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Biological signalling supports biotechnology – Pulsed electric fields extract a cell-death inducing factor from *Chlorella vulgaris*

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ABSTRACT

Compared to mechanical extraction methods, pulsed electric field (PEF) treatment provides an energy-efficient and gentle alternative. However, the biological processes involved are poorly understood. The unicellular green microalga *Chlorella vulgaris* was used as model organism to investigate the effect of PEF treatment on biological cells. A viability assay using fluorescein diacetate measured by flow cytometry was established. The influence of developmental stage on viability could be shown in synchronised cultures when applying PEF treatment with very low specific energies where one part of cells undergoes cell death, and the other part stays viable after treatment. Reactive oxygen species generation after similar low-energy PEF treatment could be shown, indicating that PEFs could act as abiotic stress signal. Most importantly, a cell-death inducing factor could be extracted. A water-soluble extract derived from microalgae suspensions incubated for 24 h after PEF treatment caused the recipient microalgae to die, even though the recipient cells had not been subjected to PEF treatment directly. The working model assumes that low-energy PEF treatment induces programmed cell death in *C. vulgaris* while specifically releasing a cell-death inducing factor. Low-energy PEF treatment with subsequent incubation period could be a novel biotechnological strategy to extract soluble proteins and lipids in cascade process.

1. Introduction

In the face of climate change and a growing world population, the search for new and economically competitive sources for feed and food has begun. Microalgae could provide part of the answer to this problem, since they fix carbon dioxide, while not competing for arable land with traditional crop plants [1]. Depending on the species, microalgae are rich in proteins, lipids as well as other valuable components and, therefore, harbour potential for numerous applications. *Auxenochlorella protothecoides* contains high amounts of lipids and is interesting as source for biofuel [2,3]. The cyanobacterium *Athrospira platensis* (more commonly known as Spirulina) which has gained attention for its nutraceutical properties, additionally produces high amounts of phycocyanin, the only natural source for blue food colorants [4], and has been certified as GRAS (Generally Recognised As Safe), the standard needed for use in food industry. The only other microalga with a GRAS status is the chlorophyte *Chlorella vulgaris*, which is very rich in protein. In this species, up to 60% of dry weight consist of protein [5]. This has attracted considerable interest for *C. vulgaris* as food supplement in the context of a protein-rich diet. However, unprocessed biomass would just pass through the human digestion system without yielding any valuable nutrients, since eukaryotic microalgae possess a rigid cell wall [6]. As to extract intracellular compounds, a cell disintegration step is necessary. Since chemical processing often interferes with a subsequent use for food industry, physical methods are preferable. These approaches include high-pressure homogenisation (HPH), bead milling or ultrasonication, however especially HPH and bead milling usually require considerable energy input to be effective [7–9]. Disintegration by pulsed electric field (PEF) treatment provides an energy-efficient and gentle alternative. Furthermore, this method can process wet biomass in high concentrations of up to 100 $g \cdot l^{-1}$ biomass by pumping the microalgae suspension between two electrodes, while applying a pulsed electric field. Depending on the applied specific energy, this treatment leads to irreversible electroporation of the cell membrane [10,11]. Although the cell wall retains integrity, soluble intracellu-

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lar components begin leaking out and subsequently accumulate in the supernatant by mere incubation. Since PEF treatment does not cause small cell debris, separating this supernatant with the value-giving compounds is easy to achieve by centrifugation or filtration. Moreover, the remaining sediment harbours lipid components such as oil droplets that are then easily accessible for solvent extraction (e.g., in biofuel applications). Therefore, PEF treatment as extraction method provides the possibility to recover several valuable components such as soluble proteins and lipids in a cascade process. Other applications for PEF treatment utilise the inactivation of microorganisms for instance for bacterial decontamination of hospital wastewater or nonthermal food pasteurization [11].

While PEF treatment of *C. vulgaris* induces leakage of cytoplasmic proteins [12], a recent study [13] was able to detect also proteins of nuclear, chloroplast, and mitochondrial origin in the water-soluble extract after PEF treatment, indicating a breakdown of these cell organelles. This was difficult to explain from the relatively minor specific energy of PEF treatment. A second surprise came when the DNA of the cellular residue was analysed and found to show clear DNA laddering from as soon as 2 h after PEF treatment. These are indications for a cell death that is not acute but regulated. While DNA laddering of mammalian cells is a hallmark of apoptosis, linked with the activity of cysteine proteases such as the caspases, laddering can also occur during necrosis induced by mitochondrial uncoupling, albeit through different proteases, targeting serines [14]. In cycling tobacco cells, DNA laddering accompanies cell-cycle arrest at the G2/M transition [15], and is accompanied by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) staining [16]. The terminology is ambiguous and not smoothly transferrable between plants and animals (for conceptual reviews see [17,18]). The term necrosis often describes an uncontrolled and chaotic mode of death, resulting from acute cellular injury without specific induction of gene expression. In contrast, programmed cell death (PCD) describes a sequence of biochemical events leading to a controlled and organised destruction of the cell [19]. However, in plants, necrotic cell death can also be part of an adaptive response, for instance when older leaves under salt or drought stress enter cell death after having mobilised energy resources and protective molecules towards the younger leaves that will re-initiate development, once the stress period has finished (reviewed in [20]). Likewise, salinity-induced programmed cell death of the root tip allows the formation of lateral roots that will then explore the neighbourhood for regions of reduced salinity [21].

At first, PCD in mutually competing unicellular microalgae is unexpected. However, in bacteria and yeasts, self-mediated cell death has been observed as well [22]. One possible evolutionary driver could be "individual altruism" for the sake of "collective benefit". PCD would remove damaged and/or aging cells and by this means provide the surviving cells with organic compounds, such as nutrients [23]. The autolytic processes facilitating release of valuable contents from *C. vulgaris* under relatively low-energy PEF treatment might derive from PEF induced PCD [13], a phenomenon already described for yeast [24–26]. In mammalian cells, nanosecond PEF (nsPEF) induced cell death has been proposed as cancer treatment by combining necrotic and apoptotic modes of cell death [27]. One study has shown interaction of nsPEF with voltage gated ion channels, promising tumour ablation where only cancer cells expressing voltage-gated ion channels are sensitive to the treatment [28].

To determine and monitor factors regulating cell death in response to PEF treatment, a robust method was established to monitor cell viability after PEF treatment by using cell sorting based on fluorescein diacetate (FDA), a viability probe for plant cells. In the next step, the experimental system was calibrated to a point, where a set ratio of cells undergoes cell death after treatment and the other part stays viable. This allowed standardising the response to a degree that would allow for quantitative physiology. *C. vulgaris* multiplies asexually by the formation of two to four (occasionally up to 32) daughter cells inside the mother cell, a process called auto-sporulation [29]. The behaviour under PEF treatment of this microalga is most likely dependent on the developmental state and thus, on the way of cultivation. Therefore, for the purpose of standardisation, a defined developmental state is mandatory. This is possible in synchronised cultures of *C. vulgaris* achieved by applying a light–dark cycle, where the cell division is limited to a short period, just before the start of the light cycle. With these tools in hand, the cell-death response to PEF treatment can be dissected on a quantitative base. This response is strongly dependent on the time point within the synchronisation cycle. A water-soluble factor released by PEF treated cells inducing untreated cells to die can be detected. Both, the release of this factor by the donor cells, as well as the responsiveness to this factor by the recipient cells, is dependent on the progression of the respective cell through the synchronisation cycle.

These observations suggest that it might be a biotechnological strategy to administer PEF treatment at lower energy as a signal to deploy PCD, rather than to use energy-intensive PEF treatments to breach membranes electrically. The biological aspects of PEF treatment might thus help to achieve energy-efficient extraction from *C. vulgaris*. For this purpose, the cellular mechanisms behind cell death must be understood.

