ($n = 3$). For plasma treatment no viable *Legionella* were counted after 12.5 min and 25 min, respectively. Comparing plasma and PEF treatment, statistical significance was observed for $t = 12.5$ min ($p = 0.0003$). The CFU was determined after 5 days of incubation at 34 °C. Detection limit: 10 CFU/ml.

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

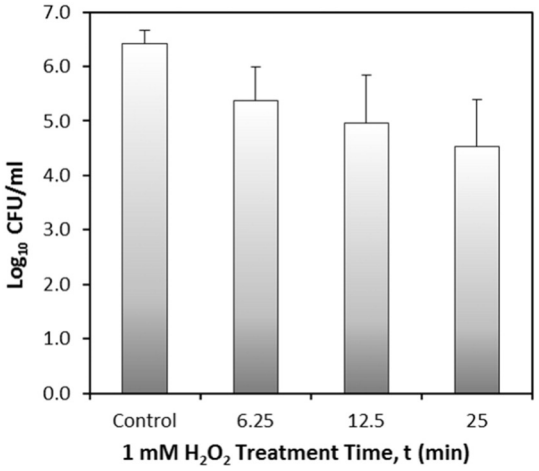

Fig. 5. Incubation of *Legionella* suspension with 1 mM H_2O_2. Samples were taken for time points that were similar to plasma treatment and PEF treatment, respectively. In general, a small but statistical significant decrease in bacteria concentration was observed in comparison to controls after 6.25 min ($p = 0.02$), 12.5 min ($p = 0.01$) and 25 min ($p = 0.01$). Each bar represents the mean of 5 independent experiments with standard deviation.

4. Discussion

Antibacterial effects of pulsed corona plasma and pulsed electric fields for decontamination of water containing *Legionella pneumophila* Philadelphia 1 JR32 as model microorganism were investigated. Increasing incidences of legionellosis motivate the development of alternative methods for water disinfection such as application of plasma or pulsed electric fields. Understanding the processes responsible for bacterial killing may help to increase efficiency of these methods. In this study both methods have been directly compared with the same experimental setup when the same energy was dissipated.

Both methods in common is the application of short high voltage pulses, which have been shown to be potentially more efficient in comparison to longer pulses for PEF treatment [22]. Supplied energy for PEF and plasma treatment can be dissipated in different ways. For PEF treatment energy will be consumed mainly for building up the electric field and the associated current flowing through the medium which increases water temperature due to Joule heating. These effects also occur during plasma treatment but in addition shockwaves, UV-radiation and reactive species are formed.

Energy per pulse by plasma or PEF treatment was calculated from current voltage measurements (Table 1). In both cases fast rising high voltage pulses with almost similar peak voltages of 80 kV were applied. Voltage pulses had an exponentially decay and pulse length was measured as full width at half maximum (FWHM). When plasma filaments are forming in the discharge volume the overall resistance of the reactor is slightly decreasing, resulting in a shorter duration of the voltage pulse that is observed (Fig. 2). Simultaneously overall current is increasing and therefore the pulse energy dissipated into plasma and/or electric field, was in the order of 1.03–1.16 J per pulse. Conductivity of *Legionella* suspension stayed almost constant during plasma and PEF application. The initial pH-value of 6 slightly decreased to a value of 5.5 during plasma treatment. Conversely, no change of pH-value was detected and the conductivity did not change during PEF treatment.

A complete killing of *L. pneumophila* was achieved after 12.5 min with plasma treatment, whereas log 2.54 CFU/ml were still alive after 12.5 min for only PEF exposure. In this case viable *Legionella* were still determined on agar plates even after 25 min of PEF treatment. Taking into account eradicated number of bacteria (CFU/ml), treated volume, energy per pulse, efficiencies can be calculated for both methods (Table 2).

For the investigated experimental setup, 124 kJ are necessary to kill *Legionella* in one liter of water (log 5.4 CFU/ml) using pulsed corona plasma. Conversely, for pulsed electric fields only, >221 kJ per liter are required for a complete killing of *Legionella*. In case of plasma, less energy is required than for thermal treatment. For thermal treatment, the temperature of water with a heat capacity of $c_W = 4.2$ kJ kg^{-1} K^{-1} has to be increased to 70 °C [33]. If starting temperature is around 25 °C this change in temperature ($\Delta T = 45$ K) requires approximately 188 kJ/l.

Peak voltage (80 kV) and pulse shape were almost the same for both treatments and all experiments. For the given electrode geometry the electric field was the same for both methods and only polarities of the applied high voltage pulses were different. Accordingly, application of pulsed corona plasma using a coaxial electrode geometry seems to be superior to a PEF treatment of equal energy input. Apparently, plasma mediated effects enhance bacterial killing using the applied energy more effectively.

