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Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Cold-loving bacteria: The effect of temperature on psychrotolerant exoelectrogen Shewanella loihica in microbial fuel cells

Olivier Nouwen

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Jean MANCA

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*Running title: *Cold-loving bacteria*

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ABSTRACT

Microbial fuel cells (MFCs) are battery-like devices that use microorganisms called exoelectrogens to oxidise organic matter and release electrons onto electrodes, producing bioelectricity. The transfer of electrons can occur through direct or indirect extracellular electron transfer. An important, highly variable parameter affecting MFCs is temperature. The effect of temperature on MFC technology has hardly been investigated, and therefore, the applicability of MFCs at different temperatures remains undetermined. Psychrotolerant exoelectrogens (PTEs), able to thrive in cold environments, can be used for low-temperature MFC operation. It is expected that the performance of PTE *Shewanella loihica* in MFCs will increase as the temperature decreases, by influencing its growth, morphology, and extracellular electron transfer. *S. loihica* was anaerobically cultured at 5°C, 15°C, and 25°C. MFCs operating with *S. loihica* were set up at the same temperatures and the output potential was monitored for 30 days. The highest growth rate and cell density was observed at 25°C, followed by 15°C. At 5°C, no growth was perceived. Cells were largest in the exponential and stationary phase, and smallest in the lag and death phase. The highest maximum electrical output was obtained at 25°C. However, 15°C showed the highest long-term average. An extremely low electrical potential was observed at 5°C. At 25°C, the MFCs with the highest electrical potential coincided with the MFCs with the highest relative amount of mediators. It can be concluded that *S. loihica* may have benefits for broadening the thermal window of operation of MFCs towards lower temperatures.

INTRODUCTION

The 21st century is defined by the eruption of global warming as a household term. The use of fossil fuels and other polluting energy sources have caused many negative consequences to the planet that may ultimately become catastrophic [1]. Additionally, with increasing human population, the energy demand increases as well [2]. Moreover, as fossil fuels are finite and unsustainable, advancements in renewable sustainable energy technology are of utmost importance [3].

As a result, bioelectrochemical systems (BES) which represent a form of sustainable energy, have gained increasing interest. BES make use of enzymes and/or microorganisms to catalyse oxidation-reduction reactions [2] [4]. BES can be categorised according to different desired endpoints, such as electrogenesis systems, electrohydrogenesis systems, and microbial electrosynthesis systems [2].

BES have been used in the form of microbial fuel cells (MFCs), since first studied by Michael Potter in 1911 [5]. Potter was the first to observe electrical current generation by microorganisms. MFC technology was revisited a few times in the

20th century, even in the framework of the space programme of NASA. However, interest decreased due to rapid advancements in other emerging energy technologies such as photovoltaics [6]. On account of efforts in the field by various researchers in the 1980s and 1990s the technology caught the attention of a larger audience [7]. MFCs are electrochemical systems that make use of microorganisms to generate electrical energy from the chemical energy available within organic substrates [3].

Generally, MFCs consist of an anode and a cathode, often separated in two chambers. Within this separation, a proton exchange membrane is placed, to allow solely for the transport of protons [8] [9]. The anode and cathode are connected via an external electrical circuit. In the anode chamber (Fig. 1b), microorganisms are used to catalyse the oxidation of organic substrates, functioning as a primary electron donor. The freed electrons are then released onto the anode and travel to the cathode through the electrical circuit (Fig. 1e), where they are consumed by a terminal electron acceptor in the cathode chamber (Fig. 1f) [3]. This relocation of electrons generates a current and thus bioelectricity [10].

As these microorganisms are able to capture electrons from various organic substrates in aqueous media, MFCs can act as an innovative method for wastewater treatment additional to energy production [3] [10]. As an illustration, in the case of lactate as a primary electron donor in the MFC, the following two half-reactions take place:

$$\text{Lactate} \rightarrow \text{pyruvate} + 2H^+ + 2e^- \quad (1)$$

$$2H^+ + 2e^- + 2O_2 \rightarrow 2H_2O \quad (2)$$

The oxidation of lactate takes place in the anode chamber (Eq.1). The oxidation causes the release of two electrons per lactate molecule. These two electrons travel to the cathode chamber, by means of an external electrical conductor, where oxygen will be reduced and water is formed (Eq. 2) (Fig. 1).