2. Material and methods

2.1. Cultivation of Chlorella vulgaris

Chlorella vulgaris (SAG strain 211–12, University of Göttingen) cells were inoculated in 1 × Tris-Acetate-Phoshphate (TAP) medium (0.02 M Tris, 1 × TAP salts [30], 1 mM potassium phosphate buffer, 1 × Hutner's trace elements [31], 0.001% (v/v) acetic acid, pH 7.0) and grown in Erlenmeyer glass flasks. After inoculation to an OD_{750} of 0.1, the suspension culture was cultivated at 23 °C for 7 days post inoculation (dpi) under constant exposure to light (photosynthetically active radiation (PAR) 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and shaking at 150 rpm till harvest at stationary growth-phase (OD_{750} of 1.8–2.0, pH 8.5). Cells could be counted using a cell counter (CASY Model TT System, Roche Diagnostics Gmbh, Germany) and representative size distribution of *C. vulgaris* in stationary phase 7 dpi is shown in **Supplementary Figure S1**. The average cell size was around 3 μm and the slight right shoulder of the peak in the size distribution as well as microscopic imaging additionally shows the formation of some cell aggregates. For synchronisation, *C. vulgaris* was inoculated in TAP medium to a cell count of $2 \cdot 10^6$ - $cells \cdot ml^{-1}$ ($\approx OD_{750}$ 0.05) and then cultivated under a light–dark cycle (12 h : 12 h). Hereby, cells grew under a lower PAR ($65\ \mu mol \cdot m^{-2} \cdot s^{-1}$), and were diluted to the initial density ($2 \cdot 10^6$ $cells \cdot ml^{-1}$) prior to onset of each light period [32,33], again under shaking at 150 rpm and 23 to 25 °C. After 2 weeks of these cycles, the culture was sufficiently synchronous. Samples were taken at different time points in the synchronisation cycle (see Fig. 1**a**). Cells were harvested by centrifugation at 10,000 g for 2 min at 23 °C (Heraeus Primo Centrifuge, fixed-angle rotor, Thermo Scientific), and the suspension adjusted to the desired concentration. The cell dry weight (CDW, $[mg \cdot g_{sus}^{-1}]$) was determined according to Akaberi et al. 2019 [34] using a circulation air oven (85 °C; ULP 500, Memmert, Germany) to dry 5 ml of cell suspension as well as supernatant on an aluminium plate for 3 h. The samples were weighed before and after drying with a fine balance (AE163, Mettler-Toledo, Switzerland). Taking the density of water

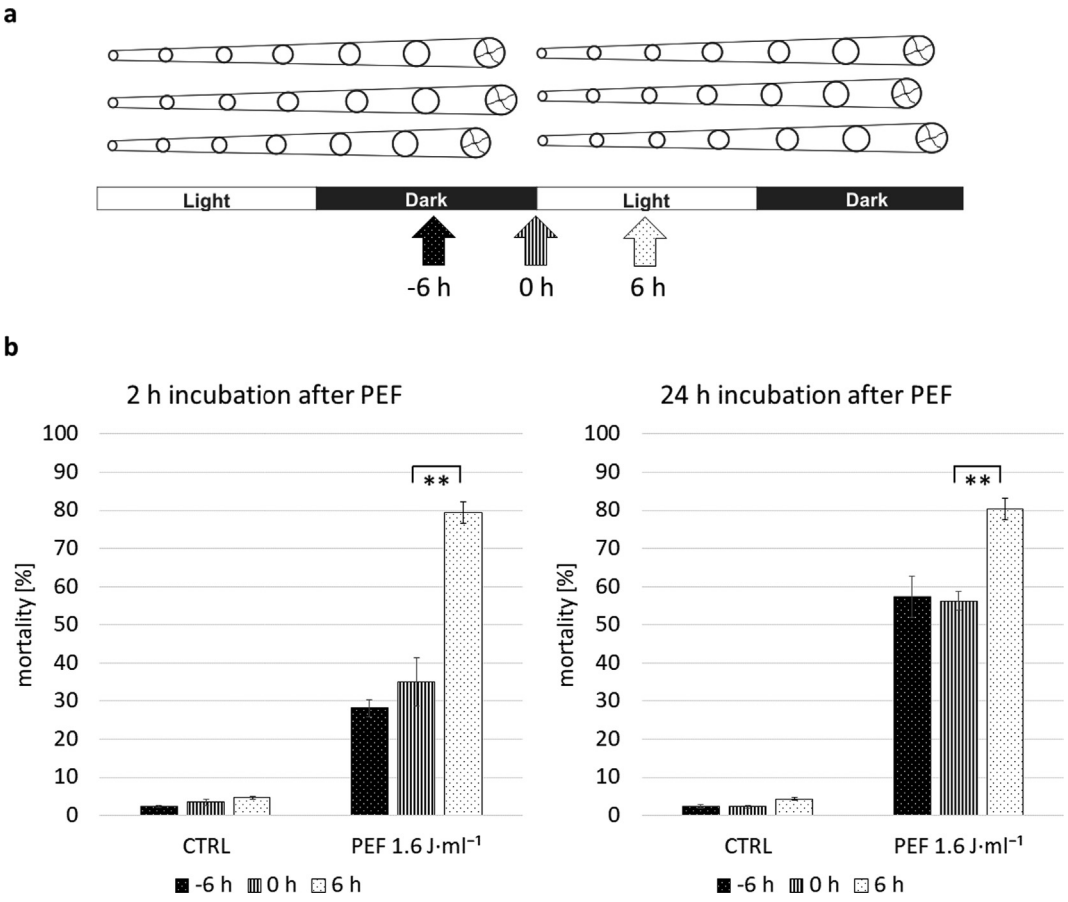

Fig. 1. Mortality in response to PEF treatment depends on the time in the synchronised cell cycle. (**a**) Diagram showing synchronised cell cycle with different time points for sampling at -6 h, i.e. in the dark (black), 0 h, i.e. at the onset of light (striped) and + 6 h, i.e. in the light period (white), redrawn from Prescott (1972, p. 304) [40] (**b**) Cell mortality after PEF treatment at different cell cycle stages. *C. vulgaris* from synchronised cultures at different times of the cell cycle were pulsed with a specific energy of 1.6 J·ml^{-1}. Viability was monitored for 2 and 24 h via the FDA assay (50 μM). CTRL: control without PEF treatment. Data represent averages and standard errors from three biological replicates. Brackets indicate differences that are significant at $P \leq 0.01$ (**), using two-sample *t*-test assuming equal variances.

and thereof derived the algae suspension (ρ = 1 g·ml^{-1}) into account, CDW can be expressed in mg·g$_{sus}^{-1}$ ≙ mg·ml^{-1} – this unit will be used throughout the paper.

2.2. Mechanical cell disruption and PEF treatment

For mechanical cell disruption, high-pressure homogenisation (HPH) was employed. The cell suspension was passed through a high-pressure homogeniser (EmulsiFlex-C3 homogeniser, Avestin, Canada) at 2000 bar with 5 passages under constant cooling on ice. Afterwards cell debris was removed by centrifugation (10,000 g, 10 min, 4 °C, Heraeus Megafuge 8R, fixed-angle rotor, Thermo Scientific). This method had been demonstrated to allow complete diagnostic access to the entire protein content of the sample [13].

To disrupt the cells by PEF treatment, there were two set ups. Depending on the sample volume and the desired electroporation parameters, PEF treatment was performed in continuous flow or in batch treatment with commercial cuvettes. Table 1 shows exemplary parameters for each set up. In either configuration, the algal suspensions had an initial temperature of 21 °C, while during pulse treatment with a maximum specific energy of 36.8 J·ml^{-1}, the output temperature never exceeded 30 °C. For large volumes the PEF treatment was conducted in continuous flow in a uniform-field flow chamber with a volume of 525 μl, enclosed by two planar stainless steel electrodes with a gap of 2 mm in-between [35]. The conductivity of the algal suspension (at stationary phase around 1.2 mS·cm^{-1}) was adjusted to 1.5 mS·cm^{-1} by adding the necessary amount of NaCl with an end concentration of 2.7 mM for both untreated and PEF treated samples, however the osmolarity was not balanced. For treatment the suspension was pumped through silicon tubing by means of a peristaltic pump (MS-4/12–100 ISMATEC, Cole-Parmer GmbH, Wertheim, Germany). The flow rate was set to 3 ml·min^{-1} resulting in a retention time of 10.5 s in the flow treatment chamber. A transmission-line pulse generator delivered square pulses of 1 μs length. From a preparatory study adjusting treatment energies, the parameters were set to a field strength of 20 kV·cm^{-1} and frequency of 1.5 Hz resulting in a specific treatment energy of 9.4 J·ml^{-1}. This specific energy was sufficient to induce subsequent cell death. For batch treatment,

Table 1
Exemplary parameters of PEF treatment in contionus flow and batch treatment.

	Gap	Field strength	Pulse duration	Conductivity	Pump speed	Frequency	Number of pulses	Specific energy
Continuous flow	2 mm	20 kV·cm^{-1}	1 μs	1.5 mS·cm^{-1}	3 ml·min^{-1}	1.5 Hz	16	9.4 J·ml^{-1}
Batch treatment	2 mm	20 kV·cm^{-1}	1 μs	2.0 mS·cm^{-1}	–	–	2	1.6 J·ml^{-1}

commercially available electroporation cuvettes (Electroporation Cuvettes Plus, BTX Harvard Apparatus, Holliston MA, USA) with a treatment volume of 420 μl and a gap of 2 mm between the aluminium electrodes were used. In this set-up, for both untreated and PEF treated samples the conductivity of the concentrated algal suspension was adjusted to 2 $mS \cdot cm^{-1}$, using the necessary amount of NaCl with an end concentration of maximal 7.3 mM without balancing the osmolarity. The field strengths were set to either 20 $kV \cdot cm^{-1}$ or 40 $kV \cdot cm^{-1}$. Again, the electric field distribution in the treatment cuvette was uniform. Here, the specific energy was adjusted through the number of pulses, whereby each pulse was 1 μs long, delivered by a transmission-line pulse generator. Using a field strength of 20 $kV \cdot cm^{-1}$, the specific energies ranged from 0.8 $J \cdot ml^{-1}$ with 1 pulse up to 36.8 $J \cdot ml^{-1}$ with 46 pulses, while at 40 $kV \cdot cm^{-1}$, the specific energies ranged from 3.2 $J \cdot ml^{-1}$ (1 pulse) up to 150.4 $J \cdot ml^{-1}$ (47 pulses). The specific energy can alternatively be expressed in $kJ \cdot kg^{-1} \triangleq J \cdot ml^{-1}$ when the density of water and thereof derived of the algae suspension (ρ = 1 $g \cdot ml^{-1}$) is factored in again.

