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PII: S0960-8524(18)31377-4
DOI: https://doi.org/10.1016/j.biortech.2018.09.124
Reference: BITE 20540

To appear in: Bioresource Technology

Received Date: 31 August 2018
Revised Date: 23 September 2018
Accepted Date: 24 September 2018

Please cite this article as: Buchmann, L., Frey, W., Gusbeth, C., Ravaynia, P.S., Mathys, A., Effect of nanosecond pulsed electric field treatment on cell proliferation of microalgae, Bioresource Technology (2018), doi: https://doi.org/10.1016/j.biortech.2018.09.124

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Effect of nanosecond pulsed electric field treatment on cell proliferation of microalgae
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Abstract
Photoautotrophic microalgae based biorefinery concepts are currently not competitive compared to other established production systems. Therefore, innovative upstream processes need to be developed to increase the competitiveness of photoautotrophic microalgae biorefinery concepts. Abiotic sub-lethal stress induction via nanosecond pulsed electric field (nsPEF) treatment might be a viable process to increase the efficiency of photoautotrophic microalgae cultivation. In this work, an increased cell growth
after nsPEF treatment was observable. Application of nsPEF to highly proliferating cells in a repetitive process resulted in statistical significant increase in cell growth ($p = 0.009$). The effect was most pronounced after five days wherefore cellular structures and processes were analyzed to reveal a possible mechanism. Within this work, a protocol for increased cell proliferation with a possible mechanism was derived, which improves competitiveness of photoautotrophic microalgae biorefineries in the future. However, based on the derived mechanism, the results are also relevant for other microorganisms.

**Keywords**

abiotic stress; nanosecond pulsed electric field; microalgae cultivation;

*Arthrospira platensis*; cell proliferation.

**1. Introduction**

The constant growth of human population causes new challenges regarding energy supply, food security, human health and biodiversity. Agriculture causes 30% of global greenhouse gas emission and 85% of the water footprint (Smetana et al., 2015). Biorefinery concepts, implementation of innovative technologies as well as a shift from animal toward more plant-based foods are possible solutions to tackle those challenges (Chaudhary et al., 2018). In this context, the exploitation of microalgae have become a field of great interest (Golberg et al., 2016; Rocha et al., 2018). They can serve as raw material for biofuels or agricultural biostimulants but at the same time are a promising source for food and feed production due to their high proportion of proteins and micronutrients (Colla et al., 2007). Besides these, microalgae can be cultivated on non-arable land and fixate CO$_2$ if cultivated photoautotrophically (Jacob-
lopes et al., 2018). However, the cultivation of photoautotrophic microalgae is currently not competitive in comparison to many other plant sources (Smetana et al., 2017). High production costs resulting from the growth medium, energy supply and extraction of valuable compounds demands further improvement in the up- and down-stream processing of microalgae (Golberg et al., 2016).

Nanosecond pulsed electric fields (nsPEF) are a promising technology to increase the viability of microalgae cultivation systems. Among the possible applications of nsPEF are sub-lethal stress induction as well as targeted microbial flora control (Buchmann et al., 2018b; Buescher and Schoenbach, 2003; Eing et al., 2009). In nsPEF applications on eukaryotic cells, an increased growth was observable after the treatment (Eing et al., 2009; Gusbeth et al., 2013).

The underlying theory assumes an abiotic stress induction on a sub-cellular level. Based on mathematical models an increased electropermeabilization effect on intracellular structures compared to the cell membrane was observed for angular frequencies $\omega$ (rad s$^{-1}$) above $10^7$ (Kotnik and Miklavčič, 2006; Vernier et al., 2006). The double-shell model of a biological cell and its resulting reference circuit supports this theoretical analysis (Schoenbach et al., 2004).

