**Developing a reversible electroporation model of bacteria based on rate permeabilization measurements of hydrophilic and hydrophobic molecules in a moderate electric field**

**1. Scientific Background**

**1.1 Pulsed electric field**: Applying an external pulsed electric field (PEF) to microbial cells leads to an increase in their membrane permeability, a phenomenon which is termed electroporation (Sale and Hamilton, 1976). Based on theoretical studies and experiments, it was suggested that this phenomenon appears when the external electric field exceeds the capacity of the cell membrane potential. This leads to mechanical changes and the creation of hydrophilic pores where water molecules can enter through the membrane lipid bilayer, causing the polar head groups of adjacent phospholipids to face toward the water (Gehl, 2003; Kotnik et al., 2015; Neumann et al., 1999). The pore creation occurs in less than a second, while resealing has been reported to occur over a range of minutes or even hours (Kinosita and Tsong, 1977; Lee et al., 1992).

The influence of PEF treatment on bacterial cell electroporationdepends on three main parameters and their sub-parameters: 1) electrical parameters, which include the electric field strength and treatment duration; pulse number and amplitude; pulse width and shape; pulse frequency and unipolar or bipolar pulse modes; the electrode configuration, treatment chamber geometry; and the continuous or batch treatment affect (Donsì et al., 2005; Raso et al., 2016); 2) the targeted cell type, which depends on whether it is a eukaryote or prokaryote, the type of genus and species, and whether they are in a vegetative or spore state (Pillet et al., 2016). Other cell type sub-parameters, such as cell growth phase, size, shape, orientation in the electric field, and cell concentration also affect PEF efficiencies (Raso et al., 2016); and 3) the treatment medium and its composition, pH, temperature, and conductivity values, which also influence PEF efficiencies (Álvarez et al., 2002; Raso et al., 2016). After PEF treatment, the combination of these parameters influences cell susceptibility to reversible membrane permeability for a period of time until the membrane returns to its original state: Or, certain combinations lead PEF treatment to irreversible permeabilization and cell death (Mahnič-Kalamiza et al., 2014; Raso et al., 2016)**.**

The electric fields are divided into four ranges according to the membrane electroporation characterization (Yarmush et al., 2014): 1) no detectable electroporation – below a threshold of a specific electric field strength, regardless of the duration, no detectable electroporation is produced (Kramar et al., 2007; Pucihar et al., 2014); and 2) reversible electroporation – this electric range is characterized by pore formation which leads to the transport of molecules in and out of the cells. In this range, the phenomenon of pore resealing, where most of the electroporated cells retain their viability, may occur depending on the electric parameters and environmental conditions; 3) non-thermal irreversible electroporation – the pores reseal too slowly or not at all, leading to the release of cell contents, and; 4) irreversible electroporation with thermal damage – the electric current increases the temperature which leads to a denaturation of the released proteins (Kotnik et al., 2015).

**1.2 Viable but non-culturable (VBNC) cells:** The evaluation of bacterial viability is commonly based on their ability to replicate using the viable count assay (Espina et al., 2016). The lack of replication indicates the nonviability or nonexistance of microbial life in a sample (Emerson et al., 2017). However, for many microorganisms, such as bacteria, the designation as strictly a dead or livecellis not clear. In addition, the complete processes involved with cell life to cell death, along with how cells repair after injury, remain to be elucidated (Davey 2011; Schottroff et al., 2018). Viable but non-culturable (VBNC) cells, as well as sub-lethally injured microorganisms, are important cell states that may be induced by stressful conditions, such as heat treatment, ultraviolet radiation, hydrostatic pressure, cold plasma, pulsed light, and PEF treatment (Colwell, 2009; Schottroff et al., 2018). It is important to be able to identify and differentiate when a cell is injured or when it is in a state of VBNC. VBNC cells are characterized as being able to maintain membrane integrity while exhibiting low gene expression and reduced metabolic activity. However, in this state, the cell’s ability to create colony forming units (CFUs), the golden standard of characterizing cell viability, is inhibited (Ayrapetyan and Oliver, 2016). Suitable environmental conditions may lead to cell recovery from the VBNC state (Ramamurthy et al., 2014). In contrast to VBNC cells, sub-lethally injured cells can still replicate and form CFUs, but they do so very slowly and only when provided with more nutrient rich, non-selective growth media (Li et al., 2014). After being exposed to more suitable conditions, the cell recovery mechanisms activate and can restore the cells’ ability to function and grow normally again (Espina et al., 2016).

Research indicates that gram-positive bacteria may be more resistant than gram-negative bacteria to PEF treatment (Qin et al, 1995). It has been suggested that the thick peptidoglycan layerof gram-positive bacteria protects them from PEF damage (Hülsheger et al., 1983; Schottroff et al., 2017). However, after testing several types of gram-negative and gram-positive bacteria, García et al. (2005) reported that the bacterial types most resistant to PEF depended on the medium pH. They found that the gram-negative bacteria *Escherichia coli* O157:H7 exhibited higher resistance to PEF treatment at pH 4, while gram-positive *Listeria monocytogenes* showed higher resistance to PEF treatment at pH 7 (García et al., 2005). Due to their larger cell sizes, yeasts are more sensitive to PEF than are gram-positive bacteria. However, yeasts are more resistant to PEF effects than are gram-negative bacteria, probably due to the enrichment of disulfide bonds in the yeast walls that appear to stabilize the cells against PEF (Qin et al., 1995; Shamtsyan, 2012). Thus, it is important to understand the impact of the cell wall on the electroporation process. This is one major focus of the proposed research.