To expand the possible applications of BES technology, many studies focus on the optimisation of the electrode materials and the used microorganisms to overcome shortcomings of the technology [10]. The microorganisms used in MFCs are called exoelectrogens (Fig. 1a). A special feature exoelectrogens exhibit is that they are capable of long-range electron transfer. In this process, the electrons are relocated from available electron donors to available electron acceptors. This is done as a means for the exoelectrogens to gain energy. Only when there are no soluble electron acceptors present in their direct environment, they are able to transfer the electrons onto solid mineral electron acceptors, in a process also known as extracellular electron transfer (EET) [7] [4]. In MFCs, this process is exploited and solid minerals are replaced with electrodes, allowing for the harvesting of bioelectricity.

In the past, discussion has arisen of how exactly these transfer mechanisms work in different exoelectrogen species. Two main mechanisms can be distinguished; direct and indirect EET.

For direct EET to happen, direct contact between the electrode and the bacteria is necessary [11]. Key players in this process are the outer membrane cytochromes, which are redox proteins that are able to transfer the electrons to a terminal electron acceptor [11]. These can be present either on the cell surface, cell appendages, or assembled in nanowires (Fig. 1c). Through a chain of redox reactions occurring through the cytochromes, the electrons can be transferred to the anode and ultimately to the final electron acceptor.

Contrary to the aforementioned mechanism, no contact is needed for indirect EET. The indirect transfer of electrons to the anode surface makes use of mediating molecules (Fig. 1d). These can either be naturally present in natural systems (exogenous, e.g. mediators present in the environment by excretion of non-exoelectrogenic bacteria, humic substances [11]), artificially added to MFCs to enhance the electrical output (exogenous, e.g. methylene blue, neutral red [12]), or the result of microorganism activity (endogenous, e.g. quinones and flavines). These redox mediators function as redox carriers, acting as a shuttle for the electrons [11]. They accept electrons inside the cell, and in a reduced state, travel to the anode where the electrons are released, causing the mediators to revert back to their oxidised form [3] [11]. This process is repeatable.

Temperature is an important environmental parameter affecting microbial activity and thus plays a crucial role in the functioning of MFCs [13]. Unlike other environmental parameters such as pH, temperature is one of the few factors that cannot be regulated by bacteria [14] [15].