Hence, nsPEF treatments affect sub-cellular compartments. This aspect results from the charging time constant of the plasma membrane (Vernier et al., 2006). The charging time constant is dependent on the conductive (intra- and extracellular fluids) and the dielectric (cell membrane) properties of a cell in an electric reference circuit (Vernier et al., 2006). A pulse duration below the charging time constant will result in an electric field conveyed through the
intracellular space (Schoenbach et al., 2004). In addition, intracellular effects are most pronounced in proliferating cells compared to stationary phase cells (Schoenbach et al., 1997).

Induced intracellular effects could be Ca$^{2+}$ release from internal storages, formation of radical oxygen species and release of cytochrome c from mitochondria among others (Batista Napotnik et al., 2016; Schoenbach et al., 2007).

The induced effects are confirmed by numerical simulation in which nsPEF increased the number of minimum size pores compared to conventional PEF (Gowrishankar et al., 2006). Hence, nsPEF treatments enable the transport of primarily small molecules across the membrane by either direct transport or voltage gating of channels (Casciola and Tarek, 2016; Stewart et al., 2004).

However, the fundamental process characteristic to induce intracellular electro effects is not yet understood.

The influence of pulse repetition frequency (PRF), ionic strength of the medium and cell composition are currently investigated. Silve et al., (2014), introduced the concept of electro-desensitization, were an increased PRF results in reduced electroporation effects. This principle is based on different dynamics of membrane resealing (Lamberti et al., 2015). A study from Sridhara and Joshi, (2014) focused on the influence of external medium conductivity and found that high ionic strengths in the medium locally increase the electroporation process, resulting in much faster pore formation. Napotnik et al., (2012), could further demonstrate that nsPEF lead to an increase in the inner mitochondrial membrane permeability.
The lipid composition between the inner mitochondrial membrane and the prokaryotic membrane was shown to be similar (Fontanesi, 2015). Hence, the concept of electropulsation, oxidation of membrane phospholipids, could play an important role in the underlying mechanisms of pulsed electric field application (Breton and Mir, 2018). Therefore, the effects of nsPEF on mitochondrial structures might be transferrable to prokaryotic cells, and vice versa with regard to the endosymbiotic theory. However, the exploitation of nsPEF induced effects on prokaryotic cells is a new area of research.

2. Material and Methods

2.1 nsPEF treatment

The nsPEF treatments were executed in triplicates using a Blumlein generator (manufactured at the Karlsruhe Institute of Technology (KIT), Germany, for further information refer to (Kolb et al., 2006)). The cable length of 20.8 m resulted in a rectangular pulse with a pulse duration of 100 ns. A metal oxide semiconductor field-effect transistor (MOSFET) switch (manufactured at the Karlsruhe Institute of Technology (KIT), Germany) was connected to the Blumlein and triggered with a frequency generator (YOKOGAWA FG 300, Yokogawa, Musashino, Japan). The system was charged to 1 kV by incorporating a high voltage power supply (Model 205A-05R, Spellmann High Voltage Electronics Ltd., Pulborough, UK) (Figure 1).

The resulting electric field strength $E$ (kV cm$^{-1}$) was 10 kV cm$^{-1}$ as experiments were conducted using a plate-plate 1 mm polycarbonate treatment chamber (manufactured at the Karlsruhe Institute of Technology (KIT), Germany), for further information regarding the experimental setup refer to (Goettel et al.,
2013). The pulse duration and electric field were kept constant due to experience from previous studies (Eing et al., 2009) and processing capabilities.

The microalgae suspension was pumped through polypropylene-based tubes (SC0319A, Cole-Parmer GmbH, Wertheim, Germany) connected to the treatment chamber. A peristaltic pump (MS-4/12-100 ISMATEC®, Cole-Parmer GmbH, Wertheim, Germany) was used at 15 rpm to pump the suspension through the treatment chamber. The electrical efficiency of the process is around 90% and higher, in dependence of the treatment homogeneity (Buchmann et al., 2018a, 2018b) In order to achieve matched load conditions the plate-plate treatment chamber was adjusted to the conductivity \( \sigma \) (mS cm\(^{-1}\)) of the microalgae suspensions. The relation between conductivity and impedance can be derived from Equation 1, with the total impedance \( Z_{\text{tot}} \) (\( \Omega \)) equal to the fraction of the inverse resistance, with the conductivity \( \sigma \) (mS cm\(^{-1}\)), electrode surface area \( A \) (m\(^2\)) and electrode distance \( d \) (m), added by the system's admittance \( Y_c \) (S)

\[
Z_{\text{tot}} = \frac{1}{(\sigma \cdot A/d + Y_c)} \quad (1)
\]