**1.3 Electroporation** **applications**: The application of irreversible electroporation technology for bacterial disinfection has been widely employed for the disinfection of liquid-based foods and in industries involving water purification. This technology is considered a "clean" method which does not use heat and thereby does not change food taste, flavor, or color (Amiali et al., 2006; Puertolas et al., 2009; Sobrino-López and Martin-Belloso, 2009; Evrendilek et al, 2013). In addition, irreversible electroporation has been shown to be useful for the extraction of compounds from cells. For example, in Gateau et al. (2020), the team used PEF to extract 46% of the total protein amount from the microalga *Haematococcus pluvialis.* This was done under a field strength of 1 kV cm-1 and a majority of the extraction occurred within five min after PEF treatment (Gateau et al., 2020). In another study, Pankiewicz et al., (2020) enriched the probiotic *Lactobacillus rhamnosus* B 442 bacterial strain with calcium ions by applying a PEF electric field strength of 3.0 kV cm-1. The calcium-enriched *L. rhamnosus* B 442 was used to prepare ice cream with a high content of protein, carbohydrates, and fat, along with the lowest melting rates (Pankiewicz et al., 2020). Reversible electroporation has also been used for lipid extraction from the green algae *Chlorella pyrenoidosa* (Han et al., 2019).

**1.4 Modeling of reversible electroporation in bacterial cells**: While the molecular mechanisms of PEF which lead to membrane permeabilization have not been fully explored, experimental and theoretical studies have demonstrated that PEF forms pores in the microbial lipid bilayer membrane. Relatively simple electroporation models are based on dynamic diffusion-driven transmembrane transport caused by electropermeabilization (Pucihar et al., 2001; Puc et al., 2003; Henslee et al., 2014; Tylewicz, 2020). Puc et al. (2003) presented a pharmacokinetic model of diffusion-driven transmembrane transport describing how this process, caused by electropermeabilization, facilitates stuyding molecule uptake as a function of elapsed time, voltage and pulse duration small molecules. They also showed cases where the permeabilization process was divided into a short permeabilizing phase during the pulse, and a longer resealing phase beginning after the end of the pulse.

The most significant factor in the basic electroporation process is the transmembrane potential difference induced by the electric field (REFs). The basic models use the spherical shape cell approximation to analytically find the induced transmembrane potential difference (REFs). Comprehensive electroporation models that provide a more extensive view of the pore formation process are molecular dynamics (MD) simulations and the continuum model (REFs). Although MD models are a powerful tool for studying systems on the molecular level, their use is limited by high costs associated with large computational requirements (Rems 2017, 2019). The continuum models describe the electric field, flow field, and temperature distributions in a pulsed electric field (PEF) treatment chamber by solving for the continuity, momentum, energy, and electric field equations of different geometries (Salengke et al., 2012; Knappert et al., 2020).

The theoretical study in the proposed project is based on two modeling efforts. The models will describe the fraction of the electroporated gram-negative and gram-positive bacteria as a function of the electric field strength and will include an experimental examination of rate permeabilization of hydrophilic and hydrophobic molecules with different molecular weights (MW). The models will also describe the pore size until resealing of the bacterial cells.

The first proposed model aims to analyze the experimental results. The model will evaluate the electric field effect by estimating the transmembrane transport rate and the membrane pore sizes using a kinetic model. The model will be used to predict the transmembrane transport caused by electropermeabilization using the experimental results of the cursor penetration into cells as a function of time. The model is based on a dynamic model for the cursor concentration outside and inside the cell. The transfer rate parameters will be correlated with the cursor concentration from experimental results. The model will be used for estimating parameters affecting the flow between intracellular and extracellular space, such as the membrane opening size dynamics. An example for a similar kinetic model approach is presented in Puc et al. (2003).

The second and more comprehensive model aims to study the effect of the field variables (e.g., electrical field strength, fluid velocity concentration and temperature) on the treatment process dynamics. This model will be based on a continuum model by using numerical simulations to calculate the local treatment conditions (electric field strength, temperature, flow field) inside a treatment chamber, as described in a recent study by Knappert et al. (2020). The numerical model will be implemented in a Computational Fluid Dynamic (CFD) simulation using the COMSOL® Multiphysics® modeling software, an interactive environment for modeling and simulating scientific and engineering problems. This second model will be developed concomitantly with experimental work to determine the most appropriate model parameters. The measured concentrations history of the cursor outside and inside the cell will be compared to the model predictions. Such a model will allow for a more fundamental understanding for designing the experiments and analyzing the results.

**2. Research Objectives & Expected Significance**

**2.1 Research goal:** The overall research goal of this study is to develop a reversible electroporation model of bacteria based on rate permeabilization measurements into the cells of hydrophilic and hydrophobic molecules in a moderate electric field.