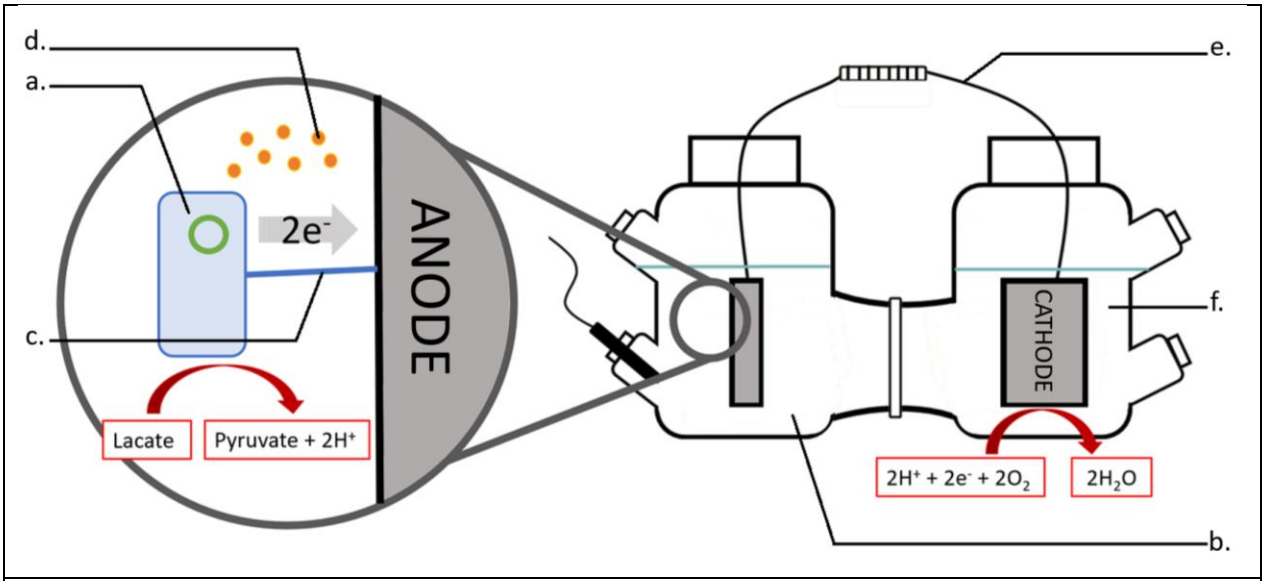

Fig. 1 – A schematic overview of the working mechanism of a MFC using lactate as an electron donor and oxygen as a final electron acceptor. Exoelectrogens (a) present in the anode chamber (b) oxidise lactate, resulting in the formation of pyruvate and the release of 2 electrons. The electrons are transferred onto the anode through direct contact (c) with the use of outer membrane cytochromes on the cell body, appendages or nanowires, or indirectly through the use of mediators (d). After relocating through the external electrical circuit (e), the reduction of oxygen takes place in the cathode chamber (f).

At high temperatures, the cells are no longer viable. At low temperatures, microorganisms tend to have a lower metabolic rate due to lower kinetic energy associated with decreasing temperatures [16]. As a result, the bacterial metabolism is no longer able to sustain the MFC. Aside from the importance of temperature on the metabolic activity of bacteria, temperature also strongly affects the Gibbs free energy, shifting the equilibrium of certain processes (Eq. 4) [17].

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Where:

ΔG = change in free energy
ΔH = change in enthalpy
T = Temperature
ΔS = change in entropy [18]

In the context of MFCs, the Gibbs energy per electron is especially relevant, as they set the limits for the electrical potential and coulombic efficiency of the system [19].

Because of the aforementioned reasons, new ways to overcome this constraint need to be considered. Furthermore, operating MFCs at lower temperatures allows for certain advantages, such as a higher coulombic efficiency, a larger temperature window of operation with a higher electrical output resulting in a broader possible applicative range [17].

Because of this dependency of temperature on MFCs, extremophiles have been studied in several types of BES to overcome malfunctioning at low or high temperatures. Extremophiles are species able to live in physiological conditions that are harmful to most life on Earth [20]. Among extremophiles are psychrophiles, able to live, grow, and have a sustainable metabolism in temperature conditions well below 15°C [21].

This adaptation to cold of psychrophiles has several physiological explanations. Firstly, their genome has a high GC content. Secondly, their enzymes are more flexible than those of species that grow optimally under moderate temperature conditions, also known as mesophiles. Psychrophilic enzymes also tend to have more catalytic sites to compensate for slower diffusion rates at lower temperatures in comparison with the

enzymes of their mesophilic counterparts. Thirdly, they have an increased amount of unsaturated and shorter-chain fatty acids in the plasma membrane to maintain its fluidity. And lastly, they possess genes associated with overcoming cold shock and anti-freeze proteins, which are special proteins or sugars that prevent the development of damaging ice crystals [14]. Interestingly, biofilms may also have a self-heating mechanism that possibly allows them to keep up with lower temperatures [17].

On the other hand, psychrotolerant microorganisms, also known as psychrotrophs, do not have as extensive capacities as psychrophiles for their adaptation to cold. However, they are still able to tolerate colder temperatures than mesophiles, as these species grow optimally around 15°C [17].

Interestingly, even exoelectrogens may be psychrophilic or psychrotolerant. This means that they are able to execute EET at lower temperatures than mesophilic exoelectrogens [17]. The unique characteristics of psychrophilic or psychrotolerant exoelectrogens can be advantageous in BES, as their use is especially interesting for the treatment of waste streams given that no heating would be required. However, only few studies on real life settings have been executed [17]. Psychrotolerant exoelectrogens (PTEs) may have a greater temperature range provided that the functioning at higher temperatures is not compromised.