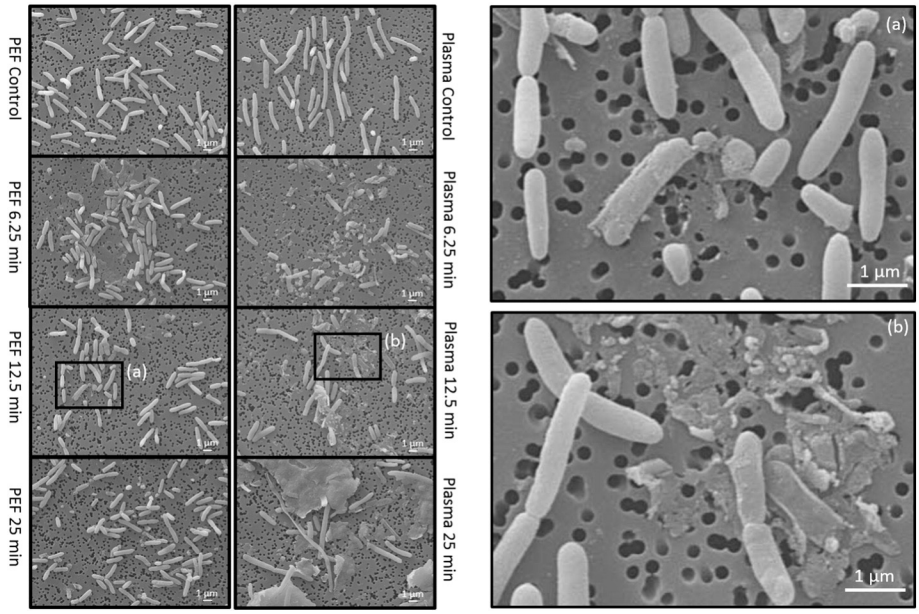

Fig. 6. Scanning electron microscopy pictures of *L. pneumophila* after plasma and PEF treatment, respectively.

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

Table 1
Pulse and energy parameters (MEAN) with standard deviation (SD) after application of 30,000 consecutively applied high voltage pulses, which is equal to a treatment time of 25 min. High voltage pulses were applied with a repetition rate of 20 Hz.

	Rise time in ns	Pulse-width in ns	Peak-voltage in kV	Peak-current in A	Pulse-energy in J
Plasma (mean)	16	145	80.86	128.10	1.16
SD	±0.05	±3.97	±0.61	±5.05	±0.05
PEF (mean)	19	228	−80.65	−143.45	1.03
SD	±2.08	±27.90	±0.22	±5.74	±0.17

Nevertheless, killing effects mediated by the electric field itself (log 3.4 CFU/ml) have to be considered as an integral part of plasma treatment contributing to overall killing of log 5.4 CFU/ml. Formally this circumstance can be expressed as a ratio of 63% PEF and 37% plasma based on observed log reduction. However, it has to be kept in mind that nature is most likely more complicated and it can be assumed that killing of log 3.4 is much easier than killing of log 5.4. Also findings should not be generalized for other microorganisms and are dependent on the experimental setup, pulse shape and the applied electric field.

For pulsed electric field exposures, the killing of bacteria can be explained by the irreversible poration of cell membranes. The at least required electric field for membrane poration can be estimated from Schwan's equation [34]. Hereby the assumption of a spherical shape is in fact a poor approximation for the shape of *Legionella pneumophila*. For elongated (prolate) cell shapes have Gimsa and independently Kotnik shown, that the induced (peak) transmembrane potential for an orientation of the major axis of the cell in parallel to the applied electric field will be more than twice as high at the poles for prolate cells in comparison to spherical cells of the same volume [35,36]. Further away from the poles is the membrane potential higher (by at most a factor of 2) for spherical cells. Altogether the cumulative effect on the cell membrane as estimated for prolate cells in comparison to spherical cells is therefore probably very similar, especially when taking into account that the poration at the poles together with the continuously changing orientation of the cells is probably sufficient for the killing of bacteria. Accordingly Schwan's equation is providing a reasonable approximation determining the effective volume around the high voltage electrode where an adverse effect on the cells can be expected. The estimate of an effective volume is arguably more strongly affected by the decay of the electric field with distance from the high voltage electrode than by the more gradual differences arising from the cell shape.

The minimal field strength E_{min} necessary for electroporation can be described by the general first order formulation of Schwan's equation (Eq. (2)) for the induced transmembrane potential, $\Delta\Psi$ [35]:

$$\Delta\Psi = 1.5 * E * r * \cos\theta * \left(1 - e^{\frac{-t}{\tau_m}}\right) \quad (2)$$

where t is the time of the pulse and $\tau_m = (0.5 * (\rho_a + \rho_b)) * C * r$ the characteristic charging time and with ρ_a and ρ_b are conductivities of the solvent and the cytoplasm, C the capacity of the cell membrane and r the radius of the cell.