The specific energy input \( W_S \) (J kg\(^{-1}\)) can be calculated according to Equation 2, with the electric field \( E \) (V m\(^{-1}\)), pulse width \( \tau_p \) (ns), conductivity \( \sigma \) (mS cm\(^{-1}\)), and pulse number \( n \) (-)

\[
W_S = E^2 \cdot \tau_p \cdot \sigma \cdot n \quad (2)
\]

The pulse number can be derived from Equation 3, with the frequency \( f \) (Hz), the treatment duration \( t \) (s) which can be expressed as the treatment chamber's volume \( V_0 \) (m\(^3\)) divided by the volumetric flow rate \( \dot{V} \) (m\(^3\) s\(^{-1}\))

\[
n = f \cdot t = f \cdot V_0 / \dot{V} \quad (3)
\]
Further information on these theoretical backgrounds could be found elsewhere (Buchmann et al., 2018a, 2018b, Pirc et al., 2017; Reberšek et al., 2014). Based on an in-depth nsPEF system characterization (Buchmann et al., 2018a, 2018b), the specific energy input could be calculated for all experiments.

2.2 Arthrospira platensis cultures

The strain *Arthrospira platensis* SAG 21.99 was cultivated in a modified Zarrouk medium (Aiba and Ogawa, 1977). The cultures were incubated at 25 ± 0.2 °C, 70% relative humidity, 150 rpm, ambient CO₂, and continuous illumination with a mean photosynthetically active photon flux density (PPFD) of 32 µmol photons m⁻² s⁻¹ using warm white LED lamps in a shaking incubator (Multitron Pro shaking incubator, Infors AG, Bottmingen, Switzerland). The growth of *A. platensis* was monitored by OD measurements at 750 nm. Prior and after every experiment, the conductivity and pH of the *A. platensis* suspensions were measured using a Seven compact conductivity meter (Mettler-Toledo International Inc., Columbus OH, USA) and pH meter (827 pH lab, Metrohm, Herisau, Switzerland) that was connected to a primatrode (Metrohm, Herisau, Switzerland).

2.3 Microalgae growth and pigment assessment

Assessing microalgae growth was done by gravimetical determination of the dry substance (DS) (g L⁻¹) using an analytical balance (LA 214i, VWR, Leuven, Belgium). The gravimetical analysis was conducted after microalgae harvesting by use of a vacuum filtration system (SciLabware Ltd., Staffordshire, UK). Glass microfiber filters with a pore size of 1.2 µm (GF/C, GE Healthcare, Chicago IL, USA), were used to collect the dry substance. Based on the gravimetical
analysis a conversion factor to the optical density (OD) at 750 nm was determined. OD measurements were conducted in triplicates with a UV/VIS spectrophotometer (Cary 100, Agilent Technologies, Santa Clara CA, USA). The conversion of OD to DS was conducted in accordance with equation 4:

$$DS = 0.689 \pm 0.039 \cdot OD_{750} \quad (4),$$

where the conversion factor (0.689 ± 0.039) was obtained empirically. The C-phycocyanin (cPC) and allophycocyanin (aPC) content of the *A. platensis* suspensions was assessed by correcting the measured absorption values with the absorption at 750 nm. Based on Yoshikawa and Belay, (2008) aPC and cPC concentrations (g L⁻¹) could be determined according to equations 5 & 6:

$$aPC = 0.180 \cdot A_{650} - 0.042 \cdot A_{620} \quad (5)$$

$$cPC = 0.162 \cdot A_{620} - 0.098 \cdot A_{650} \quad (6).$$

An independent T-test at a confidence interval of either 95% or 99% was used to assess statistical significance of the results. The results were obtained using the software IBM SPSS Statistics (IBM Corp., Armonk NY, USA).