**2.2. Research objectives**

1. To obtain basic data on the duration of the PEF-induced pores until resealing, a fluorescent dye, Lucifer Yellow (LY), will be applyed to gram-negative and gram-positive bacteria. The LY-positive cells will be quantified using a flow cytometer. The PEF treatment will be performed in a moderate electric field (1 - 4 kV cm-1) and variable physical parameters (e.g., pulse number, current density and total specific energy) will be measured. These experiments will be conducted immediately after exposure to PEF treatment and will continue until the LY-positive cells are reduced to near zero.

2. To examine the permeabilization rate into the cells of hydrophilic and hydrophobic molecules after exposure of gram-negative bacteria (*Pseudomonas putida*) to PEF treatment, as described in clause. These experiments will be conducted immediately after exposure to PEF treatment and will continue until the examined molecule concentrations in the supernatant are reduced to zero.

3. To investigate the permeabilization rate into the cells of hydrophilic and hydrophobic molecules after exposure of gram-positive bacteria (*Staphylococcus aureus*)to PEF treatment as described in clause 1. These experiments will be conducted immediately after exposure to PEF treatment and will continue until the examined molecule concentrations in the supernatant will be reduced to zero.

4. To understand the impact of the bacterial cell wall on electroporation efficiencies, the permeabilization of Lucifer Yellow and selected hydrophilic and hydrophobic molecules into PEF-treated bacterial protoplast cells will be examined (PEF parameters as described in clause 1).

5.To elcucidate the electroporation recovery processes of *P. putida* and of *S. aureus*,the bacterial proteome of the PEF-treated and untreated cells will be examined using mass spectrometry analysis (MS). Samples will be taken immediately after the PEF treatment and during cell recovery.

6. To develop a reversible electroporation model for bacteria by describing the transport affinity of hydrophilic or hydrophobic molecules across the pores of the bacterial cell and respective protoplast membranes. The model will be used to predict the kinetics of pore formation and size and kinetics of pore resealing caused by electropermeabilization using the experimental results of the cursor penetration into cells as a function of time.

7. To develop a numerical simulation for the temperature, flow and electric field distributions within the batch PEF treatment chambers using Computational Fluid Dynamic (CFD) software. The experimental results model will be used to elucidate the parameters of the dynamic model and to demonstrate the validity of the proposed models.

**2.3 Expected significance:** This research seeks to develop electroporation models for evaluating and analyzing experimental results along with predicting experimental outcomes. The results of these models will be used to optimize experimental protocols. Therefore, this model needs to accurately predict the physical parameters for pore formation and resealing that are accessible via experimental measurements. The ability to create reversible electroporation of relatively small molecules (78.12 - 716.604 kDa) is an important method in many fields, including: bioremediation of recalcitrant molecules considered as environmental pollutants; molecule extraction from bacterial cells; and genetic engineering processes. In addition, such a model will provide a basic theoretical understanding of the processes involved in PEF technology. Furthermore, our work attempts to rule out a possibe thermal effect on the bacterial cells which may influence on the results.

**3. Detailed Description of Proposed Research**

Pore formation and resealing in electroporated-mammalian, plant, yeast, and bacterialcellsare well-known phenomena. Existing models describing electroporation are mostly based on mammalian cells or artificial membranes, and some attempt to characterize permeabilization using fluorescent dyes like propidium iodide (PI) applied to the electroporated target. However, PI transport is influenced by many factors, such as the cell physiology state and the medium properties. The bacterial cell wall, its small cell size compared to mammalian cells, and the bacterial cytoplasmic membrane composition may also influence pore formation and resealing. Exposure of bacterial cells suspended in a phosphate buffered saline (PBS) solution applied to PEF-treatment may lead to pore formation. However, transferring the treated bacteria to a more nutrient-rich medium, such as brain heart infusion medium (BHI) may enhance the chances of cell recovery and pore resealing. While pore resealing may occur within a minute to hours, pore size and its affinity to hydrophilic or hydrophobic molecules until resealing are not well understood. The internal treatment chamber dimensions for this research were designed as a continuum media where the laws of physical conservation can be implemented.

**3.1** **Working hypothesis**

Pore duration and size as well as the physical and environmetal conditions for pore resealing in PEF-treated bacterial cells are not well understood. The cell wall constituents, like peptidoglycan, may influence pore formation of exposed bacterial cells. We hypothesize that based on collected data on varying physical parameters (such as electric field intensity, current density, total specific energy, pulse number and duration), a dynamic model of diffusion transmembrane transport in a moderate electric field (1 – 4 kV cm-1) can be developed. The data we will measure for our models include the permeabilization rate of a fluorescent dye as well as the quantities of hydrophilic and hydrophobic molecules in sample supernatants of electroporated gram-positive and gram-negative bacteria, as well as bacterial protoplasts.

**3.2 Research design and methods**

**3.2.1 Examination of the pore size and resealing time in electroporated gram-negative bacteria**:Abacterial suspension (106 CFU mL-1) of *Pseudomonas putida* F1 (6899 DSMZ, Germany) will be exposed to a pulsed electric field (PEF) with physical parameters of 1– 4 kV cm-1 with a frequency of 100 Hz, square pulse shape, and a duration of 10 µs. A pulse variation number between 1,000 to 10,000 in a continuous series of trains with 500 pulses each will be applied. The duration of a train will be 5 s with a 2 s interval between trains, which will be delivered in a polar mode.