At a sufficiently high voltage across the cell membrane which is generally assumed to be on the order = 1 V [37], pores are formed and the cell membrane becomes permeable. Dependent on field strength, pulse duration and number of pulses applied, a pore can be formed permanently or only temporarily. As a consequence, pores can induce leakage and possibly cell death [12,22,38,39].

However, in the chosen geometry the electric field is not homogeneous and only a fraction of cells in the vicinity of the high voltage electrode will be exposed to the required field. To estimate field strength alongside the high voltage electrode with respect to reactor dimensions the following equation was employed (Eq. (3)):

$$E = \frac{V_0}{\ln \frac{b}{a}} * \frac{1}{R} \quad (3)$$

where V_0 is the applied voltage, a and b the radii of the wire and reactor. R is the chosen distance between the wire and the reactor wall. For an applied voltage of 80 kV the electrical field strength E close to the high voltage tungsten wire is 2745 kV/cm and decreases exponentially to 8.1 kV/cm close to the outer electrode (Fig. 7).

According to the exponential decrease of the electrical field strength, induced membrane potential is decreasing with increasing distance from the high voltage electrode. The necessary electric field, E_{min}, for inducing a transmembrane potential difference of $\Delta\Psi = 1$ V, was calculated with 30.4–31.2 kV/cm for a solvent conductivity of 60–62 μS/cm. With these assumptions and the applied peak voltage of 80 kV the necessary field strength is achieved up to a distance of about 0.45 cm from the wire surface. Thus, effects of PEF were mediated in a cylindrical zone around the high voltage electrode with a volume of 4.1 to 4.3 cm^3.

It has to be noted that the analysis is not taking into account the decreasing value of the applied voltage with time as shown in Fig. 2. Therefore, the interaction volume might actually be much smaller for the threshold voltage that is equivalent to the minimum required electric field. The problem is alleviated by the flow system together with the high number of applied high voltage pulses. As consequence a large fraction of bacteria will eventually pass through the interaction zone, most likely repeatedly, and be prone to poration accordingly. Conversely, the killing rates that are achieved in the coaxial geometry for the dissipated energy are certainly still much lower than could be expected for the exposure to a homogeneous electric field and the efficiencies reported in Table 2 could be further increased. However, the objective of the study was not optimizing the PEF exposure but instead to compare them with the competing approach exploiting the generation of plasma filaments, for the same energy input per applied high voltage pulse. In addition, to the electric field around the wire, high electric fields are also formed around the plasma filaments.

Table 2
Energy efficiency for plasma treatment and PEF treatment for killing of *Legionella pneumophila* Philadelphia 1 JR32. Each column represents the mean of three independent experiments (n = 3). Comparing plasma and PEF treatment killing effects statistical significance was observed for 12.5 min (p = 0.004).

	Killing after 12.5 min in CFU/ml	Killing after 25 min in CFU/ml	Energy per pulse applied in J	Efficiency per log reduction in kJ/l	Efficiency for eradication in kJ/l
Plasma (mean)	log 5.4	log 5.4	1.16	23.03	124.44
SD	±0.15	±0.15	±0.02	±0.85	±5.15
PEF (mean)	log 3.42	log 4.82	1.03	46.97	>221.15
SD	±0.46	±0.57	±0.08	±5.90	±35.60

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

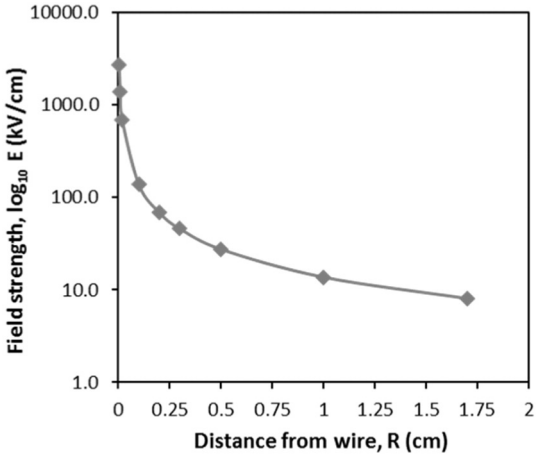

Fig. 7. Estimated electric field strength *E* as a function of distance from the wire at the center for an application of a high voltage of 80 kV. Field strength was calculated with respect to reactor dimensions, electrode curvature and applied voltage.

More importantly the mechanisms that are provided by the plasma can further be directed towards the killing of bacteria in addition to the electric field. Several studies proposed that in particular the generation of reactive species by the interaction of a plasma with water provides a most effective means for the killing of bacteria [40–43]. Chemical reactions responsible for the formation of reactive species in a plasma are often quite complex. They are dependent on many parameters like plasma source, gas mixture and/or immersion in a liquid. As a result of this reactive species for a variety of plasma sources may differ.