2.3. Incubation conditions

After PEF treatment, the algae suspension remained at 23 °C to allow for release of proteins up to 24 h for a time response experiment. As to analyse the dependence of biomass concentration on viability, a part of the algal suspension was diluted with sterile supernatant directly after PEF treatment and then both sets were incubated in parallel at 23 °C. The HPH extract served as positive control. To ensure comparability, this extract remained as well for 24 h, but at 4 °C to reduce proteolysis. After incubation, viability was measured in the suspension and optionally the cells were centrifuged (10,000*g*, 10 min, 23 °C, Heraeus Primo Centrifuge, fixed-angle rotor, Thermo Scientific), such that the protein content in the supernatant could be quantified and the extract be used for further experiments.

2.4. Viability assay by FDA staining

Fluorescein diacetate (FDA) is a non-fluorescent, cell permeating esterase substrate for the intracellular esterases of living cells that cleave FDA to fluorescein, which yields a fluorescent readout for viability and cell membrane integrity by retention of the fluorescent product. This assay has allowed to measure algal esterase activity in several microalgae including *C. vulgaris* [36,37]. A stock solution of 1 mM FDA (Thermo Fisher Scientific) was prepared in 100% DMSO and kept at 4 °C until use.

To quantify viability, the following FDA staining method was established. The algae suspension was diluted to an OD_{750} of 0.1–0.2 using sterile supernatant. FDA staining was achieved using a working concentration of 50 μM FDA in 5% DMSO. The stained sample was incubated for 5 min in the dark and after incubation the sample was diluted by factor 10 with Attune™ Focusing Fluid 1× (Thermo Fisher Scientific). Then, the fluorescein signal was quantified using a flow cytometer (Attune™ Nxt, Thermo Fisher Scientific) equipped with a blue argon ion laser (excitation at 488 nm). Cell debris particles were excluded from analysis by gating the homogenous population visible in the forward scatter (FSC) versus side scatter (SSC) histogram, one way to visualise the size and complexity of the measured particles. Furthermore, cell aggregates were excluded by plotting a histogram of the forward scatter area signal (FSC-A) versus the forward scatter height signal (FSC-H) in order to use area scaling to gate only individual particles for analysis. Chlorophyll red fluorescence is also excited by the blue laser and can be detected using a far-red bandpass filter of 695 ± 40 nm (BL3). A positive chlorophyll α fluorescence allowed to select microalgae cells for analysis. Summing up, only single cells of the correct size with a positive chlorophyll signal were used for further analysis of the fluorescein signal. Green fluorescence from fluorescein was recorded through a bandpass filter of 530 ± 30 nm (BL1). The sample volume yielded more than 100,000 events for analysis with a flow rate of 1000–2000 events per second. After data collection, the data was displayed as histograms showing BL1-H (height of fluorescein fluorescence signal in logarithmic mode) versus count (linear mode). Cells with a signal higher than 10,000 (BL1-H) were defined as viable and the percentage of viable cells over the total analysed cell number was calculated as readout for viability (in %). The mortality rate (in %) can be calculated by subtracting the value for viability from 100 and is used throughout this paper.

2.5. Protein quantification

Protein concentrations in the extract supernatant was determined with a modified protocol of the Lowry Assay (Detergent Comatible, DC™, BioRad, Munich) according to the manufacturer's protocol. Using this assay, colour development is primarily due to several reduced species which have a characteristic blue colour. The protein concentration could be determined by measuring the absorption at 750 nm photometrically (Genesys 10S UV–VIS, Thermo Scientific). The readout was calibrated using a standard curve with a serial dilution of BSA (bovine serum albumin).

2.6. Measurement of reactive oxygen species (ROS)

Malondialdehyde (MDA) is generated by lipid peroxidation of polyunsaturated fatty acids during oxidative stress. MDA reacts with thiobarbituric acid (TBA) to a product that serves as photometrical readout [38]. Superoxide is generated as first stress signal after disruption of photosynthetic electron flow, but the molecule is very unstable. Hydrogen peroxide (H_2O_2) is a breakdown product from superoxide dissipation. Steady-state levels of the more stable hydrogen peroxide report, therefore, the oxidative homeostasis of the chloroplast. Hydrogen peroxide reacts with potassium iodide (KI), giving rise to a coloured educt that can be determined photometrically [39]. Samples were generated using synchronised cultures that were 50 × concentrated, prior to PEF treatment in cuvettes. The specific energies used were 0.8 $J \cdot ml^{-1}$, 1.6 $J \cdot ml^{-1}$ and 8.0 $J \cdot ml^{-1}$. Directly after PEF treatment, the algal samples were re-diluted by the factor 50 to ensure a stable survival ratio over time. After 3 h of incubation, viability was determined via the FDA assay. Afterwards, the algal suspension was centrifuged (10,000 g, 10 min, 23 °C, Micro Star 17R, fixed-angle rotor, VWR), and after discarding the supernatant and determining the fresh weight (fw), the algal sediment was frozen in liquid nitrogen. For processing, the algal precipitates were ground (TissueLyser; Retsch) with a 4 mm steel bead in 2 ml of 3% trichloroacetic acid (TCA) in the reaction tube (twice for 30 s at 2 Hz). After removing the bead, the suspension was centrifuged at 10,000*g* for 1 min and MDA and H_2O_2 levels were measured in the supernatant. MDA content was determined by mixing 750 μl of the supernatant with 750 μl of TBA (0.5% in 20% TCA) and 75 μl of butylated hydroxytoluen (BHT, 4% in absolute ethanol). This mixture was heated at 95 °C for 30 min, quickly cooled on ice, and then centrifuged (10,000*g*, 30 s, 23 °C) before measuring the absorptions at 532 and 600 nm. Hydrogen peroxide content was determined by mixing 750 μl of the supernatant with 750 μl of 1 M potassium phosphate buffer (pH 7.0) and 1.5 ml KI. After incubation in the dark for 3 h, absorption at 390 nm was measured. For calibration, a serial dilution of H_2O_2 was measured and the MDA concentration was determined by dividing the difference in absorbance (A(532 nm) – A(600 nm)) by the molar extinction coefficient (155 $mM \cdot cm^{-1}$). The results were normalised to 1 g of fresh weight.

2.7. PEF and HPH extract experiments

PEF extract was generated using *C. vulgaris* grown under continuous light concentrated to a CDW of 7 to 10 mg·ml^{-1}. PEF treatment was administered either in cuvettes with specific energies of 0.8 J·ml^{-1}, 1.6 J·ml^{-1}, 8.0 J·ml^{-1} and 36.8 J·ml^{-1} or, when bigger sample volumes were necessary, in a continuous flow with a specific energy of 9.4 J·ml^{-1}. After 24 h of incubation, viability of the treated sample, as well as of a control sample without PEF treatment, was measured via the FDA assay. The PEF-treated and the control sample were centrifuged at 10,000 g for 10 min at 23 °C (Heraeus Primo Centrifuge, fixed-angle rotor, Thermo Scientific) and the sediment discarded. In the following, these extracts will be called PEF supernatant (PEF SN) and control supernatant (CTRL SN), respectively. HPH extract (HPH SN) was used as positive control since it contained the entire content of the cell. For further experiments, the extracts were subjected to different treatments, such as heating at 23, 40 and 50 °C for 2 h, treatment with a 1 × cOmplete™ protease inhibitor cocktail (PI, Roche) as well as a dilution series of PEF extract. To test the effect of these donor extracts, viable synchronised cells were used as recipients. Without further concentration, synchronised *C. vulgaris* provided a biomass of around 0.15 mg·ml^{-1} at the beginning of the light cycle after dilution (time point 0 h, see Fig. 1**a**). 900 µl of living recipient cells were centrifuged (14,000*g*, 30 sec, 10 °C, Micro Star 17R, fixed-angle rotor, VWR) and the supernatant discarded. Subsequently, different donor extracts were added to the remaining sediment. As negative control one sample remained with the original supernatant after centrifugation (called medium). Next, the sediments were resuspended using a vortex mixer and incubated the suspensions for up to 24 h at either 23 °C or 4 °C while following viability with the FDA assay.

2.8. Statistical data analysis

All data represent mean and standard errors from at least three to six independent experimental series. Statistical significance was determined using two-sample *t*-test assuming equal, or when necessary, unequal, variances.