Shewanella loihica, is a species within the *Shewanellaceae* Family, that has psychrotolerant characteristics. This gram-negative bacterial species was isolated from microbial mats in deep sea hydrothermal vents from mount Loiha in the Pacific Ocean. Similarly to other species within the *Shewanellaceae*, it is facultative anaerobic, meaning that it is able to survive in anoxic and oxic environments, obtaining an orange like colouration when exposed to oxic conditions [22]. Moreover, there are more similar characteristics to other species belonging to the same Family, for instance, a rod shape and a single polar flagellum [22].

As this species is a psychrotolerant exoelectrogen and has close ties to the well-studied model organism *Shewanella oneidensis*, often used in MFC studies, it is an ideal candidate to study their adaptation to cold as a means for low temperature MFC operation. This study will establish pure cultures of PTE *Shewanella loihica* in the MFC set-up to be tested according to different temperatures. The effect of temperature, and how they are able to overcome low temperatures will be investigated.

It is hypothesised that temperatures slightly below 25°C will increase the electrical output of MFCs inoculated with *Shewanella loihica* by influencing its growth, morphology, and direct and indirect EET mechanisms. This will be done to determine the exact impact of temperature on MFCs inoculated with *Shewanella loihica*. Additionally, it will be examined how this knowledge can be used to broaden possible applications of MFC technology and increase the resilience thereof.

With increased resilience, MFC technology may be used for local niche energy applications such as micropower generation for fuelling autonomous electrical systems, and for environmental sensing in the context of the Internet of Things for environmental on-line monitoring networks [23] [24]. For environmental sensing, temperature is especially of importance as environmental exposure will have a significant impact on this parameter of the system.

EXPERIMENTAL PROCEDURES

Medium preparation and culturing - *Shewanella loihica* strain PV-4 (DSMZ, Braunschweig, Germany) was routinely cultured in Hungate tubes filled with 9 ml Luria Bertani medium (Appendix 1), flushed with N_2 gas to achieve anaerobic conditions [25]. After flushing, the Hungate tubes were sealed with a rubber septum and screw cap, and 7 ml of N_2 gas was added to the headspace to overpressurise for autoclavation. Afterwards, the Hungate tubes were acclimatised in the climate chamber (VWR international, Leuven, Belgium) to their respective incubation temperatures (25°C, 15°C, and 5°C), together with the cultures used for inoculation. Following acclimatisation, the Hungate tubes were inoculated with 1 ml of the original culture. As *S. loihica* can make use of lactate as an electron donor, agrobacterium (AB) minimal medium with a concentration of 20 mM (Appendix 2) was prepared for use in the MFCs [26].

MFC preparation and operation - To test the effect of temperature on the electrical output of *S. loihica,* MFCs were set up in triplicate per

temperature condition (25°C, 15°C, and 5°C), according to the protocol of O'Brien and Malvankar [8]. The set-up consists of two glass vessels with each one GL45 aperture on the top, two GL14 apertures on the side, and one flange on the side. Nafion membrane (Fuel cell store, Texas, USA) was boiled in milliQ and cooled down to room temperature. Subsequently, the Nafion was placed over the flange, which is used to connect the two glass vessels. The Nafion membrane was held in place with an O-ring, and the two glass vessels are held together with a clamp. Once the Nafion membrane is in place, and the two vessels are attached, the glass vessels were filled with Milli-Q to maintain Nafion membrane integrity, and to prevent it from drying out. A graphite plate, functioning as the anode (5X2.5X0.5 cm, Mersen, Wemmel, Belgium), was buffed with sandpaper and titanium wire was attached. Graphite felt (5X7.5 cm, Mersen, Wemmel, Belgium), functioning as the cathode was also attached to the titanium wire. Both were sealed in their respective vessels, one functioning now as the anode chamber and the other as the cathode chamber. 25G needles were inserted in the upper GL14 apertures and covered with aluminium foil, to prepare for autoclavation.

After autoclaving for 15 min (or automatic programme Liq. A), the Milli-Q is replaced with medium. AB minimal medium with lactate was placed in the anode chamber, and AB minimal medium without lactate was placed in the cathode chamber.