In pulsed corona plasma generated directly in water, hydroxyl radicals seem to be the dominant transient species [19]. Although hydroxyl radicals have only a lifetime of a few nanoseconds in aqueous solution they can form long-lived chemical products such as H_2O_2 [29]. Increased concentration of hydrogen peroxide was observed for plasma experiments conducted in this work. As an uncharged species H_2O_2, with its ability to penetrate membranes, is causing oxidative stress to compounds also inside the cell. Together with the applied electric field and the associated membrane poration, reactive species could enter cells more easily.

After 12.5 min, i.e. the time point where no viable colonies could be found, a concentration of 1 mM H_2O_2 was determined. Presumably this concentration can lead to severe damage due to oxidation of the DNA of the bacteria by generation of 8-oxo-guanidine or at proteins by generation of disulfide bridges [44,45]. However, when *Legionella* was exposed to 1 mM H_2O_2 only (i.e. without the associated generation of corona plasma or pulsed electric fields), a log reduction of only log 1.47 (CFU/ml) after 12.5 min was observed. Due to its own catalase and alkyl hydroxide reductase *L. pneumophila* is able to convert H_2O_2 into non-toxic compounds when applied in low concentration [46]. It should be mentioned that catalase activity is different in several Legionella strains [47].

However, we cannot exclude that other bacteria react less sensitive to H_2O_2 exposure than *Legionella*. A striking example is the opportunistic pathogen *Streptococcus pneumoniae*, which does not produce a catalase but is highly resistant against reactive oxygen species including H_2O_2 [48]. Meanwhile, hydrogen peroxide alone cannot explain increased cell disruption during plasma treatment when compared to PEF. No cell damage of *L. pneumophila* was detected by scanning electron microscopy when *Legionella* was exposed to 1 mM hydrogen peroxide (Fig. S2).

Although, the generation of reactive species, including hydrogen peroxide seems to be primarily responsible for the killing of *Legionella*, some other corona plasma characteristics could also contribute. These are in particular UV emissions, shockwaves and local heating generated directly within the plasma filaments. For example shear forces induced by shockwaves or water vapor expansion due to local heating during streamer propagation may be an explanation for increased cell damage observed during plasma treatment [31]. However, these effects are most likely only effective in close vicinity of the filaments while long-lived chemical species could be found throughout the liquid.

Altogether the combination of plasma induced mechanism, such as the generation of reactive species, with pulsed electric fields that are provided in the investigated configuration result in a much more efficient killing of *Legionella* than the exposure to pulsed electric fields alone.

5. Conclusion

The aim of this study was an unambiguous comparison of two promising emerging methods for the elimination of *Legionella pneumophila* from water. The experimental setup was designed to generate pulsed electric fields or pulsed corona plasma by changing the polarity of the applied high voltage pulses. It could be shown that killing of *L. pneumophila* was possible with both methods. However, pulsed corona plasma seems to be more efficient. Herby the effects of pulsed electric fields and the generation of reactive species are combined. Electroporation of the cell membrane due to the established electric field might favor the uptake of reactive species such as H_2O_2 into the *Legionella*. Once inside the cell radicals can develop their cytotoxic effects resulting in an enhanced bacterial killing. Based on these findings the application of pulsed corona plasma can be further optimized. Conversely, water treatments that are currently conducted by pulsed electric fields could be improved by a combination with plasma induced chemistry. This way, it might be possible to reduce the duration of applied electric fields and increase the energy efficiency of the treatment.

Acknowledgment

The authors would like to thank Birgit Rietow for excellent technical assistance and Rabea Schlüter from the Ernst Moritz Arndt University of Greifswald for performing the electron microscopy. *Legionella pneumophila* JR32 was provided by Michael Steinert from the Institute of Microbiology, TU Braunschweig. The authors appreciate the support of the Federal Ministry of Education and Research of Germany (BMBF) under contract no. 13N13638.

Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bioelechem.2016.05.006.