3. Results and discussion

3.1. Effect of cell cycle on the mortality induced by PEF

To test, whether the cellular response to PEF treatment depends on the cell cycle, a constant energy input (1.6 J·ml^{-1}) was used but the time of treatment over the synchronised culture cycle varied (Fig. 1**a**). Cell division ensues just before the onset of light, during the light period cells increase in size, but do not divide. Samples were taken, therefore at the time point −6h (i.e., in the dark phase before division), at the time point 0 h (i.e., just after completed division), and at 6 h after the onset of lighting (i.e., during the non-cycling phase, when cells increased in volume). The mortality in response to the pulse treatment was strongly dependent on the time point. The cells pulsed at −6h and 0 h displayed a substantially (more than a factor of 2) reduced mortality, especially for the rapid response, 2 h after pulsing (Fig. 1**b**, left). When measuring mortality at 24 h after the pulse, the values were still significantly lower for the cells pulsed at −6h or 0 h, but the difference was less pronounced compared to that at 2 h. One plausible explanation for the substantially reduced mortality just after completed division at 0 h could be the small cell size. The induced transmembrane potential is proportional to the size of the organism, in this case the cell radius. The field strengths required for electroporation of smaller organisms like bacteria is higher than for bigger organisms like microalgae and yeast [10,11]. The cell radius of synchronised *C. vulgaris* increases around 10% in the time period from 0 h to 6 h. However, this is not sufficient to generate a higher mortality of more than a factor 2. Furthermore, the effect of a higher transmembrane potential and subsequently higher mortality should then also be visible when comparing the mortality of the time points 6 h and −6h, since the increase of cell size in this period is even greater. Indeed, in the dark phase before division (-6h) the mortality is reduced to the same level when comparing with 0 h, even though the cells are significantly bigger in size. This leads to the conclusion that the mortality in response to PEF treatment depends on the cell cycle.

3.2. Effect of PEF on the redox homeostasis of C. vulgaris

To assess potential oxidative stress in response to PEF treatment, lipid peroxidation as readout for oxidative membrane damage and hydrogen peroxide steady state levels as reporter of photosynthesis-related oxidative stress were quantified. Synchronised cells were treated with different specific energies establishing a range of viabilities (2%, 50% and 90%, respectively) and then subsequently the MDA and H_2O_2 content was measured (Fig. 2). Since MDA is the terminal product, these levels report the integral of oxidative stress over the 3 h after PEF treatment. In contrast, H_2O_2 reports steady-state levels from oxidative burst and dissipation by catalase and, thus, leads to a snapshot of oxidative stress at 3 h after PEF treatment. From the combination of both parameters, it can be concluded that PEF treatment at the lowest specific energy of 0.8 J·ml^{-1} (with a viability of 90%) did not cause a significant perturbance of redox homeostasis. When the specific energy was doubled to 1.6 J·ml^{-1} (reducing viability to 50%) both parameters were increased. A specific energy of 8.0 J·ml^{-1} leading to almost complete cell death (viability 2%) produced a significantly high MDA readout, while H_2O_2 steady-state levels at 3 h were not significantly different from untreated controls. This indicates that the accumulation of peroxide is an active process requiring that the cells were alive, while the MDA levels report that these cells had experienced considerable oxidative stress before death. One key signalling component of PCD is generation of ROS [41]. Lower doses of ROS are employed as signal to activate stress responses while high concentration of ROS can lead to PCD. The signal-related production of ROS in plants is executed by NADPH oxidases (respiratory burst oxidase homologues, RboHs) that are located in the cell membrane (reviewed in [42]). These enzymes generate superoxide anions which later are converted to the signal molecule H_2O_2. In parallel, lipoxygenation can occur, leading to the generation of MDA. This lipoxygenation can be of either non-enzymatic or of enzymatic nature. Lipoxygenases cannot only generate oxylipins including the jasmonates, central stress hormones in land plants, but also superoxide [43], which dissipates to the more stable hydrogen peroxide in the presence of superoxide dismutases. Lipoxygenases able to generate the precursors of jasmonates, have been purified from the related species *Chlorella pyrenoidosa* [44]. Whether green algae harbour jasmonates, is unclear and under dispute. At least in *Chlamydomonas reinhardtii*, homologues for all relevant steps of the pathway have been recovered, as well as most relevant members of the signalling complex [45].

On the other hand, the central factor of signalling, the JAZ proteins, seem to be absent [45]. From these considerations, two alternative scenarios emerge. The PEF treatment might disrupt the integrity of the thylakoid membrane in the chloroplast, leading to perturbed electron transport resulting in non-enzymatic lipid peroxidation (giving rise to MDA), while the superoxide resulting from transfer of excess electrons on the oxygen resulting from water-splitting would be dissipated by the plastidic superoxide

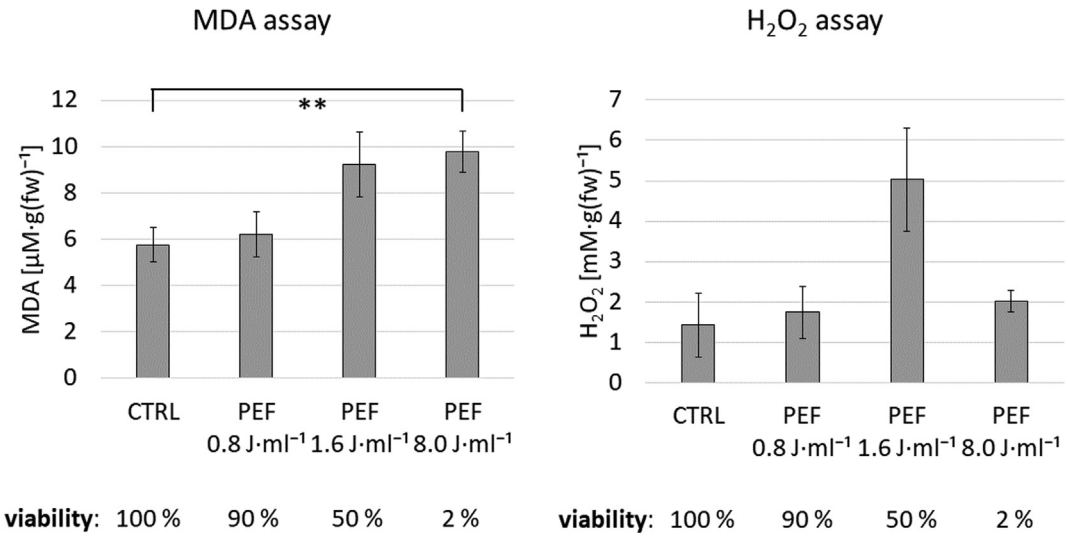

Fig. 2. Generation of ROS in response to PEF treatment. *C. vulgaris* from synchronised cultures (time point 0) were pulsed with three specific energies establishing viabilities of around 90% (0.8 J·ml^{-1}), 50% (1.6 J·ml^{-1}) or 2% (8.0 J·ml^{-1}), respectively, as determined via FDA assay 3 h after PEF treatment. The generated algae sediments were analysed for MDA and H_2O_2 levels normalised to fresh weight (fw) accordingly. CTRL: control without PEF treatment. Data represent averages and standard errors from six biological replicates. Brackets indicate differences that are significant at $P \leq 0.01$ (**), using two-sample *t*-test assuming equal variances.

dismutase. The alternative scenario would ascribe the increase in H_2O_2 content to enzymatic origin (RboH, plastidic lipoxygenase) and the accumulation of MDA as well. In this second scenario, the low-energy PEF treatment would, therefore, trigger stress signalling, and the perturbed redox homeostasis would not be the cause, but the consequence.

In previous experiments, where the release of plastid proteins was monitored in response to a much stronger (150 J·ml^{-1}) PEF treatment, the release required 4 h to become relevant, indicating that the thylakoid integrity in response to this around 100-fold stronger pulse was well retained [13]. This renders the non-enzymatic "damage scenario" for the current study rather unlikely. Therefore, the "stress-signalling scenario" was tested by subsequent experiments.

3.3. Effect of cell density on the mortality induced by PEF

PEF treatment as extraction method requires high biomass concentrations to render the technology cost effective. This set of experiments aimed at analysing the influence factor of cell density on mortality after PEF treatment (Fig. 3). Control samples (CTRL) with the same biomass concentration and changed osmolarity were incubated in parallel to PEF treated samples and showed no reduced viability. It can be observed that, at higher cell densities, mortality in response to PEF treatment differed considerably compared with lower cell densities. While for high energies (36.8 J·ml^{-1}), the mortality was already almost close to 100% at the earliest time point, independently from cell density, significant differences for the lower energy (0.8 J·ml^{-1}) could be observed. Here, the mortality increased drastically (from 30% to almost 90% for 0.8 J·ml^{-1}, and from 70% to around 95% for 1.6 J·ml^{-1}), when the pulsed cells remained at high density rather than undergoing dilution after PEF treatment. This significant increase of mortality is a slow response since it required 24 h to become manifest. At 2 h and 4 h after pulsing, mortality is not significantly different, no matter, whether the cells were at high or low density, albeit mortality at these time points is already around three times higher than in the control. Since the cells had been separated only after pulsing, the difference cannot come from physical parameters during the PEF treatment itself but must arise from biological processes occurring after the pulse. This has been followed up in a subsequent experiment.