**2.4 Single cell electrical impedance spectroscopy**

Impedance measurements of microalgae cells were conducted using a custom-made electrical impedance spectroscopy (EIS) platform for single cell analysis. The platform is composed of a microfluidic chip made of polydimethylsiloxane (PDMS) and bonded to a glass substrate with patterned platinum electrodes. The PDMS chip consists of one inlet and outlet connected by a 3 mm straight channel of 300 x 50 µm² (width x height) dimensions. The channel features a constriction at the center with 50 µm width and height. The 150 nm thick
platinum electrodes are deposited on a glass substrate patterned via a lift-off process. A pair of coplanar electrodes of 150 µm width and spacing between the electrodes is aligned with the channel constriction to enable EIS measurements of single flowing cells. The solution was injected with a flow rate of 2.5 µL min$^{-1}$ using a syringe pump (Pump 11 Elite, Harvard Apparatus, Holliston MA, USA) connected to the inlet port. The chip was contacted via a custom-made PCB to route the connections from the impedance spectroscope to the electrodes. Impedance measurements were performed using a HF2-LI impedance spectroscope (Zurich Instruments AG, Zurich, Switzerland). EIS measurements were taken at six frequencies logarithmically spaced between 100 kHz and 10 MHz. An AC voltage of 300 mV at each selected frequency was applied between the coplanar electrodes. The current flowing through the system was then converted to voltage through a trans-impedance amplifier with 1 kΩ feedback resistor and sampled by the HF2-LI with a sampling frequency of 225 Hz. The signal was post processed in Matlab (MATLAB 2016b, The Mathworks Inc., Natick MA, USA). The passage of each microalgae cell induces a transient reduction of the voltage-converted current between the electrodes pair resulting in a peak. The transient peak height was extracted by measuring the local peak-to-baseline value for all simultaneously recorded frequencies. Untreated as well as nsPEF treated *A. platensis* cells were analyzed. More than 100 cells per condition were analyzed to provide significant statistics for measuring the average peak-to-baseline signal variations caused by the passage of the cells over the electrodes. All measurements were carried out in
the same modified Zarrouk medium (Aiba and Ogawa, 1977) to avoid signal differences caused by different medium conductivity.

2.5 Proteomic analysis

A shotgun analysis liquid chromatography and mass spectrometry (LC-MS) was conducted at the Functional Genomics Center Zurich (FGCZ), Switzerland. Protein/peptide identification and characterization was performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS). Sample preparation was conducted by precipitation and proteolytic digestion. The nsPEF treated \textit{A. platensis} cells were harvested and the pellet was resuspended (10 g L\textsuperscript{-1}) in a 1:5 diluted solution I of the Zarrouk medium (Aiba and Ogawa, 1977). In a next step trichloroacetic acid (TCA, Sigma-Aldrich, St. Louis MO, USA) precipitation was conducted using 10 μL of sample with 90 μL of water and 100 μL 20% TCA. The sample was washed twice with cold acetone. The dried pellets were afterwards dissolved in 45 μL buffer (20 mM Tris + 2 mM CaCl\textsubscript{2} at pH 8.2) and 5 μL trypsin (100 ng μL\textsuperscript{-1} in 10 mM HCl) for digestion, which was carried out in a microwave instrument (Discover System, CEM, Matthews NC, USA) for 30 min at 5 W and 60 °C. Subsequent samples were dried in a Savant SpeedVac (Thermo Fisher Scientific, Waltham MA, USA). For LC-MS/MS analysis the samples were dissolved in 0.1% formic acid (Romil Ltd., Cambridge, UK) and an aliquot of 4% was analyzed on a nanoAcquity UPLC (Waters Inc., Milford MA, USA) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham MA, USA) equipped with a Digital PicoView source (New Objective, Woburn MA, USA). Peptides were trapped on a Symmetry C18 trap column (5
µm, 180 µm · 20 mm, Waters Inc., Milford MA, USA) and separated on a BEH300 C18 column (1.7 µm, 75 µm · 150 m, Waters Inc., Milford MA, USA) at a flow rate of 250 nl min⁻¹ using a gradient from 1% solvent B (0.1% formic acid in acetonitrile, Romil) / 99% solvent A (0.1% formic acid in water, Romil) to 40% solvent B / 60% solvent A within 90 min. Mass spectrometer settings were: Data dependent analysis. Precursor scan range 350 – 1500 m z⁻¹, resolution 70’000, maximum injection time 100 ms, threshold 3e⁶. Fragment ion scan range 200 – 2000 m z⁻¹, resolution 35’000, maximum injection time 120 ms, threshold 1e⁵.