References

[1] D. García, N. Gómez, P. Mañas, S. Condón, J. Raso, R. Pagán, Occurrence of sublethal injury after pulsed electric fields depending on the micro-organism, the treatment medium ph and the intensity of the treatment investigated, J. Appl. Microbiol. 99 (2005) 94–104.
[2] M. Swanson, B. Hammer, Legionella pneumophila pathogenesis: a fateful journey from amoebae to macrophages, Annu. Rev. Microbiol. 54 (2000) 567–613.
[3] B.S. Fields, R.F. Benson, R.E. Besser, Legionella and Legionnaires' disease: 25 years of investigation, Clin. Microbiol. Rev. 15 (2002) 506–526.
[4] C. Joseph, Legionnaires' disease in Europe 2000–2002, Epidemiol. Infect. 132 (2004) 417–424.
[5] P. Stöcker, B. Brodhun, U. Buchholz, Legionärskrankheit in Deutschland unter besonderer Berücksichtigung der im Krankenhaus oder in einer Pflegeeinrichtung erworbenen Erkrankungen, 2004–2006, Bundesgesundheitsbl. Gesundheitsforsch. Gesundheitsschutz 52 (2009) 219–227.
[6] K. Neil, R. Berkelman, Increasing incidence of legionellosis in the United States, 1990–2005: changing epidemiologic trends, Clin. Infect. Dis. 47 (2008) 591–599.
[7] Y.S. Lin, J.E. Stout, V.L. Yu, R.D. Vidic, Disinfection of water distribution systems for *Legionella*, Semin. Respir. Infect. 13 (1998) 147–159.
[8] J.M. Kuchta, A.M. McNamara, R.M. Wadowsky, R.B. Yee, Susceptibility of *Legionella pneumophila* to chlorine in tap water, Appl. Environ. Microbiol. 46 (1983) 1134–1139.

[9] Q. Zhang, J. Zhuang, T. von Woedtke, J.F. Kolb, J. Zhang, J. Fang, K.-D. Weltmann, Synergistic antibacterial effects of treatments with low temperature plasma jet and pulsed electric fields, Appl. Phys. Lett. 105 (2014) 104103.

[10] S.J. Beebe, P.M. Fox, L.J. Rec, K. Somers, R.H. Stark, K.H. Schoenbach, Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis induction and tumor growth inhibition, Plasma Sci. IEEE Trans. 30 (2002) 286–292.

[11] R. Nuccitelli, U. Pliquett, X. Chen, W. Ford, R. James Swanson, S.J. Beebe, J.F. Kolb, K.H. Schoenbach, Nanosecond pulsed electric fields cause melanomas to self-destruct, Biochem. Biophys. Res. Commun. 343 (2006) 351–360.

[12] S. Toepfl, V. Heinz, D. Knorr, High intensity pulsed electric fields applied for food preservation, Chem. Eng. Process. Process Intensif. 46 (2007) 537–546.

[13] H. Akiyama, T. Sakugawa, T. Namihira, K. Takaki, Y. Minamitani, N. Shimomura, Industrial applications of pulsed power technology, Dielectr. Electr. Insul. IEEE Trans. 14 (2007) 1051–1064.

[14] X. Chen, J.F. Kolb, R.J. Swanson, K.H. Schoenbach, S.J. Beebe, Apoptosis initiation and angiogenesis inhibition: melanoma targets for nanosecond pulsed electric fields, Pigment Cell Melanoma Res. 23 (2010) 554–563.

[15] P. Lukes, M. Clupek, V. Babicky, P. Sunka, Ultraviolet radiation from the pulsed corona discharge in water, Plasma Sources Sci. Technol. 17 (2008) 024012.

[16] M. Dors, E. Metel, J. Mizeraczyk, E. Marotta, Coli bacteria inactivation by pulsed corona discharge in water, Int. J. Plasma Environ. Sci. Technol. 2 (2008) 34–37.

[17] A.T. Sugiarto, M. Sato, Pulsed plasma processing of organic compounds in aqueous solution, Thin Solid Films 386 (2001) 295–299.

[18] B. Jiang, J. Zheng, S. Qiu, M. Wu, Q. Zhang, Z. Yan, Q. Xue, Review on electrical discharge plasma technology for wastewater remediation, Chem. Eng. J. 236 (2014) 348–368.

[19] R. Banaschik, P. Lukes, H. Jablonowski, M.U. Hammer, K.-D. Weltmann, J.F. Kolb, Potential of pulsed corona discharges generated in water for the degradation of persistent pharmaceutical residues, Water Res. 84 (2015) 127–135.

[20] V. Joubert, C. Cheype, J. Bonnet, D. Packan, J.-P. Garnier, J. Teissié, V. Blanckaert, Inactivation of *Bacillus subtilis* var. *niger* of both spore and vegetative forms by means of corona discharges applied in water, Water Res. 47 (2013) 1381–1389.

[21] P. Sunka, V. Babický, M. Clupek, P. Lukes, M. Simek, J. Schmidt, M. Cernák, Generation of chemically active species by electrical discharges in water, Plasma Sources Sci. Technol. 8 (1999) 258.

[22] K.H. Schoenbach, F.E. Peterkin, R.W. Alden III, S.J. Beebe, The effect of pulsed electric fields on biological cells: experiments and applications, Plasma Sci. IEEE Trans. 25 (1997) 284–292.

[23] A. Abou-Ghazala, S. Katsuki, K. Schoenbach, F. Dobbs, K. Moreira, Bacterial decontamination of water by means of pulsed corona discharges, pulsed power plasma science, PPPS-2001. Dig. Tech. Pap. IEEE 2001 (2001) 612–615.