3.4. In response to PEF treatment, C. vulgaris releases a cell-death inducing factor

To understand, why cells incubated at high density were more sensitive subsequently to PEF treatment, one possible explanation could be that the cells release a water-soluble factor that would induce cell death in the neighbouring cells. This led to the design of experiments, where the supernatant from donor cells (defined as PEF SN) incubated for 24 h after PEF treatment was added to living recipient cells and then the resulting viability, converted into mortality, was scored (Fig. 4**a**). As control, mortality in recipient cells was determined in response to a supernatant from non-pulsed donor cells (defined as CTRL SN). Additionally, mortality was analysed in recipient cells that remained in medium that had not been in contact with any donor cell (defined as medium). A strong and rapid mortality of the recipient cells in response to treatment with PEF SN could be observed (Fig. 4**b**). Independently of the specific energy, mortality rose to more than 70% after 3 h incubation in PEF SN and grew even further over 24 h. Neither in the CTRL SN sample, nor in the control containing medium, could any significant mortality be observed. Since the release of the cell-death inducing factor did not change in the range of 0.8 to 36.8 J·ml^{-1}, for the subsequent experiments the specific energy of 8.0 or 9.4 J·ml^{-1} were chosen for generating the PEF extract with donor cells. This experiment led to the conclusion that *C. vulgaris* releases a cell-death inducing compound in response to PEF treatment. A cell-death inducing factor generated by *C. vulgaris* can be dated back to the 1940 s, when the group of Pratt et al. already described a compound inhibiting multiplication of cells [46]. This substance called *Chlorellin* was later found to be a mixture of fatty acids responsible for inhibiting growth [47]. Cell death in relation to PEF treatment has been proposed to be an enzyme-driven process, where autolysis is responsible for protein liberation [13]. This concept of autolytic processes associated with cell death has already been described for yeast cells [24,25]. Principally, the cell-death inducing compound might be a protein or a lipid-derived compound released in consequence of perturbed membrane integrity.

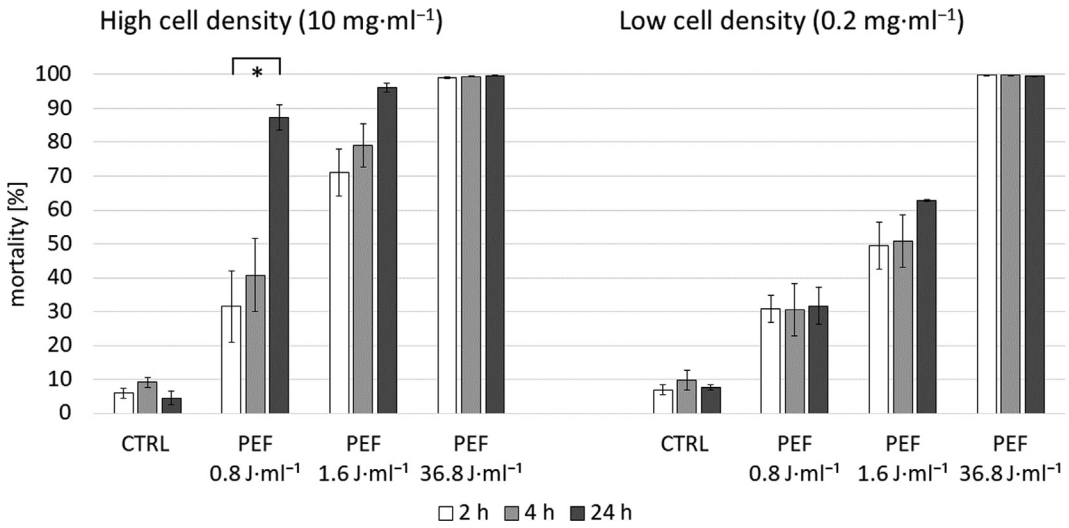

Fig. 3. Influence of cell density on the mortality induced by PEF treatment with different energies. *C. vulgaris* from cultures grown under continuous light (7 dpi) were concentrated to a high cell density of 10 mg·ml^{-1} and pulsed with three specific energies chosen to establish around 70% (0.8 J·ml^{-1}), 50% (1.6 J·ml^{-1}), or 0% viability (36.8 J·ml^{-1}), respectively. Immediately after PEF treatment, the sample was divided. One set remained at high density, while the other set was diluted by a factor of 50 by adding sterile medium. Then, in both samples viability was monitored over 24 h via FDA assay (50 μM). CTRL: control without PEF treatment. Data represent averages and standard errors from three biological replicates. Brackets indicate differences that are significant at $P \leq 0.05$ (*), using two-sample *t*-test assuming equal variances.

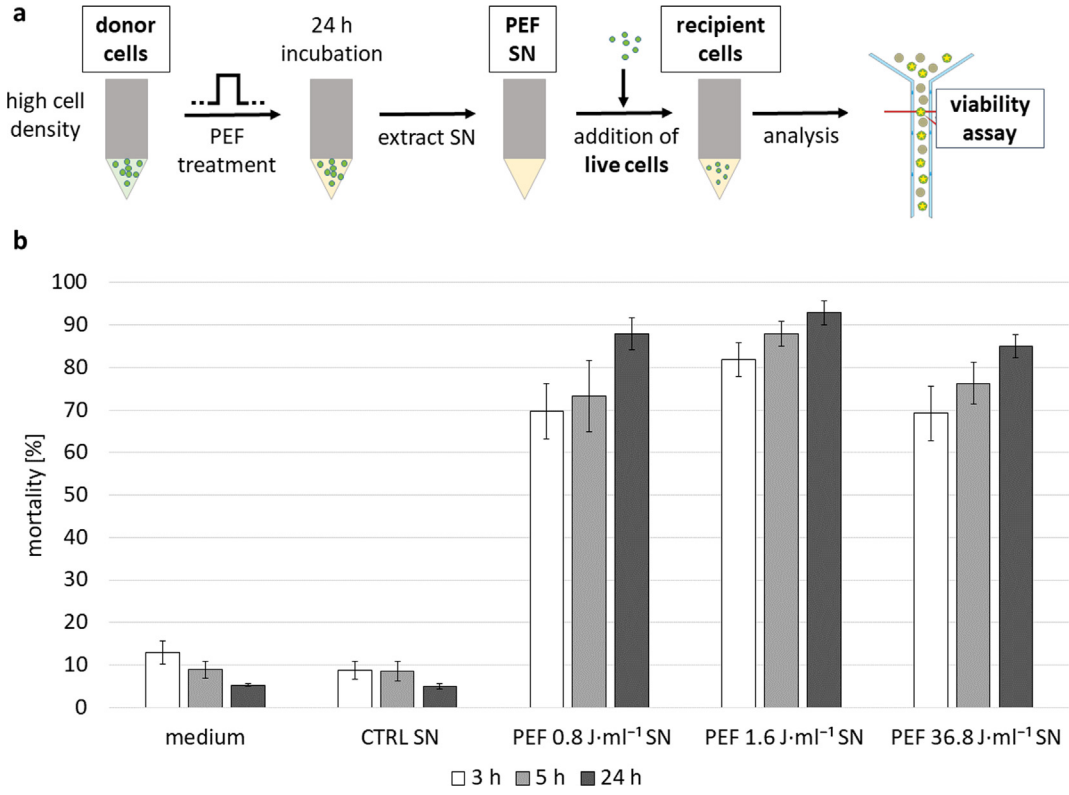

Fig. 4. Cell mortality in response to supernatant of PEF treated cells. (**a**) Schematic figure showing experimental set up. SN = supernatant (**b**) *C. vulgaris* cells from cultures grown under continuous light (7 dpi) were concentrated to a high cell density of 10 mg·ml^{-1}, as donor cells pulsed with three specific energies (0.8 J·ml^{-1}, 1.6 J·ml^{-1} and 36.8 J·ml^{-1}) and incubated for 24 h. After centrifugation, the water-soluble extract in the supernatant of the PEF treated donor cells was added to living synchronised recipient cells (time point 0) that were not subjected to direct PEF treatment and viability was monitored over additional 24 h via the FDA assay. Medium: recipient cells without exchanging supernatant. CTRL SN: recipient cells treated with supernatant from donor cells that had not been subjected to PEF. PEF (specific energy) SN: recipient cells treated with supernatant from donor cells that had been subjected to PEF treatment at given specific energies. Data represent averages and standard errors from three biological replicates.