*P. putida* F1 suspended in PBS will be diluted in ultra-pure (UP) water (0 – 24.22 mM PBS), which leads to current densities between 0.02 ± 0.01 to 5.2 ± 0.1 A cm-2.The conductivity before adding the bacteria in the UP water and PBS will be 1 and 155 – 4058 µS cm-1, respectively. Immediately after the PEF-treatment of the bacterial suspension in PBS (six replicates), the treated bacterial suspension will be diluted into brain heart infusion (BHI), a rich medium which was shown to reseal the pores. The BHI medium will contain: a) a fluorescent dye, Lucifer Yellow (LY) (457.25 Da), in an attempt to receive basic ~~i~~nformation on the pores’ duration of opening until resealing. It has been found that LY enters injured cells and the percent of LY-positive cells can be easily measured using flow cytometry (FCM); b) hydrophobic molecules from one aromatic hydrocarbon to 10 rings which include seven aromatic hydrocarbons and three rings of five carbons which include benzene, naphthalene, anthracene, pyrene, benzo[e]pyrene and decacyclene, with molecular weights of 78.12, 128.174, 178.23, 202.256, 252.3 and 450.5 g/mol, respectively; c) and relatively hydrophilic moleculesincluding phenol, bisphenol A, ellagic acid, epigallocatechin gallate, procyanidin B2 and theaflavin-3-gallate with molecular weights of 94.11, 250.275, 302.197, 458.372, 578.52 and 716.604 g/mol, respectively.

It is important to note that the concentration of each examined molecule will be less than the concentration thresholds which are known to cause cell damage and that most of the chosen hydrophobic and hydrophilic compounds are considered environmental contaminants and were reported to be biodegradable. Part of these molecules are characterized by slow biodegradation rates (Fernández-Luqueño et al., 2010 to add to references). The control of these experiments will be non-PEF treated suspensions of *P. putida* F1, but treated with the same environmental parameters.

**3.2.2 Methods and calculations:** After the suspension of the PEF-treated bacteria in BHI, the permeabilization rate of each molecule will be examined using High Performance Liquid Chromatography (HPLC) at differing time intervals (e.g., every 15 min) until the permeabilization rate will reduce to zero. The measurements will be conducted on the supernatant. To understand the level of the bacterial enzyme activity during the pore formation and resealing, the degradation rate for two selected molecules (hydrophobic and hydrophilic) will be examined. For this, the bacterial sediment will be sonicated (name, version, manufacturer, location) and the intermediate metabolites of the degraded molecules (hydrophilic and hydrophobic) will be measured using HPLC. CFUs will be examined immediately after the suspension into BHI and at the same intervals as described in paragraph 1.

The total specific energy (WT) will be calculated as described by Raso et al. (2016). Equation 1 calculates the specific energy input per pulse (*W*), with *W* the integral over time of the recorded pulse shape of voltage and current that was measured in the treatment chamber during the pulse (τ).

**Equation 1:**

where *m* is the sample mass, *U(t)* is the voltage, and *I(t)* is the current measured in the PEF chamber during load pulse (τ). The total specific energy (*WT*) for each treatment will be determined in Eq. 2 by multiplying the pulse number (*n*) with specific energy per pulse (*W*).

**Equation 2:**

**3.2.3 Gram-positive bacterial experiments:** The same experiments as performed with *P. putida* F1 will be conducted with *Staphylococcus* *aureus*, a gram-positive bacterium (25923 ATCC, USA).

**3.2.4 Influence of bacterial cell wall on electroporation kinetics:** To understand the influence of the bacterial cell wall on the electroporation kinetics, the permeabilization of the bacterial protoplast from one hydrophilic and one hydrophobic molecule will be examined. The protoplast will be exposed to PEF and nontreated protoplasts will serve as controls. The experiments will be performed as described in section 3.2.1, except that only two molecules and select physical parameters will be examined. For preparing protoplasts, the peptidoglycan of *P. putida* as well as *S. aureus* will be digested using murein hydrolases where the commonly used enzyme is the white lysozyme in hen eggs (to add ref). Since the outer membrane of gram-negative bacteria mostly prevents entry of enzymes towered the peptidoglycan, these types of cells require pre-treatment with a chelating agent (e.g. EDTA) or detergent (e.g. Triton X-100) for removing the outer membrane (Salazar and Asenjo, 2007).The protocol for preparing protoplasts is described by Figueroa et al., 2018.

**3.2.5** **Bacterial pore resealing following the electroporation:** To understand if and how bacterial pore resealing occurs following the electroporation process, analysis of the proteome will be conducted. At select times, electroporated bacteria and the control, non-treated bacteria will be collected and centrifuged. The proteins from the washed sediment will be sonicated and treated with urea, ammonium bicarbonate, and DTT as described in our previous study (Emanuel et al, 2019). Mass spectrometry analysis will be performed at the Smoler Proteomics Center at the Technion, Israel. Details of this processes are described in Emanuel et al., 2019. The data will be quantified by label-free analysis using the same software, based on extracted ion currents (XICs) of peptides, thus enabling quantification from each LC/MS run for each peptide identified in the experiments.