Proteins were identified using the Mascot search engine (version 2.5.1.3, Matrix Science, Boston MA, USA). Mascot was set up to search the SwissProt and the Trembl (bacteria only) database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.030 Da and a parent ion tolerance of 10.0 ppm. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (Proteome Software Inc., Portland OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they achieved a false discovery rate (FDR) of less than 0.1% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they achieved an FDR of less than 1.0% and contained at least 2 identified peptides.

Subsequent results are based on the Trembl (bacteria) search. The database contains around 80 million different proteins and a fully sequenced A. platensis strain with 6630 open reading frames (NIES-39, National Institute of Technology and Evaluation, Tokyo, Japan). The data was analyzed applying very stringent settings, (protein threshold 99%, min # peptides 2 and peptide threshold 95%).
Statistical analysis was conducted by a T-test with normalization, on a 99% confidence interval.

3. Results and Discussion

According to the theory of an increased nsPEF effect on fast proliferating cells (Schoenbach et al., 1997), the influence of cell growth stage on the increased cell proliferation was investigated. Based on literature data different inoculation intervals were analyzed. The effect of nsPEF treatments after 12, 36 and 60 hours of inoculation was assessed (Figure 2).

The obtained results (Figure 2) correspond with the theory where an increased effect was expected on highly proliferating cells (Schoenbach et al., 1997). Therefore, experiments were conducted using a time interval of 36 h between inoculation and nsPEF treatment.

Based on the concept of electrosensitization (Pakhomova et al., 2011), the nsPEF treatment was applied repetitively. A single treatment did not result in a statistical significant effect and neither did a single repetition of the treatment. The increased cell proliferation was observable if the treatment was repeated three times as described in the methods section 2.1. In-between the single nsPEF treatment was a time interval of three hours.

Based on the obtained results, the assessment of the influence of treatment energy on the cell proliferation was examined after 36 h and after three subsequent nsPEF treatments. We found that nsPEF treatments lead to an increase in DS in A. platensis suspensions. The various analyzed energy inputs were achieved by increasing the PRF while maintaining all other pulse parameters (Table 1).
Based on findings from Buchmann et al. (2018a), varying only the PRF ensured an reproducible nsPEF treatment. Furthermore allowed this alteration of the system to analyze the effects of PRF and energy input on the obtained results. A PRF of 9 Hz resulting in an energy input of $256.22 \pm 67.53 \text{ J kg}^{-1}$ was found to be most effective (Figure 3A) ($p = 0.009$). The increase in DS was $13.1 \pm 1.6 \%$. The other tested energy inputs did not increase cell proliferation significantly ($p > 0.05$). The treatment energy is always stated for an individual replication of the nsPEF treatment (Figure 3A). The energy input in-between experiments did not vary significantly, as no influence on external medium conductivity was detectable.

With regard to aPC and cPC, an even increased difference between treated and untreated samples was found (Figure 3B). The increase was $18.8 \pm 5.5 \%$ and $19.5 \pm 6 \%$ for aPC and cPC, respectively. The observation was in accordance with the overall results. Energy inputs resulting in a non-significant cell proliferation increase did also not affect phycocyanin concentrations significantly.