The MFCs were put in a climate chamber together with the mother culture in the climate chamber for acclimatisation before the inoculum was inserted. Once acclimatised, the MFCs were circuited with 330 Ω resistors and 10 ml of culture, with cells in the exponential phase was inserted into the MFCs. Measuring was initiated with the DAQ6510 Data Acquisition and Logging Multimeter System (Keithley instruments, Ohio, United States) for a period of 30 days.

After MFC operation, a single MFC from each temperature condition was uncircuited to determine the open circuit potential. Afterwards, power density and polarisation curves were made with a Nev4 potentiostat (Nanoelectra, Madrid, Spain). Internal resistance was calculated by means of following equation (Eq. 5):

$$P=V^2/R \quad (5)$$

Where:

P= power
V= potential
R= resistance

Optical density analysis - To determine the growth of *S. loihica* an optical density analysis was carried out. After acclimatisation to their respective temperature conditions (25°C, 15°C, and 5°C), Hungate tubes were inoculated with 1 ml from the culture, and the absorbance at 600 nm of the cultures were routinely measured every hour with a spectrometer (Novaspec II, Pharmacia LKB, Little Chalfont UK), relative to a sterile blank. This was repeated for each temperature condition.

Atomic Force Microscopy - To investigate the morphology during the different stages of growth, a NaioAFM Atomic Force Microscope (AFM) (Nanosurf, Liestal, Zwitserland) was used. Samples of 1 ml were removed from the Hungate tubes and put in Eppendorf tubes. Subsequently, they were centrifuged with a Minispin (Eppendorf, Hamburg, Germany) for 8 min at 5,000 rpm. Afterwards, the supernatant was removed, and the pellet washed with 1 ml sterile deionised water, and the sample was centrifuged again. This step was repeated two more times. Silver paint (Micro to Nano, Haarlem, Netherlands) was used to adhere a mica wafer (10mm Ø, Micro to Nano, Haarlem, Netherlands) to a steel wafer (15mm Ø, Micro to Nano, Haarlem, Netherlands). The fully washed sample was placed in small droplets on a mica wafer and left to airdry. In case the samples could not be prepared immediately, the samples were centrifuged a single time and the supernatant was removed and the pellet was submerged in 1.5 ml of 30% glycerol solution and shortly vortexed for storage in the freezer (-80°C) for conservation. The Tap190AI-G cantilever (Budget sensors, Sofia, Bulgaria) in dynamic mode was used to investigate the sample. Features as length, width, thickness, and the presence of flagella and appendages on the cell were investigated using Gwyddion software version 2.58.

Medium analysis - When the power output of the MFCs started to decrease, 25 ml of spent medium was removed, and 25 ml of new AB minimal medium with a higher concentration of

lactate (80 mM) was added. During this process, the anode chamber was continuously flushed with N_2 gas, and samples of the spent medium were taken and used for further investigation of the morphology of planktonic cells with AFM. Additionally, the medium was also used for measuring the levels of mediators using PTI QuantaMaster 8000 series fluorometer (Horiba Scientific, Kyoto, Japan). For the investigation of the medium using fluorescence spectroscopy, the medium needed to be cell free. The medium samples were centrifuged for 20 min at 12,000 rpm and filtered via 0.22 µm filters to obtain this. Afterwards, the wavelength emission spectrum was recorded between 375-600 nm, at an excitation wavelength of 360 nm to detect the relative amount of redox mediators such as quinone and flavin derivatives [27].