[24] E. Van Heesch, A. Pemen, P.A. Huijbrechts, P.C. van der Laan, K. Prasinski, G.J. Zanstra, P. De Jong, A fast pulsed power source applied to treatment of conducting liquids and air, Plasma Sci. IEEE Trans. 28 (2000) 137–143.

[25] R. Banaschik, F. Koch, J.F. Kolb, K.D. Weltmann, Decomposition of pharmaceuticals by pulsed corona discharges in water depending on streamer length, Plasma Sci. IEEE Trans. 42 (2014) 2736–2737.

[26] B. Sun, M. Sato, J.S. Clements, Optical study of active species produced by a pulsed streamer corona discharge in water, J. Electrost. 39 (1997) 189–202.

[27] M.A. Malik, A. Ghaffar, S.A. Malik, Water purification by electrical discharges, Plasma Sources Sci. Technol. 10 (2001) 82.

[28] E. Wintermeyer, B. Ludwig, M. Steinert, B. Schmidt, G. Fischer, J. Hacker, Influence of site specifically altered Mip proteins on intracellular survival of *Legionella pneumophila* in eukaryotic cells, Infect. Immun. 63 (1995) 4576–4583.

[29] B.R. Locke, K.-Y. Shih, Review of the methods to form hydrogen peroxide in electrical discharge plasma with liquid water, Plasma Sources Sci. Technol. 20 (2011) 034006.

[30] G. Eisenberg, Colorimetric determination of hydrogen peroxide, Ind. Eng. Chem. Anal. Ed. 15 (1943) 327–328.

[31] J. Kolb, R. Joshi, S. Xiao, K. Schoenbach, Streamers in water and other dielectric liquids, J. Phys. D. Appl. Phys. 41 (2008) 234007.

[32] S. Handtke, R. Schroeter, B. Jürgen, K. Methling, R. Schlüter, D. Albrecht, S.A. van Hijum, J. Bongaerts, K.-H. Maurer, M. Lalk, *Bacillus pumilus* reveals a remarkably high resistance to hydrogen peroxide provoked oxidative stress, PLoS One 9 (2014).

[33] B. Kim, J. Anderson, S. Mueller, W. Gaines, A. Kendall, Literature review—efficacy of various disinfectants against legionella in water systems, Water Res. 36 (2002) 4433–4444.

[34] V.H. Pauly, H. Schwan, Über die Impedanz einer Suspension von kugelförmigen Teilchen mit einer Schale, Z. Naturforsh. B 14 (1959) 125–131.

[35] T. Kotnik, D. Miklavčič, Analytical description of transmembrane voltage induced by electric fields on spheroidal cells, Biophys. J. 79 (2000) 670–679.

[36] J. Gimsa, D. Wachner, Analytical description of the transmembrane voltage induced on arbitrarily oriented ellipsoidal and cylindrical cells, Biophys. J. 81 (2001) 1888–1896.

[37] T. Kotnik, W. Frey, M. Sack, S.H. Meglič, M. Peterka, D. Miklavčič, Electroporation-based applications in biotechnology, Trends Biotechnol. (2015).

[38] N. Rowan, S.J. Macgregor, J. Anderson, R. Fouracre, O. Farish, Pulsed electric field inactivation of diarrhoeagenic *Bacillus cereus* through irreversible electroporation, Lett. Appl. Microbiol. 31 (2000) 110–114.

[39] S. Spilimbergo, F. Dehghani, A. Bertucco, N.R. Foster, Inactivation of bacteria and spores by pulse electric field and high pressure CO_2 at low temperature, Biotechnol. Bioeng. 82 (2003) 118–125.

[40] F. Liu, P. Sun, N. Bai, Y. Tian, H. Zhou, S. Wei, Y. Zhou, J. Zhang, W. Zhu, K. Becker, Inactivation of bacteria in an aqueous environment by a direct-current, cold-atmospheric-pressure air plasma microjet, Plasma Process. Polym. 7 (2010) 231–236.

[41] K. Oehmigen, M. Hähnel, R. Brandenburg, C. Wilke, K.D. Weltmann, T. von Woedtke, The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids, Plasma Process. Polym. 7 (2010) 250–257.

[42] Z. Machala, L. Chládeková, M. Pelach, Plasma agents in bio-decontamination by dc discharges in atmospheric air, J. Phys. D. Appl. Phys. 43 (2010) 222001.

[43] N. Bai, P. Sun, H. Zhou, H. Wu, R. Wang, F. Liu, W. Zhu, J.L. Lopez, J. Zhang, J. Fang, Inactivation of *Staphylococcus aureus* in water by a cold, He/O_2 atmospheric pressure plasma microjet, Plasma Process. Polym. 8 (2011) 424–431.