3.5. The cell-death inducing activity is heat-labile and dose-dependent

As to analyse the nature of the cell-death inducing compound found in the extract after PEF treatment, the effect of preheating, protease inhibition (Fig. 5**a**), cooling during the incubation period (Fig. 5**b**), as well as the effect of dilution (Fig. 5**c**) was investigated. Incubation of fresh PEF extract at 40 °C and 50 °C for 2 h prior to addition to recipient cells almost completely disables the cell-

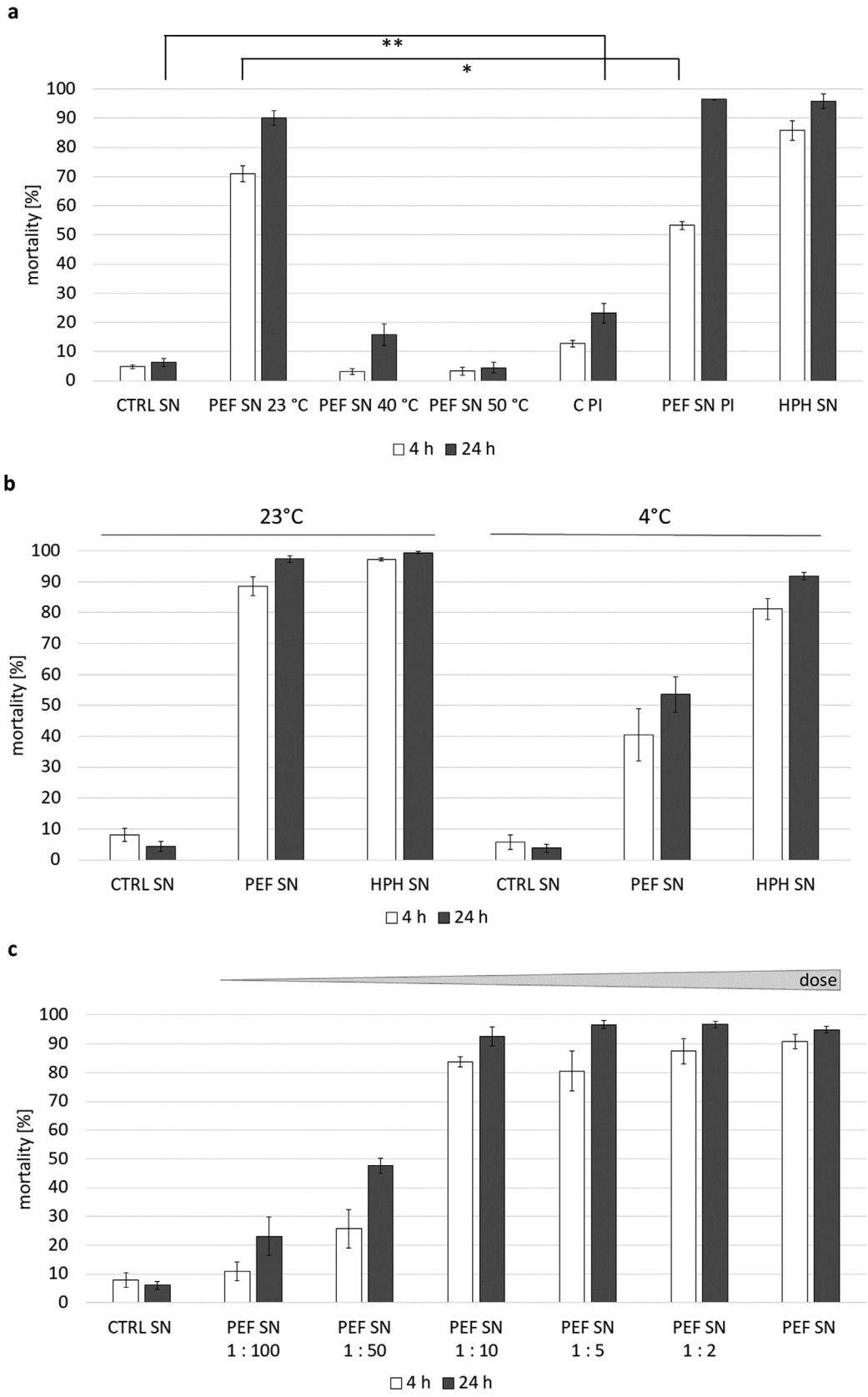

Fig. 5. Cell mortality after addition of PEF supernatant. *C. vulgaris* from cultures grown under continuous light (7 dpi) were concentrated to a high cell density of 10 mg·ml^{-1}, as donor cells pulsed with a specific energy of 9.4 J·ml^{-1} and incubated for 24 h. After centrifugation, the water-soluble extract in the supernatant was added to live synchronised recipient cells (time point 0) and viability was monitored at 4 h and 24 h via FDA assay (50 μM). **a)** Before addition to live cells, the water-soluble extract was treated for 2 h in water baths with temperatures at 23 °C (PEF SN 23 °C), 40 °C (PEF SN 40 °C) and 50 °C (PEF SN 50 °C). The effect of protease inhibitor cocktail (PI) was tested on control samples (C PI) and on samples with PEF supernatant (PEF SN PI), additionally the effect of HPH supernatant (HPH SN) was tested. **b)** Incubation period after addition of different supernatants was performed either at 23 °C or 4 °C. **c)** Dilution series of PEF supernatant with sterile medium previous to addition to live recipient cells. CTRL SN: extract without PEF treatment. Data represent averages and standard errors from three biological replicates. Brackets indicate differences that are significant at $P \leq 0.05$ (*) and $P \leq 0.01$ (**), using two-sample *t*-test assuming equal variances.

death inducing effect of supernatant compared to incubation of extract at 23 °C (Fig. 5**a**). Since this would be consistent with the working hypothesis that the cell-death inducing compound might be a protein, or would activate proteins in the recipient cell, and since PCD is often executed by caspases (animals) or metacaspases (plants), the effect of a protease inhibitor cocktail on the extract was tested. Protease inhibition led to a slightly but significantly increased mortality in control samples (C PI). However, in combination with supernatant generated from donor cells after PEF treatment (PEF SN PI), protease inhibition delayed the manifestation of mortality, since there was a minor (around 20%), but significant decrease of the cell-death inducing effect after 4 h incubation, while after 24 h the mortality in presence of the protease inhibitor equalled that of cells treated with a PEF extract without protease inhibitors. Thus, proteins are either acting as cell-death signal generated by the donor cells, or in response to the cell-death signal in the recipient cells. Interestingly, the supernatant of total cell extract after high-pressure homogenisation (HPH SN) shows cell-death inducing effect comparable to PEF extract. Therefore, the mechanical cell extraction method HPH leads to release of the cell-death inducing compound as well, leading to the conclusion that the signal is extracted rather than generated since HPH treatment immediately destroys the cell and does not allow any cellular response to the treatment. With this experiment PEF treatment related side effects can be excluded as well. These include the possible cell-death inducing effects of metallic ions released due to electrode degradation or water electrolysis. It additionally shows that mechanical disruption does not destroy the signal component. Incubation of recipient cells in PEF extract at 4 °C decelerates the cell-death inducing effect in comparison with incubation of recipient cells at 23 °C (Fig. 5**b**). This delay was substantial, since the mortality after 4 h was less than around half of that seen at 23 °C. Still, in both cases, there was a rapid increase in the time interval till 4 h, while the incremental mortality in the much longer period between 4 h and 24 h was small (around 10%) and comparable for both conditions. When comparing the rate of mortality progression in the rapid early phase, a drop of temperature by around 20 °C reduces the rate by a factor of 2.36, corresponding to a Q10 of $\sim$ 1.2, which is indicating a diffusion-driven rather than an enzymatic process. This is consistent with the small delay in mortality observed with the protease inhibitor (Fig. 5**a**). The dose–response relation of the cell-death inducing compound was further tested by diluting the PEF extract from the donor cells, while keeping the cell count of the recipient cells constant (Fig. 5**c**). The PEF extract from the donor cells retains comparable cell-death inducing effect down to a dilution of ten times. If this extract is diluted further, the mortality starts to decrease significantly, and a dilution of 1:100 yields a mortality that is only insignificantly higher as that seen in untreated control samples. This dose-dependency shows that the reception of the cell-death inducing factor is at first saturated independent of dilution with factor 2, 5 or 10. This can be explained by a model involving a receptor that accepts the cell-death inducing signal but can be saturated with signal at high PEF SN concentrations. When diluting the extract by factor 50 this putative receptor does not seem to be saturated anymore, leading to reduced signalling resulting in reduced mortality in the recipient cell.