Based on the experimental procedure the effect of PRF and energy input on the nsPEF induced effect was studied. The obtained results indicate that the effect of nsPEF on the cell proliferation of *A. platensis* is primary energy dependent. Alternatively, an increased cell proliferation would have to be observed with PRFs of 11 and 13 Hz (Table 1). As illustrated in Figure 3A, this dependency on PRF was not detectable.

However, regarding the importance of the repetitive nsPEF application the concept of electrosensitization (Pakhomova et al., 2011) appears to be valid for
nsPEF induced effects on cell proliferation. This concept was further tested by applying the energy input equal to a repetitive experiment in one single experiment. These experiments resulted in a significant difference in DS (p = 0.000003) if the energy was applied in a single, compared to a repetitive treatment (Figure 4). Hence, the repetitive abiotic stress induction appears to be critical for increased cell proliferation due to nsPEF. However, even the application of the whole energy input did not statistically significant reduce the DS of *A. platensis* (p > 0.05). Further studies should focus on the optimized treatment repetition/frequency to use the full potential of nsPEF treatments based on the concept of electrosensitization.

### 3.1 Analysis of nsPEF treatment mechanisms

Prior and after every experiment, the conductivity of the *A. platensis* cultures was measured. There was no significant difference detectable between treated and untreated cultures. In addition, no cell swelling was observable for the *A. platensis* cells during the experimental period. Moreover, there was no difference in the pH of *A. platensis* solutions detectable. Concisely, short-term electroporation effects were not detectable by these analytical methods. However as already described by Bai et al., (2017) the effect of nsPEF was most pronounced 5 days after the nsPEF treatment (Figure 5). Detectable changes after 5 days let to the hypothesis that cellular structures/processes were altered by the nsPEF treatment. Therefore, the cellular structure was analyzed after 5 days using a non-invasive technique, such as EIS. Impedance cytometers, realized by integrating a set of electrodes within a microfluidic channel, have been used for multi-parametric assessments across
different frequencies for differentiation of single cells, based on cell size, membrane integrity and internal properties of cells (Sun and Morgan, 2010). Impedance characterization at low frequencies (<1 MHz) provides information on cell volume/size due to the non-conducting cell membrane barrier, while intermediate frequencies (0.5-10 MHz) are used to investigate membrane capacitance due to increases in capacitive coupling across the cell barrier (Cheung et al., 2005; McGrath et al., 2017). EIS can therefore be used to assess morphological variations of A. platensis after exposure to different treatments. The acquired EIS spectra did not show significant differences (p > 0.05) between treated and untreated cells in the tested frequency range (Figure 6). EIS characterization therefore support the hypothesis that nsPEF treatment does not induce morphological changes on a plasma membrane level. Hence, the applied rectangular 100 ns pulses seem to primarily affect the subcellular structures (Kotnik and Miklavčič, 2006). Given the similarity between mitochondrial cell membranes and prokaryotic cell membranes (Fontanesi, 2015), prokaryotes may allow for analysis of nsPEF induced subcellular effects. Thereby, the increased cell proliferation in prokaryotic cells might be transferable to eukaryotic cells.

This hypothesis was tested with a shotgun LC-MS analysis of the harvested biomass. Thereby, 956 proteins were identified corresponding to 12268 peptide spectra. Based on the database search as described in section 2.5, two significant different proteins were determined. The first protein is the Na-Ca exchanger/integrin-beta4 OS=Arthrospira sp. PCC 8005 OX=376219 GN=ARTTHRO_430061 PE=4 SV=1 (p = 0.0069). The heterodimeric protein
integrin is involved in transmembrane cell communication and regulation of cell behavior (Rédei, 2008). The protein is present in the cytoplasmic domain of Na-Ca exchanger and mediates the bi-directional transfer of signals (Schwarz and Benzer, 1997). Thereby, integrins have been attributed to growth promotion as they signal to guanine nucleotide-binding proteins (Harburger and Calderwood, 2009). The overexpression of these proteins correlates with the abiotic stress response of plants, which involves Ca\(^{2+}\) as an essential second messenger in the signaling pathway (Zhu, 2016).