Biofilm analysis - After monitoring the electrical potential of the MFCs for a period of one month, the biofilm on the anode was analysed. The anodes were removed from the anode chamber. The smaller surfaces (0.5X5 cm and 0.5X2.5 cm) of the anode were scraped off with a plastic sterile inoculation loop. The cells on the loop were washed off with sterile deionised water into Eppendorf tubes and centrifuged for 8 min at 5,000 rpm. This sample was later used to investigate the morphology of sessile cells on the anode with AFM. One of the large surfaces (5X2.5 cm) was scraped off and put in Eppendorf tubes with isotonic wash buffer (Appendix 3) for future qPCR analysis. The biofilm left on the other large surface was stained using acridine orange (113 µM), by placing it in staining solution for 5 min [28] [29]. Afterwards the staining was washed off in a buffer solution (Appendix 4) [28]. After removing the electrode from the wash buffer, the two sides on the horizontal surface with the biofilm were gently dried, and nail polish was used to adhere small pieces of a cover glass slide (170 ± 5 µm thick). On top of the placed glass pieces, nail polish was used again to adhere a large full cover glass slide on top of the electrode. This method allowed for the biofilm not to be compressed and wash buffer to be placed in the space between the top glass slide and the electrode surface to avoid the biofilm from drying out. Afterwards the biofilm was investigated using an inverse confocal scanning laser microscope (Zeiss, Jena, Germany), and analysis was done using ZEN 3.3 blue edition.

Statistical analysis - Statistical analysis of electrical output was done in R_studio to investigate whether there are statistically significant differences between the electrical output of the three temperature conditions. Normality was assessed by means of a Shapiro-Wilk test. If the data was normally distributed, a one-way ANOVA was done to investigate if there were significant differences between temperature conditions. Alternatively, a non-parametric Wilcoxon rank sum test was used to unravel statistically significant differences between independent groups and Wilcoxon signed rank test was used to do this within groups, if the data was not normally distributed. All tests were done with a significant level of 0.05 and corrections for multiple testing was done via Hochberg correction.

RESULTS

Growth and morphology - Results obtained from the optical density analysis are presented in figure 2. To achieve complete growth curves, multiple cultures - the amount depending on the temperature condition - were set up with 12-hour time intervals, to account for growth during night, when no measurements could be made. The fastest growth rate was observed at 25°C (slope = 0.0111), where the stationary phase, and an average cell density of 0.3525 was reached after 31 hours. After the lag phase, lasting for approximately 4 hours, an average increase of cell density of 0.3325 took place within a time window of 27 hours (Fig. 2a).

At 15°C, the start of the stationary phase, with an average cell density of 0.21375 was reached after 66 hours. This was almost double the amount of time in comparison with 25°C (slope = 0.0035). The lag phase notably took the same amount of time as observed at 25°C, and an average increase in cell density of 0.18675 in 62 hours took place afterwards (Fig. 2b). For the experiments at 5°C, no large increase in cell density was observed (slope = $2*10^{-5}$), and different growth phases could not be distinguished from one another (Fig. 2c).

Additionally, the size of five randomly selected cells during each growth phase according to temperature was determined (Fig. 3) (Appendix 5). A trend in cell length can be observed during