**3.2.6. Development of the bacterial electroporation model.** The presented theoretical work aims to build a numerical simulation of cell permeabilization by PEFs. The governing equations are based on the conservation equations for mass, momentum, energy and charge (Knappert et al., 2020). The model is composed ofthe conservation equation for mass, momentum, energy, electric potential and the transport equation for the activity of passive biological tracers, detailed in Eqs. 3 through 7 below:

**Equation 3**: Continuity equation (mass conservation):



**Equation 4**: Momentum conservation equation:



**Equation 5**: Energy conservation equation (heat transfer):



**Equation 6:** Electric potentialequation:



**Equation 7**: Transport equation (component number density balance):



where *t* is the time, **u** is the fluid velocity vector,  is the fluid density, *p* is the pressure, is the fluid dynamic viscosity and **g** the gravitational acceleration vector. *c*p is the the fluid’s heat capacity, *T* the total temperature, *k* the fluid thermal conductivity, and *F*p the permeabilized cells’ population fraction (activity of passive biological tracers). The term e represents a source term for the internal energy.

**Equation 8:**

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where  is the electrical conductivity of the fluid and *E* represents the local strength of the electric field. The electric field can be computed from Eq. 9:

**Equation 9:**

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The source term for the fraction of perforated cells isFp. A function of the electric field strength and the treatment temperature, Fp will be derived from experimental data. The numerical tool to be employed isa commercially available CFD software package (COMSOL Multiphysics®). It is used to solve numerical 3D transient models by calculating the temperature response in place and time.

**4. Preliminary Results**

**4.1 Design and construction of the electroporator****:** A high voltage generator was set to administer an electric field between 100 V and 3000 V on *P. putida* suspensions. The produced voltage pulses were under the control of a synthesized function generator (Stanford Research System DS45, 30 MHz, Sunnyvale, CA, USA). To obtain the current ICH value, the voltage UR at a resistance R=1Ω was connected in series to the current circuit (ICH=UR/R) (Figure 1A). Current density was calculated in accordance with cross section S (JCH=ICH/S).

**4.2 Construction of the electroporator chamber:** The electroporator chamber was constructed from two stainless steel plates, each with a thickness of 3 mm. Dimensions are shown in Figure 1B.



Crocodile hook area

A

B

Liquid sample

Figure 1. Schematic drawings of experimental design. (A) the experimental arrangement (electronic circuit) and (B) the electroporator chamber.

**4.3 Total specific energy and the heat transfer model**: To investigate the possible thermal effects on the bacterial viability results, energy balance and heat transfer analyses were performed to find the temperature distribution in the bacterial suspension during the PEF treatment. Measurements were taken for the highest conductive sample (1050 µS cm-1). Current and electrode potential were measured during the PEF treatment, and the results are are shown in Figure 2A. The total specific energy (WT) was calculated using Eqs. 10 and 11. *W* is the integral over time of the recorded pulse shape of the voltage and current measured in the treatment chamber during the pulse (τ).

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| **Equation 10:** |  |  **Equation 11:** |  |

where *m* is the sample mass, *U(t)* is the voltage, and *I(t)* is the current measured in the PEF chamber during load pulse (τ). The total specific energy (*WT*) for each treatment was determined in Eq. 11 by multiplying the pulse number (*n*) with the specific energy per pulse (*W*). The total specific energy for the highest conductive sample (1050 µS cm-1) was found to be 224 kJ kg-1. This value was used to represent the heat source for the heat transfer modeling. The temperature response in the system was calculated using COMSOL Multiphysics® numerical software. The calculation was performed using Eqs. 12 and 13. The 3D transient heat transfer model was based on the electrodes’ domain, the heat convection at the electrodes’ boundaries and the conduction heat transfer in the bacterial suspension. The heat generation source term was taken from the total specific energy calculations. The initial temperature of the bacterial suspension and electrodes was 22°C.

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| **Equation 12:** |  | **Equation 13:** |  |

where *T* is the temperature in the space and time T(x,y,z,t). *q'''* the heat source (W m-3), the heat diffusivity, and the heat conductivity ( = for the electrodes, or s for the sample). As a boundary condition, convection heat transfer at the electrode walls was applied, with the convection heat coefficient and the ambient temperature. Figure 2B presents the temperature map after 70 s.

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| --- | --- |
| **A** | **B** |

Figure 2. Potential and current time response during a pulse for two cases of opposite polarities. (A) Voltage input (blue line); voltage output (dashed blue line); and current output (red line). (B) Electrode sample system’s temperature map after 70 s operation time.

It was found that after an operation time of 70 s, the average temperature in the sample was approximately 35 ± 1 °C. The temperature predictions using the COMSOL Multiphysics numerical software were consistent with the experimental measurements using a multimeter thermocouple (VICHY, VC99, type-K, chromel-alumel).