[44] E.L. Domingue, R. Tyndall, W. Mayberry, O. Pancorbo, Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1, Appl. Environ. Microbiol. 54 (1988) 741–747.

[45] J.J. Foti, B. Devadoss, J.A. Winkler, J.J. Collins, G.C. Walker, Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics, Science 336 (2012) 315–319.

[46] J.J. LeBlanc, R.J. Davidson, P.S. Hoffman, Compensatory functions of two alkyl hydroperoxide reductases in the oxidative defense system of *Legionella pneumophila*, J. Bacteriol. 188 (2006) 6235–6244.

[47] L. Pine, P. Hoffman, G. Malcolm, R. Benson, M. Keen, Determination of catalase, peroxidase, and superoxide dismutase within the genus legionella, J. Clin. Microbiol. 20 (1984) 421–429.

[48] M. Saleh, S.G. Bartual, M.R. Abdullah, I. Jensch, T.M. Asmat, L. Petruschka, T. Pribyl, M. Gellert, C.H. Lillig, H. Antelmann, Molecular architecture of *Streptococcus pneumoniae* surface thioredoxin-fold lipoproteins crucial for extracellular oxidative stress resistance and maintenance of virulence, EMBO Mol. Med. 5 (2013) 1852–1870.

Please cite this article as: R. Banaschik, et al., Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing *Legionella pneumophila* as model organism..., Bioelectrochemistry (2016), http://dx.doi.org/10.1016/j.bioelechem.2016.05.006

Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Datum

Katja Zocher

Curriculum Vitae

Publications and Conference Contributions

Publications in Peer Reviewed Journals

2019

Mechanism of Disintegration of Microalgae by spark discharge Treatment for compound Extraction
Zocher, K., Rataj, R., Steuer, A., Weltmann, K.-D. & Kolb, J. F. (2019), *Journal of Applied Physics: D*, (submitted).

Profiling microalgal protein extraction by microwave burst heating in comparison to spark plasma exposures
Zocher, K., Lackmann, J. W., Volzke, J., Steil, L., Lalk, M., Weltmann, K.-D., Wende, K. & Kolb, J. F. (2019). *Algal Research*, 39, 101416.

2018

Electrical and mechanical properties of polyimide films treated by plasma formed in water and isopropanol
Sava, I., Asandulesa, M., Zocher, K., Kruth, A., Kolb, J. F., Bodnar, W., Witte, K., Ishizaki, T. & Miron, C. (2018). *Reactive and Functional Polymers*, 134, 22-30.

2016

Comparison of extraction of valuable compounds from microalgae by atmospheric pressure plasmas and pulsed electric fields
Zocher, K., Banaschik, R., Schulze, C., Schulz, T., Kredl, J., Miron, C., Schmidt, M., Mundt, S., Frey, W., & Kolb, J. F. (2016). *Plasma Medicine*, 6(3-4).

Comparison of pulsed corona plasma and pulsed electric fields for the decontamination of water containing Legionella pneumophila as model organism
R. Banaschik, G. Burchhardt, K. Zocher, S. Hammerschmidt, J.F. Kolb & K.-D. Weltmann, *Bioelectrochemistry*, 112 (2016) 83-90.

Oral Presentations

Invited talks

2019

Spark discharges as tool for the extraction of microalgal compounds
Katja Zocher, Raphael Rataj, Anna Steuer, and Juergen F. Kolb; DPG-Frühjahrstagung March 17-25 2019, Munich, Germany

Talks

2019

Microalgal protein and cell damage analyses after spark discharge treatment for downstreaming purposes
Katja Zocher, Raphael Rataj, Anna Steuer, and Juergen F. Kolb, 3rd World Congress on Electroporation & Pulsed Electric Fields in Biology, Medicine, Food and Technology, September 03-06 2019, Toulouse, France

Downstreaming of valuable compounds from microalgae with spark discharges, instigated by 100-ns high voltage pulses
Katja Zocher, Raphael Rataj, Anna Steuer, and Juergen F. Kolb, 2019 IEEE Pulsed Power and Plasma Science Conference, June 23-28, 2019, Orlando, USA

Spark discharges as tool for the extraction of microalgal compounds
Katja Zocher, Raphael Rataj, Anna Steuer, and Juergen F. Kolb; DPG-Frühjahrstagung March 17-25 2019, Munich, Germany

2018

Cell wall rupture of microalgae for the extraction of metabolites by applying spark discharges – effects and mechanism
Katja Zocher, Raphael Rataj, Jan-Wilm Lackmann and Juergen F. Kolb; 15th International Bioelectrics Symposium (BIOELECTRICS 2018), September 23-26 2018, Prague, Czech Republic