The second overexpressed protein is the elongation factor Tu OS=Halomonas daqingensis OX=419596 GN=tuf PE=3 SV=1 (p = 0.0073) belonging to the cluster of Elongation factor Tu OS=Arthrospira platensis (strain NIES-39 / IAM M-135) OX=696747 GN=tuf PE=3 SV=1 (D4ZUX7_ARTPN). The cluster is assembled using protein cluster analysis based on shared evidence. The elongation factor (EF) TU binds aminoacyl-tRNA to the ribosomal acceptor site (Zvereva et al., 2001). Aminoacyl-tRNA is an amino acid-charged tRNA at the 3’ end (Rédei, 2008). The prokaryotic EF-TU is a guanine nucleotide-binding Ras-like protein (Paduch et al., 2001; Wuichet and Søgaard-Andersen, 2014). The Ras superfamily are proto-oncogenes with various cellular functions, for example they are one of the most important turnstiles in signal transduction (Dong et al., 2007; Wuichet and Søgaard-Andersen, 2014).

The proteomics result correspond well with the observed macroscopic change in cell proliferation. Integrin signaling to a Ras-like protein is strongly related to increased cell proliferation. However, the proteomics results are only indicating a possible nsPEF induced abiotic stress response pathway, as the proteome
was solely analyzed on the fifth day. Furthermore, there was no correction for multiple comparisons executed. Nevertheless, the results were obtained in triplicates and there are only two distinct proteins overexpressed with the applied boundary conditions.

Given that the observed effect is only detectable after 5 days might also correlate with the cell doubling time. With respect to Figure 5, the doubling time is around three days after the nsPEF treatment. Therefore, the cell number is quite low to detect significant differences from the beginning and need to increase, to recognize the macroscopic effect after 5 days.

The obtained results are in good agreement with literature. A repetitive nsPEF treatment led to increased effects, which corresponds well with the concept of electrosensitization (Pakhomova et al., 2011). Furthermore, the increase in cell proliferation was most pronounced in fast proliferating cells (Schoenbach et al., 1997). Moreover, the outer cellular structure of treated and untreated A. platensis did not change. This aspect might be surprising as prokaryotes and mitochondria are similar in cell membrane composition (Fontanesi, 2015) and therefore, subcellular effects in eukaryotes should theoretically be expressed on a cellular level in prokaryotes. However, focusing the effect of nsPEF in eukaryotes on mitochondrial changes might still be valid. Prokaryotes and mitochondria are not only similar in cell membrane composition but further in the aspect that both contain similar molecular weight ribosomes (Amunts et al., 2015; Ban et al., 2014; Rédei, 2008). Therefore, nsPEF induced growth-promoting effects might be linked back to organellar ribosomes and $\text{Ca}^{2+}$ signaling. The involvement of $\text{Ca}^{2+}$ signaling pathways might be a reason for the
improved effects of nsPEF on highly proliferating cells. It was shown for
*Arabidopsis thaliana* that highly proliferating cells have an increased Ca\(^{2+}\) channel activity compared to mature cells (Very and Davies, 2000). Hence, the proposed mode of action might even be valid for nsPEF induced effects in *Arabidopsis thaliana* (Eing et al., 2009).

The obtained results indicate that Ca\(^{2+}\) could play a crucial role in the nsPEF induced upregulation of cell proliferation. Therefore, further research should be conducted with labelled Ca\(^{2+}\) to analyze flux as well as relocation of the cation. In addition, a monitoring of the changes occurring in the cell’s proteome throughout the cultivation might reveal the underlying mode of action of nsPEF induced abiotic stress.