**4.4 Effect of the solution conductivity on bacterial viability:** *P. putida* suspensions (0.01 OD 600 nm) in DI water as well as in different PBS concentrations (0.01 - 2.4 mM) were exposed to electric fields of 4.0, 2.8, 2.0 and 1 kV cm-1. The PEF-treated suspensions were incubated at 37 ºC for 1.5 h, followed by viable count analysis. The electric parameters applied to the bacterial suspensions that were treated by 1 kV cm-1 were: square pulse shape with duration of 10 µs and frequency of 100 Hz.; and 5000 pulses performed by a continuous polar series of trains, each train consisting of 500 pulses. Each train duration was 5 s with a 2 s interval between each train. The first and second columns of Figure 3A represent the CFU mL-1 of the bacteria that were suspended in DI water without PEF-treatment (control) and with PEF-treatment, respectively. The remaining columns represent samples of bacterial cells suspended in different PBS concentrations (correlated to current densities of 0.5, 1.2, 3.1 and 5.2 A cm-2)that were exposed to PEF-treatment. As seen in Figure 3A, a linear correlation (p < 0.001) was found between the bacterial viabiliy and the current density. This phenomenon was also observed when the cells were exposed to 4.0, 2.8 and 2.0 kV cm-1 (data not shown). In conclusion, a linear correlation was found between the current density (that is influenced by the solution conductivity) and bacterial eradication in all electric field strengths. When the electric field was decreased from 4 to 1 kV cm-1, the current density necessary for total eradication increased.

**4.5 Bacterial membrane permeability and relative cell size as a function of current density****:** *P. putida* F1 bacteria were treated with an electric field intensity of 1 kV cm-1 as described in section 4.4, followed by incubation of the bacterial suspension (0.01 OD 600 nm) at 37ºC for 1.5 h and the addition of PI (final concentration of 1.5 µM) for 5 min at 37ºC. Four replicates of each sample were examined via flow cytometer (CytoFLEX, Beckman Coulter, Atlanta, USA). Data were analyzed using FlowJo software (Tree Star, San Carlos, USA) and the membrane permeability of about 50,000 cells in each sample was examined. The histograms of cell number as a function of PI fluorescence intensity showed that increasing the current density led to increases in cell populations that were PI positive. This indicates that the fluorecent dye probably penterates the cells via the formed pores in the PEF-treated cells (Figure 3B). The percentage of PI-positive cells in the untreated samples (control) was 10 ± 0.9%. The membrane permeability at the lower current density of 0.02 ± 0.01 A cm-2 was found to be 14 ± 0.9% (p < 0.05). However, at a current density of 1.2 ± 0.1 A cm-2, PI positive cells were 53 ± 5.1% (p < 0.001), and at the maximum tested current density (5.2 ± 0.5 A cm-2), permeability was 65 ± 0.3% (p < 0.001) (Figure 3C). In conclusion, a linear correlation was found between increasing current density and bacterial cell permeability. This phenomenon was also observed in the electric field ranges of 2.0, 2.8 and 4.0 kV cm-1 (data not shown).

Bacterial relative cell size as a function of current density is shown in Figure 3D. The PEF-treated and untreated cells were stained with PI. Each examined sample included about 50,000 cells, so that the area under each curve was equal (Figure 3D) (except the upper curve that represents only the PI stained cells). The overlay offset graph in Figure 3D presents the different scattering of *P. putida* F1 cell size under PEF treatments relative to untreated cells. As shown, the peak of the curve of the untreated sample (control) was shown to be approximately 2 × 104 forward side scatter (FSC) (blue line). At a current density of 5.2 ± 0.5 A cm-2, the peak of the scattering relative cell size curve was ~6 × 104 FSC, this is about three times higher than the untreated cells. Additionally, a peak appeared on the left side of each graph at current densities above ~1 A cm-2. We assume that this peak represents cell debris with a ~8 × 103 FSC, ten times less than the cell size of the control cell sample. Examination of the cell size only in the PI stained cells (full red color graph) shows that most of the cells are larger than the cells in the control sample, and the subpopulation with the smaller size is barely observed.

Our study’s bacterial suspension did not contain solutes that could lead to water expulsion from the cells, and the pore creation under the applied voltage led to water uptake into the cells and cell swelling.



**A**

**B**

**A**

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**D**



**C**

**Figure 3.** **Viability, pemeability and relative cell size of PEF- treated *P. putida* F1 as a function of current density.** Viability (using viable count assay) of bacterial cells was calculated in CFU mL-1 **(A)** *P. putida* F1 membrane permeability was examined using flow cytometry. A histogram of cell number (counts of events, Y-axis) as a function of propidum iodide (PI) fluorescence intensity (X-axis) where PI-nonstained cells (control) are in gray; PI-stained cells (control) are in blue; PEF-treated cells (PI-stained) after application with a current density of 0.02 A cm-2 are in purple; current density of 1.2 A cm-2 is in green; and 5.2 A cm-2 is in red; **(B) ~~(C)~~** The percentage of PI-positive cells (Y-axis) as a function of current density (X-axis); **(C)** In subfigures Aand C, the first column (control) and the second column represent bacterial cells suspended in DI water without PEF treatment and PEF-treated cells after application with a PEF current density of 0.02 A cm-2, respectively. The columns 0.5 – 5.2 A cm-2 represent bacterial suspensions in solutions with ionic strengths of 2.42 – 24.22 mM. T-test significance tests were applied with signifying p-values of p ≤ 0.05 = \* and p ≤ 0.001 = \*\*\***;** **(D)~~(D)~~** *P. putida* F1 relative cell size was measured using flow cytometry (counts of events, Y-axis, and relative cell size – FSC as X-axis). Untreated cells (control) are indicated in blue; PEF-treated cells treated with a current density of 0.02, 1.2 and 5.2 A cm-2 are as displayed in purple, green, and red lines, respectively; only PI-positive stained cells are in solid red.