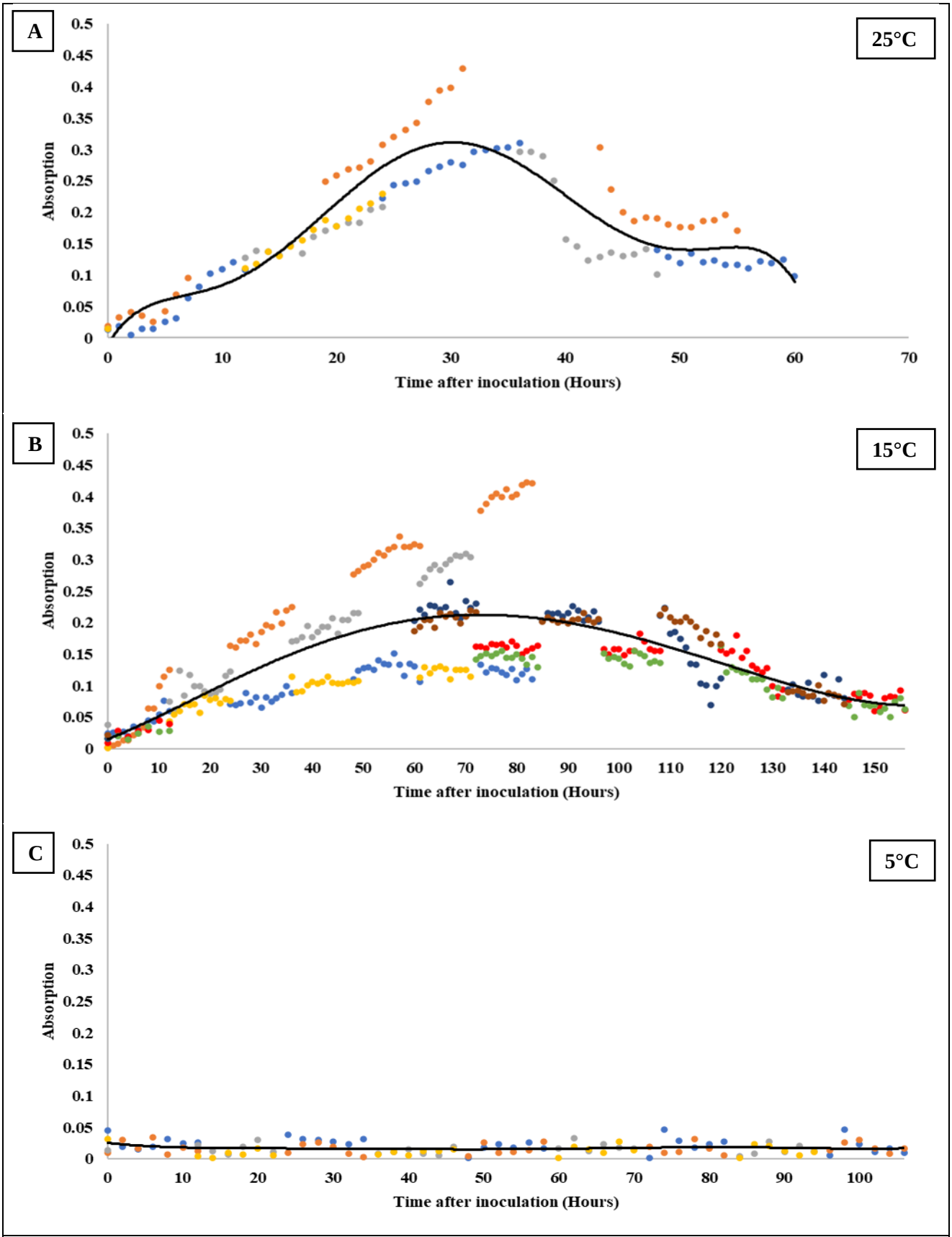

Fig. 2 – The absorption of *S. loihica* cultures in function of time (hours since inoculation), incubated at 25°C (A), 15°C (B), and 5°C (C). This growth curve is obtained from the combination of several cultures, grown at different points in time, with each culture given a distinct colour.