Since the reception of the cell-death inducing factor is of great interest, the competence of differently cultured recipient cells was tested as well. For that purpose, the results obtained from synchronised cultures at time point 0 as recipient cells were compared with recipient cells coming from non-synchronised cells that had been raised under continuous light (7 dpi). In those cells, a mixture of cells in different stages of the cell cycle as well as cells in stationary phase can be expected. The mortality in response to PEF and HPH extracts is lower, when the recipient cells come from a non-synchronised culture as compared to synchronised cells (**Supplementary Figure S2**). The reduced responsiveness of the recipient cells is observed even though their density was comparable in both set-ups. This result strengthens the concept of a signal-receptor model that is dependent on the cell cycle stage of the microalgae. Additionally, the cultures differ in the aspect that synchronised cells are perpetually in exponential phase, while the non-synchronised cells have already reached stationary phase. This difference bears on the composition of the cell wall. Following autosporulation, the cell wall consists of a fragile unilaminar layer, but later develops into a rigid thick cell wall containing a cellulosic microfibril layer [5]. This could also contribute to the observed higher resistance in non-synchronised cells since the signal has to cross the rigid thick cell wall first.

3.6. Donor cells in stationary phase release more proteins and more cell-death inducing activity

The generation of cell-death inducing extract so far had been achieved using donor cells from cultures grown under continuous light harvested 7 dpi. At this point cells have already reached stationary phase. The exponential growth takes place on day two and day three after inoculation, and with the start of day four, the stationary phase is reached. As to test, whether the ability to release the cell-death inducing activity depends on the cell state, PEF extracts from donor cells with normalised cell density at different time points of the culture cycle were generated. Here, an increasing toxicity with growing age of culture can be observed (Fig. 6). The PEF extract generated from donor cells at 2 to 3 dpi shows very small, albeit significant, cell-death inducing effect after 24 h incubation with extract. However, when using donor cells from the start of stationary phase at 4 dpi and older, the cell-death inducing effect increases with the age of the donor cells. Synchronised cells are kept in the exponential growth phase by diluting the culture continuously. When using synchronised cultures as donor cells with normalised cell density (normalised compared to the experiment shown in Fig. 6), the cell-death inducing effect of the PEF extract was completely abolished, irrespective of whether the PEF treatment was applied to the donor cells at 0 h or at 6 h of the cell cycle (**Supplementary Figure S3**). This leads to the conclusion that the cell-death inducing effect is only generated in higher concentrations when donor cells have reached stationary growth phase. Upon cultivation in TAP medium, these algae grow exclusively mixotrophically, such that in addition to limiting nutrient supply, mutual shading of the cells is leading to the characteristic sigmoidal growth curve. The physiological and biochemical characteristics of the culture vary greatly during the different growth phases [48]. These changes include protein levels, lipid and secondary compound accumulation, as well as changes in gene expression, depending on whether the cell is still undergoing division or expansion, or whether it initiates differentiation during stationary phase. To test, whether the cell-death inducing factor might be a protein, the concentrations of the proteins released by the donor cells in response to the PEF treatment were measured in parallel to the cell-death inducing activity. As a control, the total protein content of the samples as produced by the HPH extract were measured as well. It can be seen that the concentrations of proteins released by PEF treatment increased with progression of the donor cells through the culture cycle (Table 2), i.e., concomitantly with the rise of the cell-death inducing activity of the PEF extract. While total protein content evident from the HPH extract increased only by less than 20% between 2 and 5 dpi, the release of proteins by PEF treatment increased more significantly, from around 30% of total protein in the exponential phase to around 40% of total protein in the stationary phase. This increase corresponds to around 67% increase between 2 and 5 dpi. However,

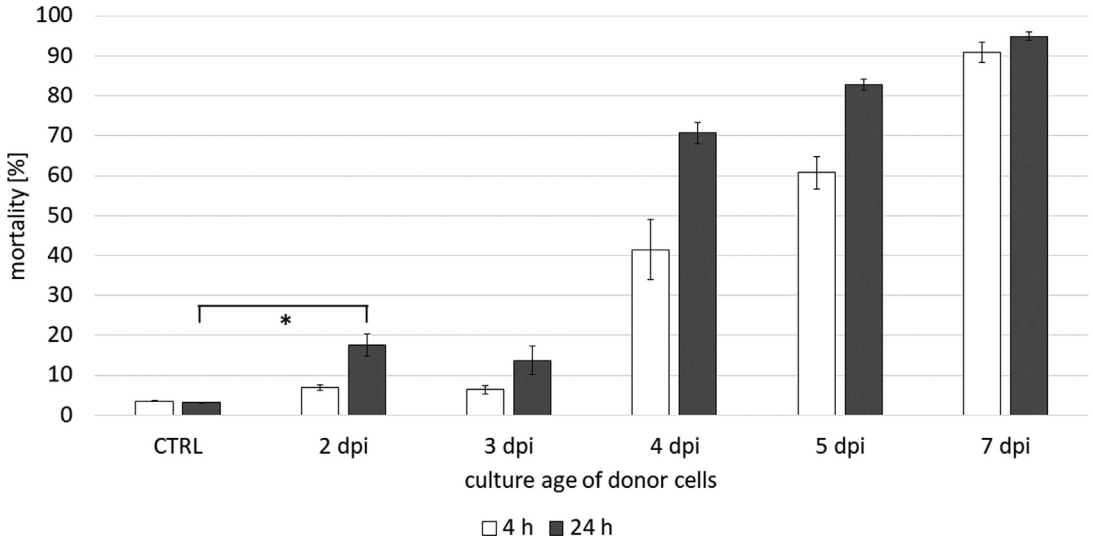

Fig. 6. Cell mortality after addition of PEF supernatant. *C. vulgaris* from cultures grown under continuous light at different time points of the growth curve were concentrated to a high cell density of 7 mg·ml^{-1}, as donor cells pulsed with a specific energy of 8.0 J·ml^{-1} and the suspension incubated for 24 h. After centrifugation, the water-soluble extracts were added to live synchronised recipient cells (time point 0) and viability was monitored at 4 h and 24 h via FDA assay (50 μM). CTRL: control sample without addition of treated supernatant. Data represent averages and standard errors from three biological replicates. Brackets indicate differences that are significant at $P \leq 0.05$ (*), using two-sample *t*-test assuming unequal variances.

Table 2
Protein content of HPH and PEF extracts after 24 h incubation measured by Lowry assay. Data represent averages and standard errors from three biological replicates. P-value indicate differences that are significant using two-sample *t*-test assuming equal variances.

	time [dpi]	Protein content [% CDW]	[%]	P-value
Total protein in HPH extract	2	44.5 ± 0.3		
	3	48.1 ± 1.7		
	4	52.9 ± 2.3		
	5	52.9 ± 0.2		
	increase 2–5		18.9	0.00005
Protein content in PEF extract	2	13.5 ± 0.2		
	3	13.6 ± 0.7		
	4	22.0 ± 1.0		
	5	22.5 ± 0.8		
	increase 2–5		66.7	0.0009

the increase in PEF-released protein abundance was not able to account for the much stronger (around 4- to 5-fold) increase of the cell-death activity during the transition from the exponential phase to the stationary phase (Fig. 6). Along with the relatively mild effect of protease treatment (Fig. 5**a**), this finding does not support a mainly proteinaceous nature of the cell-death inducing factor.

3.7. The responsiveness to the cell-death inducing factor depends on the cell cycle

Since the PEF-induced mortality depends on the cell-cycle stage in synchronised cells (Fig. 1), now the question arises, whether also the responsiveness of the recipient cells depends on the cell cycle. Therefore, mortality (at 4 h and 24 h) to PEF and HPH extracts was monitored from non-synchronised donor cells (cultivated under continuous light), when administered to recipient cells at different stages of the cell cycle (Fig. 7). Generally, the sensitivity of the recipient cells was dependent on the progression of the cell cycle. Shortly after auto-sporulation (just at the onset of the light phase, 0 h), the cells were less sensitive, compared to enlarged cells (6 h after the onset of the light phase), or compared to cells in G2 (6 h before the onset of light). In contrast, the mortality induced by a HPH extract of the donor cells, shows no difference in toxicity when comparing different cell cycle stages of the recipient cells. Therefore, the responsiveness to the cell-death inducing factor as well as the PEF-induced mortality depends on the cell cycle stage. Both these results support the concept that the cell-death inducing factor is not merely a non-specific toxin, but rather a specific signal. When comparing the response to direct PEF treatment (Fig. 1) to the response to the cell-death inducing factor (Fig. 7) one noticeable difference is the behaviour of cells in G2 (-6h in the dark phase). The cells at the time point −6h show a high mortality in response to the cell-death inducing factor, but when directly subjected to PEF treatment, the mortality of these cells stays as low as for cells shortly after auto-sporulation. Again, this difference supports a model, where direct PEF treatment at low specific energies acts as a mild abiotic stress that can be compensated, presumably through activation of adaptive gene expression. However, the same cells, when exposed to a biotic signal released from other cells (that had experienced the PEF treatment) activate a different pathway culminating in programmed cell death. The high specificity and the dependence on the cell cycle indicate a signal-receptor complex leading to activation of pathways that also ultimately lead to PCD. This putative signal-receptor complex shows either different expression levels or different activity, depending on the cell cycle. Whether the concentration of receptor varies over the cell cycle, or whether the transduction or processing of the signal deployed by this receptor is cell-cycle dependent, remains to be elucidated. One possibility to sort this out would be