**4.6 Viability of PEF-treated *P. putida*** **suspensions in rich medium vs. PBS:** In this experiment, the *P. putida* and *S. aureus* bacteria in PBS were exposed to PEF treatment (2.9 kV cm-1 at current density of 3.4 ± 0.1 A cm-2). Assessed physical parameters were the same as described in section 4.4. A PEF-treated *P. putida* suspension (0.02 OD 600 nm) was divided into three 100 µL portions each. The first portion (100 µL) was immediately taken at time ‘0’ after the PEF treatment and was examined for bacterial viability (in CFU mL-1). The second portion was suspended into 900 µL of 0.54 mM PBS and designated as PEF-treated bacteria in PBS, while the third portion was suspended into 900 µL BHI and designated as PEF-treated bacteria in BHI. The dilutions into PBS and BHI were performed immediately after the PEF treatments. The same procedure was performed on bacterial suspensions that were not exposed to PEF treatment and designated as non-treated bacteria in BHI and non-treated bacteria in PBS (control). The diluted samples of the PEF-treated and non-treated bacteria were incubated for 24 h at 37ºC, and at indicated times during 24 h,a viable count assay was performed (each examination was based on at least five replicates) (Figure 4).

At time '0', the PEF treatment led to a reduction of 4.8 log10 compared to the nontreated sample. The non-treated bacteria in PBS remained at the same concentration during the entire experiment. The non-treated bacteria in BHI continued to replicate, and reached 1.2 × 1010 CFU mL-1 at 24 h. However, no CFUs of the PEF-treated bacteria in PBS or in BHI were observed from the fourth to the sixth hours after the PEF exposure. The PEF-treated bacteria in BHI began to replicate after the sixth hour and by the eighth hour, they reached 1.18 × 104 CFU mL-1, and at the end of the experiment (24 h), the CFU countswere similar to those in the BHI control (the non-treated bacteria). The same experiment performed with the gram-positive bacteria *S. aureus* showed that this gram-positive species of bacteria were more resistant to PEF treatment, compared to the gram-negative bacteria *P. putida* (data not shown). The PEF treatment of *S. aureus* led to a reduction of approximately 3.2 log10 CFU mL-1 (at time ‘0’). In addition, the PEF-treated *S. aureus* suspended in BHI maintained a count of 1.84 × 104 CFU mL-1 for about 1.5 h, and then began to multiply (data not shown). This is an interesting contrast compared to the PEF-treated *P. putida*, which decreased to zero CFU mL-1 at the fourth h and remained there for about 2 h before beginning to multiply. It was previously noted that the thick peptidoglycan layer and structural properties of a gram-positive bacterial membrane protect them from PEF damage. We suggest that the results from the PEF-treated *S. aureus* in BHI are consistent with findings from multiple studies that demonstrated gram-positive bacteria to be more resistant than gram-negative bacteria to PEF treatment [7,54]. However, when PEF-treated *S. aureus* wassuspended in PBS, no CFU ml-1 were observed from the fifth h until the end of the experiment (24 h), similar to the results obtained for *P. putida*.

**4.7 MS analysis of the proteins from PEF-treated *P. putida* F1 suspension, compared to non-treated bacteria:** *P. putida* suspension in 0.54 mM PBS (350 µL) were PEF-treated (2.9 kV cm-1) and immediately diluted to 1:10 in BHI (three replicates). The same procedure was applied to the non-treated bacteria. The samples were taken six h after the PEF treatment. It is important to note that the growth rate of the PEF-treated bacteria in BHI from the sixth to the eighth h was 4.68 h-1. In the untreated culture, the growth rate at the beginning of the log phase was 1.08 h-1. We assume that the PEF-treated culture in BHI by the sixth h was not composed of dead cells. Rather, a large population of them were in a stressed state, which may be considered VBNC or sublethally injured cells. Thus, the appropriate control for MS analysis of the PEF-treated bacteria in BHI was nontreated cultures grown for the same time (6 h), and not a control culture at the end of the lag phase. All samples of the PEF-treated and nontreated cells were collected at the sixth h and washed in PBS (x 3) using centrifugation. The proteins in the sediment were extracted and digested in order to identify the overexpressed proteins of the PEF-treated cells in BHI compared to the non-treated samples. The MS analysis was performed at the Smoler Proteomics Center at the Technion, Israel. A little over 2,100 proteins in the MS analysis were identified with at least two unique peptides and 1% FDR in one of the triplicates. A total of 22 proteins were considered for quantification analysis, which was limited to those with at least two unique peptides in two of the three replicates, and those that were significantly (p < 0.05) over-expressed in the PEF-treated bacteria. As Figure 5 shows, the results of the over-expressed proteins in the PEF-treated cells consisted of three main groups: 55% related to stress conditions; 36% to various proteins; and 9% to uncharacterized proteins (Figure 4).