4. Conclusions

Photoautotrophic microalgae based biorefinery concepts are currently not competitive compared to other established production systems. However, nsPEF treatment led to an increased cell proliferation in prokaryotic *A. platensis*. The increase was detectable after repeated nsPEF treatment in the exponential growth phase. The effect was most pronounced five days after the treatment, and besides dry substance; an increase in pigments was detectable. Proteomic analysis revealed a possible stress response pathway. However, the role of specific cellular processes in an nsPEF induced growth stimulation needs to be further analyzed. Thereby, nsPEF treatments might enable sustainable and economical microalgae based biorefineries.

Acknowledgments
The authors gratefully acknowledge the World Food System Center (Project “NewAlgae”, grant number: 2-72235-17), the ETH Zürich Foundation, Switzerland, Prof. Dr. Erich J. Windhab, Daniel Kiechl and Bruno Pfister from the ETH Zürich Food Process Engineering Laboratory, as well as Dr. Peter Hunziker and Simone Wüthrich from the Functional Genomics Center Zurich for their support.

**Conflict of Interest Statement**

The authors declare no conflict of interest.

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**Figure Captions**

Figure 1: Reference 100 ns pulse as applied in the experiments.
Figure 2: Percentage difference in dry substance five days after the nsPEF treatment between treated and untreated *Arthrospira platensis* solutions with varying time intervals between inoculation and nsPEF treatment. The illustrated results were obtained for an energy input of $3 \cdot 256.22 \pm 67.53 \text{ J kg}^{-1}$ as shown in Table 1 and the experimental procedure stated in section 2.1.

Figure 3: Percentage difference in dry substance A) between treated and untreated *Arthrospira platensis* solutions after 5 days of cultivation. The stated energy input is only depending on the pulse repetition frequency and represents a single treatment (Table 1), which was repeated three times, with time intervals of three hours. The allophycocyanin (aPC) and C-phycocyanin (cPC) concentration difference on the fifth day for $256.22 \pm 67.53 \text{ J kg}^{-1}$ is shown in B).

Figure 4: Relative difference in dry substance after application of the same energy input by single treatment ($780.44 \pm 206.74 \text{ J kg}^{-1}$) compared to three uniform treatments ($3 \cdot 256.22 \pm 67.53 \text{ J kg}^{-1}$) performed as in section 2.1. The single energy application was achieved by a reduction of the residence time $t$ (s), while keeping all other parameters constant (Table 1).

Figure 5: Growth monitoring of *Arthrospira platensis* culture during experimental procedure, by measuring DS and OD measurement at 750nm.

Figure 6: Peak-to-baseline voltage amplitude of untreated and treated *Arthrospira platensis* cells measured between 100 kHz and 10 MHz.

Table 1: Experimental parameters used in the analyzed nsPEF treatments. With electric field strength $E$ (kV cm$^{-1}$), pulse duration $\tau_p$ (ns), conductivity $\sigma$ (mS cm$^{-1}$), frequency $f$ (s$^{-1}$), residence time $t$ (s), number of applied pulses $n$ (-), and energy input $\Delta W$ (J kg$^{-1}$). The energy input is stated for a single treatment.
### Table

<table>
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<tr>
<th>E (kV cm⁻¹)</th>
<th>τ₀ (ns)</th>
<th>σ (mS cm⁻¹)</th>
<th>f (s⁻¹)</th>
<th>t (s)</th>
<th>n (-)</th>
<th>ΔW (J kg⁻¹)</th>
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<td>100</td>
<td>11.93 ± 0.98</td>
<td>1.00</td>
<td>0.23 ± 0.06</td>
<td>0.23 ± 0.06</td>
<td>28.40 ± 7.49</td>
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<td>2.96 ± 0.78</td>
<td>370.10 ± 97.55</td>
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</tbody>
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### Graphical abstract

![Graphical abstract](image)
Highlights

- Innovative upstream process for photoautotrophic microalgae.
- nsPEF induced abiotic subcellular stress.
- Increased cell proliferation after nsPEF treatment.
- Impedance measurement of single *A. platensis* cells up to $10^7$ Hz.
- Possible stress response mechanism revealed.