2017

Microalgal protein and pigment analysis after extraction with spark discharge treatment
Katja Zocher, Kristian Wende, Johann Volzke, Juergen F. Kolb and Klaus-Dieter Weltmann; 2nd World Congress on Electroporation & Pulsed Electric Fields in Biology, Medicine, Food and Technology, September 24-28 2017, Norfolk, USA

Protein and Pigment extraction from microalgae with spark discharge plasmas
Katja Zocher, Robert Banaschik, Johann Volzke, Kristian Wende, Michael Lalk and Juergen F. Kolb; The 5th International Workshop & The 4th International Mini Workshop on Solution Plasma and Molecular Technologies, June 25-29 2017, Greifswald, Germany

Extraction of metabolites from microalgae with spark discharges
Katja Zocher, Robert Banaschik, Johann Volzke, Kristian Wende, Michael Lalk and Juergen F. Kolb; 2017 IEEE International Conference on Plasma Science (ICOPS), May 21-25 2017, Atlantic City, USA

2016

Enhancement cell wall rupture of microalgae by spark discharges
Katja Zocher, Robert Banaschik, Tilo Schulz, Camelia Miron, Juergen F. Kolb; 13th International Bioelectrics Symposium (BIOELECTRICS 2016), September 12-15 2016, Rostock, Germany

Non-thermal plasma and pulsed electric fields for the extraction of valuable substances from microalgae
Juergen F. Kolb*, Katja Zocher, Tilo Schulz, Anna Steuer, Jörn Winter, Jörg Ehlbeck, Thomas von Woedtke, Klaus-Dieter Weltmann; 1st International Workshop on Plasma Agriculture (IWOPA 1), May 15-20 2016, Philadelphia, USA; *presenter

Poster Presentations

2016

Pulsed Corona Plasma and Pulsed Electric Fields for the decontamination of water containing Legionella pneumophila
Robert Banaschik, Gerhard Burchhardt, Katja Zocher, Sven Hammerschmidt, Juergen. F. Kolb, Klaus-Dieter Weltmann, 13th International Bioelectrics Symposium (BIOELECTRICS 2016), September 12-15 2016, Rostock, Germany

Extraction of pharmaceutic compounds from microalgae with spark discharge plasmas
Katja Zocher & Juergen F. Kolb; Leibniz PhD Workshop of the section C and D on Health Technologies, September 07-08 2018, Berlin, Germany

Acknowledgment

Writing a thesis never works without the support of many people. Therefore, I would like to thank the following persons for their encouragement throughout the years:

Prof. Michael Lalk, who assumed fearlessly the supervision of this exceptional topic, who helped me whenever necessary and who taught me to be “not too strict” with myself.

Prof. Jürgen F. Kolb, who helped to develop this topic and providing ideas whenever I thought I was at a dead end; for helping me to grow as scientist and as person, and for sharing the same passion for postcards and cartoons.

Prof. Klaus-Dieter Weltmann, who gave me the opportunity to start a thesis at all and who listened to me carefully whenever I had a problem.

Dr. Robert Banaschik for his constant support, the lab jokes and his ability to help me to become a better scientist.

Dr. Jan Lackmann, for saving me when I was dangling at the edge, for sharing his exhaustless knowledge about really everything and for having the patience of answering all my Columbo-questions.

Dr. Camelia Miron, for having the idea of applying spark discharges and providing me her setup for first experiments, as well as her encouragement through the years and the kind conversation we had.

My lab buddy Raphael Rataj, for basically every help he offered, for all the encouragement and trust in my abilities, the scientific and philosophical conversations and for tolerating all my stupid questions about plasma physics.

Tilo Schulz for perfectly building my setups and the continuous supply of coffee and cookies.

The Bioelectrics group, Dr. Henrike Brust, Dr. Nicola Wannicke, Dr. Jörn Winter, Robert Bansemer (the best office mate you can get), Liane Kantz, Rüdiger Titze, Johann Volzke, Prof. Thomas von Woedtke for sharing their knowledge and making the serious science business a bit fun.

All other doctoral researchers and colleagues at the INP, the people from the pharmaceutical biology at the University of Greifswald for all help, fun and support.

My family and especially my mum for her excitement, encouragements, questions about the aim of this work and the never-ending interest in the progress of my thesis, the phone conversations and her ability to cheering me up whenever necessary. In loving memory of my grandfather, who unfortunately missed the amazing journey I was allowed to make.

All my beautiful friends and in particular, minun suomen ystavät Heike, Viia Satu, Marjatta ja Jens - kiitos paljon huomiostanne ja tuestanne.

The most important person in my life, without whom I would not even have started this journey. For being everything I needed to not to give up, to develop myself and grow in mind, for believing in me, for supporting me in tough decisions, and for cheering me on the entire racetrack. Thanks for being my best friend, soulmate, best travel buddy and survival trainer. I am really looking forward to our next adventure, which I also would have never started without your optimism. Let’s see what will happen!