**Figure 4. Viability assays and protein expression tests on PEF-treated and non-treated *P. putida* F1. (Left Figure)** Viability assays of PEF-treated and non-treated *P. putida* F1 included all samples that were collected at various time-points between 0 h and 24 h after treatment . PEF-treated bacteria at time '0' (); non-treated bacteria at time '0' (); PEF-treated bacteria in BHI (1.5 - 24 h) (); PEF-treated bacteria in PBS (1.5 - 24 h) (); non-treated bacteria in BHI (1.5 - 24 h) (); non-treated bacteria in PBS (1.5 - 24 h) ().T-tests were applied and significance of the CFU count at each examined time related to its control was designated asp < 0.001\*\*\*;significance of the CFU of the treated bacteria in PBS related to BHI, at each examined time was designated p < 0.001 ###. **(Right Figure)** Proportions of over-expressed proteins in PEF-treated *P. putida* F1 in BHI, compared to the non-treated sample. A total 22 over-expressed proteins (p < 0.05) in the PEF-treated bacteria were analyzed by MS.

**5. Available Equipment, Personnel, and Collaborations**

The microbiology experiments will be performed in the labs and under the supervision of Prof. Rivka Cahan. The lab is equipped with a high-voltage generator for applying an electric field on bacterialsuspensions with a signal generator (Stanford Research System DS45, 30 MHz) to control voltage pulses. The lab also contains a homemade electroporator chamber made of two stainless-steel plates, incubators for bacterial growth, UV-Vis spectrophotometer, analytical scale, orbital shakers, laminar safe cabinet, chemical hood, centrifuge with fix-angle and swinging bucket rotors, CG-FID, HPLC, autoclaves, refrigerators, freezers and ‐80ºC freezers. We will have access to the shared facilities of Ariel University. Major equipment includes a flow cytometer (FCM), GC/MS, SEM, AFM, confocal microscopy, and fluorescence microscope. Prof. Cahan's lab group consists of one post-doctoral fellow, four PhD students, one MSc and four undergraduate students. The microbiology aspect of the project will be conducted by PhD and postdoctoral students.

The reversible electroporation model on bacteria will be conducted by Dr. Gad Pinhasi. His lab group focusses on theoretical, experimental and numerical studies on transport phenomena, i.e*.*,fluid dynamics, heat and mass transfer. His group will perform the numerical analyses and computational fluid dynamics portion of this project and will investigate the mechanisms of two-phase flashing flow and combustion. Dr. Pinhasi’s lab group has two PhD students and five undergraduate students. The modeling aspect of the mentioned study will be conducted by a PhD student.

**Collaborations**

**6. Expected Results and Pitfalls**

Opening duration of the PEF-induced pores until resealing will be determined by applying the fluorescent dye Lucifer Yellow (LY) to gram-negative and gram-positive bacteria and the bacterial protoplasts. The LY-positive cells will be quantified using a flow cytometer. One anticipated pitfall of the proposed research is thatbecause the MW of theLY is 457.25 Da, the data on when the beginning of the pores open and the exact time of resealing will not be accurate for the low and high MW of the differing selected molecules. In addition, the time duration of pore openings may change due to the selected molecule properties. These limitations may be adressed by expanding (correlated to the data of LY) the time for examining the permeability rate of the different hydrophobic and hydrophylic molecules. The permeability rate of the hydrophilic and hydrophobic molecules will be determined by measuring the concentration of these molecules in the bacterial supernatant using HPLC analysis. Another anticipated pitfall of this project involves the fact thatsome of the selected molecules, when used in high concentrations, may cause damage to the cells.This may be solved by reducing the concentration of the selected examined molecule.

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Example of significantly increased **stress proteins** (**total 22**) are Alkyl hydroperoxide reductase and TonB-dependent siderophore receptors. Alkyl hydroperoxide reductase (A5W5H2) is an enzyme related to a large family of thiol-specific antioxidant proteins which reportedly protect bacteria from abiotic stresses [62]. The alkyl hydroperoxide reductase is a crucial enzyme for gut [bifidobacteria](https://www-sciencedirect-com.mgs.ariel.ac.il/topics/immunology-and-microbiology/bifidobacterium), helping to manage reactive oxygen species (ROS) effectively under conditions of [oxidative stress](https://www-sciencedirect-com.mgs.ariel.ac.il/topics/immunology-and-microbiology/oxidative-stress) [63]. Three types of TonB-dependent siderophore receptors were identified (A5VXD9, A5W124 and A5W341). These proteins are located in the bacteria’s outer membrane; they are known for binding and transporting ferro-chelating siderophores, vitamin B12, carbohydrates and nickel complexes. A positive correlation was reported between the expression of iron-uptake systems in *P. aeruginosa* and the response to oxidative stress [65].