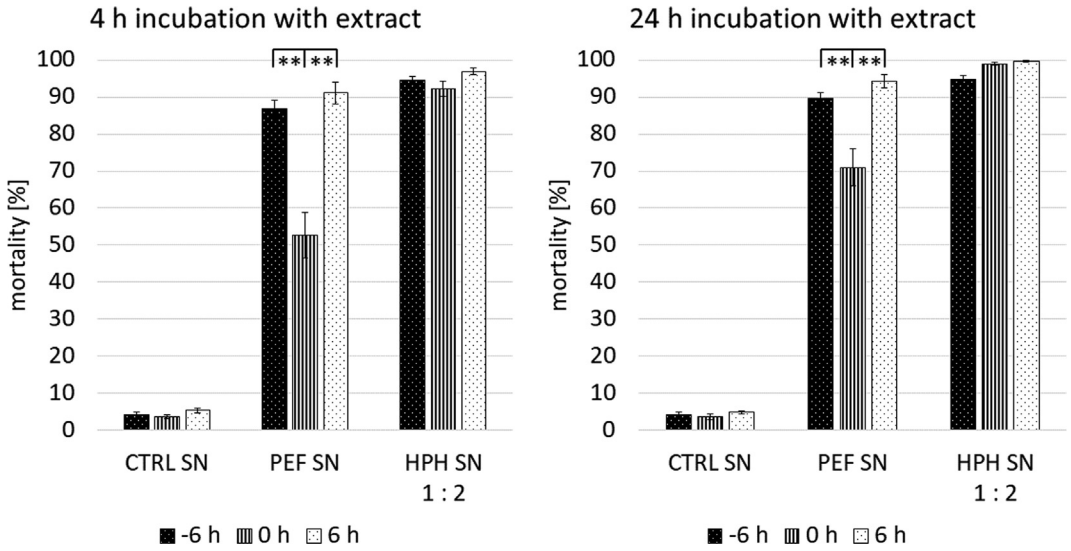

Fig. 7. Cell mortality of synchronised recipient cells at different stages of the cell cycle in response to PEF supernatant from non-synchronised donor cells. *C. vulgaris* from cultures grown under continuous light (7 dpi) were concentrated to a high cell density of 7,6 mg·ml^{-1}, one part pulsed with a specific energy of 9.4 J·ml^{-1} and the suspension incubated for 24 h. The other part was diluted by factor 2 and treated with HPH. After centrifugation, the water-soluble extracts were added to live synchronised cells at different times of the cell cycle (-6 = dark, 0 = onset of light, 6 = light) and viability was monitored at 4 h and 24 h via FDA assay (50 μM). Data represent averages and standard errors from at least three biological replicates. Brackets indicate differences that are significant at $P \leq 0.05$ (*) or $P \leq 0.01$ (**), using two-sample *t*-test assuming unequal variances.

to record dose–response curves. If they are shifted with respect to their threshold, this would support a receptor modulation, if they are not shifted, but just modulated with respect to amplitude, this would support a transductor modulation [49].

3.8. Protein recovery is already saturated by low energy input

If the release of proteins in response to PEF treatment involves active biological processes rather than being a merely physical phenomenon, it might be sufficient to trigger these processes at low energy and see the same effect as for higher energy inputs. To test this hypothesis, the dose–response relation of protein recovery over specific energy was determined, covering a range of 3.2 to 150.4 J·ml^{-1} (Fig. 8). This dose–response relation shows that protein recovery was already reaching saturation for the lowest tested energy input (3.2 J·ml^{-1}), especially for incubation periods greater than 2 h. The efficiency achieved with this very low specific energy was virtually identical to that seen with the standard energy (150.4 J·ml^{-1}) usually applied, although the energy input was around a factor of 50 lower. Since higher energies also lead to unavoidable rise of temperature in the sample and possible degradation of valuable compounds, use of lower specific energies is preferable. These findings support the hypothesis of PEF treatment acting as abiotic stress signal inducing PCD processes responsible for autolytic processes breaking down the cell. One potential building block playing into this scheme could be the putative interaction of PEF treatment with voltage-gated calcium channels, one kind of voltage-gated channel found in green algae [50]. The opening of voltage-gated calcium channels in the plasma membrane as central component of early stress signalling in plants (reviewed in

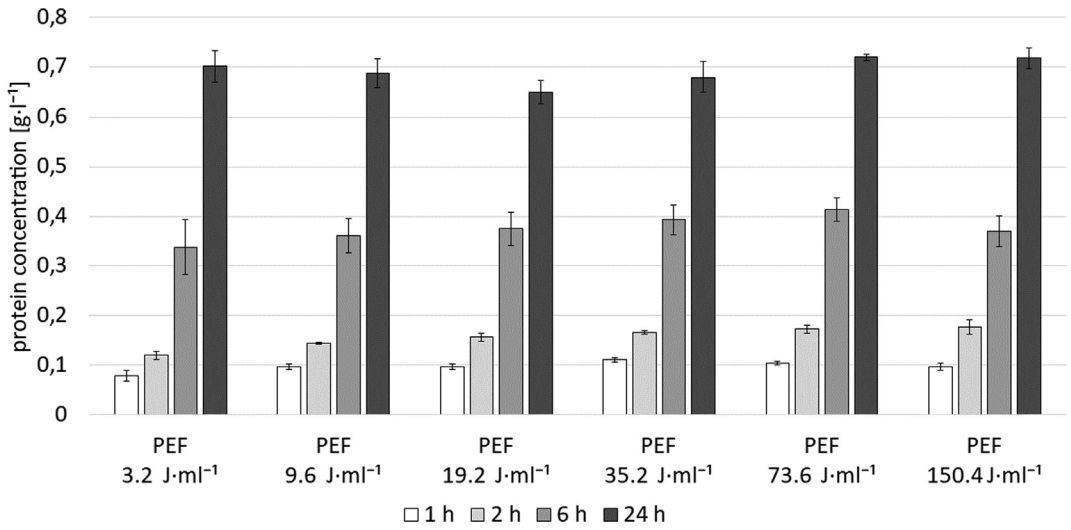

Fig. 8. Protein recovery efficiency in dependence of energy input. *C. vulgaris* from cultures grown under continuous light (7 dpi) were concentrated to a medium cell density of 5 mg·ml^{-1} and pulsed with different specific energies in the range of 3.2 to 150.4 J·ml^{-1} (field strength E = 40 kV·cm^{-1}). Protein concentration of PEF extracts was measured in the supernatant after centrifugation by Lowry assay. Data represent averages and standard errors from three biological replicates.

[51]) could be the first step to inducing PCD. Low-energy PEF treatment with subsequent incubation period could be a novel biotechnological strategy to extract first water-soluble proteins and subsequently lipids in cascade process without wasting energy on drying of the biomass or high treatment energies while still providing products safe for consumption.

4. Conclusion

This study shows that in response to PEF treatment, *C. vulgaris* in stationary phase releases a cell-death inducing factor, which is heat-labile and dose-dependent. As the responsiveness of recipient cells (as well as the release of this factor by the donor cells) depends on the cell cycle stage, this cannot be a merely physical phenomenon, but must involve a biological process. The high specificity of this process is consistent with a model, where PEF treatment deploys active signalling culminating in the observed induction of PCD.

The biological function of PCD might appear counter-intuitive in this unicellular alga. However, under conditions of vigorous clonal growth, this phenomenon provides selective advantage, when conditions become limiting, because in a clonal organism, ‘‘altruistic” behaviour would undergo kin selection. The otherwise enigmatic phenomenon that the recipient cells at time of autosporulation are less sensitive to the cell-death inducing factor, points to the same direction. Although Chlorella, like the other Trebouxiophyceae has been proposed to have lost sexuality, it still has retained the meiotic genes [52]. The auto-sporulation, thus, represents, a remnant of gametogenesis. The collective suicide of older cells for the benefit of enhanced gametogenesis would then be a manifestation of the facultative sexuality, which is widespread in many algae including Chlamydomonas [53]. The cell-death inducing factor needs to be identified so that the signal pathways can be elucidated. Since the experiments shown in this study do not necessarily support a mainly proteinaceous nature of the cell-death inducing factor, a volatile small molecule might be relevant for the cell-death inducing activity. Concerning the signal pathway in the recipient cells, involvement of the MAP kinase cascade is plausible due to the tight link with the cell cycle, leading to testable implications. Irrespective to the exciting biology behind the phenomenon described in the current study, it bears directly on biotechnological application. Even when administering PEF treatment at low energies, protein recovery efficiency is already saturated, presenting an energy-efficient way of protein extraction possibly connected to contribution of the putative cell-death inducing factor. Rather than describing the process merely in terms of physics, insight into biological signalling can help biotechnology to be more energy-efficient and, thus, more sustainable.

5. Acknowledgements

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2021.107991.

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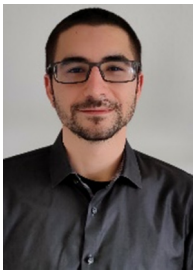

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