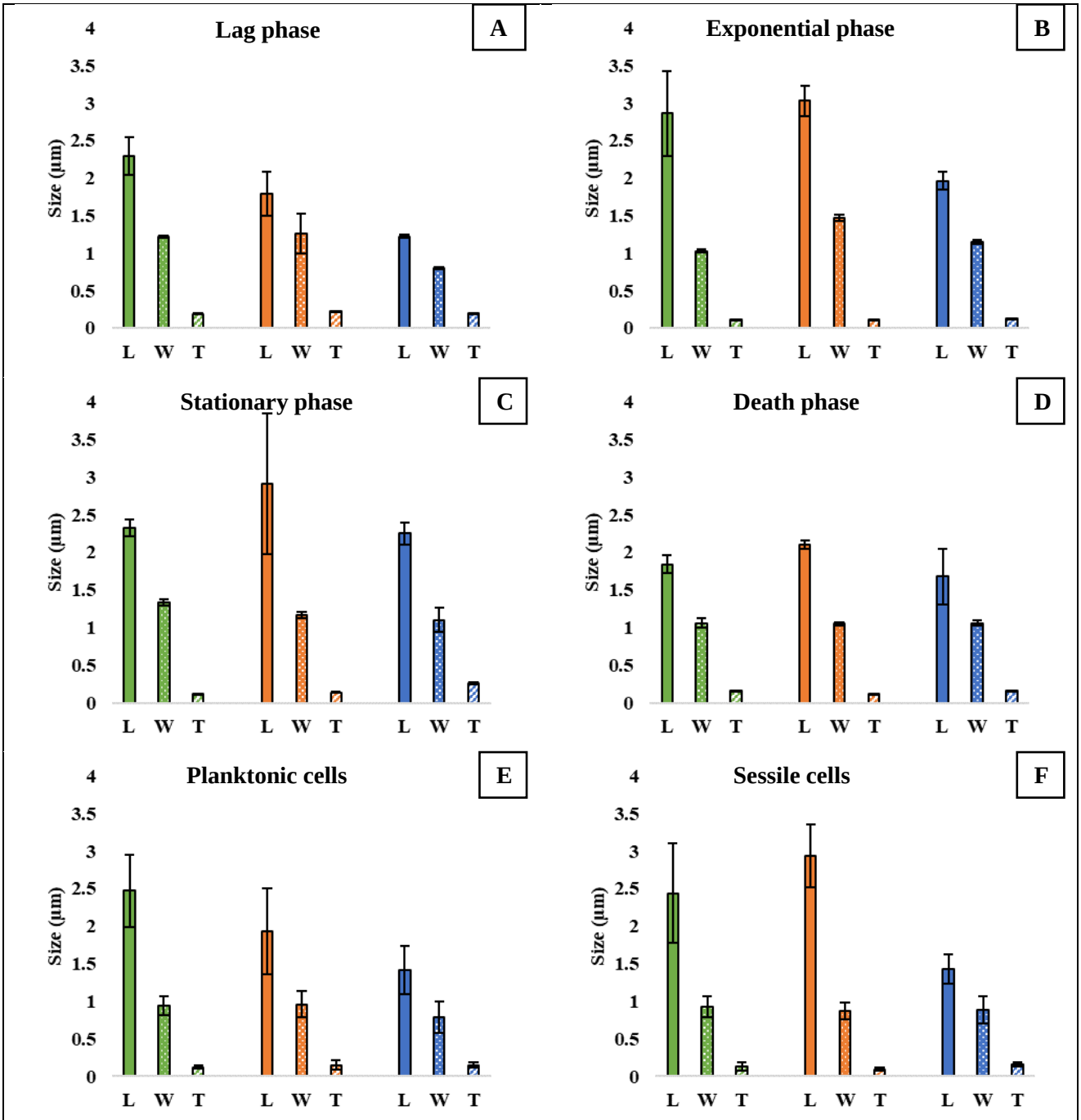

Fig. 3 – The average length (L, solid), width (W, dotted), and thickness (T, striped) (micrometres) of *S. loihica* during the lag (A), exponential (B), stationary (C), and death phase (D), in addition to planktonic cells removed from the medium (E), and sessile cells scraped off from the anode (F) at 25°C (green), 15°C (orange) and 5°C (blue).

growth. In all temperature conditions, cells are at their shortest in the lag phase (Fig. 3a) and death phase (Fig. 3d), and at their largest during the exponential phase, except for cells at 5°C (Fig. 3b). This difference in length is not as pronounced at 5°C, where there is overall very little difference in average length between the different samples.

When comparing the cells cultured at 15°C with those cultures at 25°C, cells at 15°C are on average slightly longer in all phases. Thickness and width did not follow the same trend and only minimal differences between the different growth stages were observed.

The presence of flagella was only observed in a few samples during the exponential phase. Other appendages such as fimbriae on the cell body could be observed in all phases, but not in all samples (Appendix 5) (Appendix 6).

Planktonic cells removed from the medium and sessile cells removed from the biofilm increase in size as the temperature increases. Planktonic cells obtained at 5°C (1.43 ± 0.19 µm) are shorter than the planktonic cells at 15°C (2.04 ± 0.42 µm), which in turn are smaller than those at 25°C (2.44 ± 0.66 µm) (Fig. 3e) (Fig. 4). Similarly, sessile cells were also longest at 25°C (2.47 ± 0.48 µm), followed by 15°C (1.93 ± 0.57 µm) and 5°C (1.41 ± 0.32 µm) (Fig. 3f) (Fig. 4). As with the different growth phases, no notable difference between thickness and width was observed. As expected, some of the planktonic cells at 15°C were observed to have flagella (Fig. 4e), and sessile cells at 25°C were observed to exhibit fimbriae (Appendix 5) (Appendix 6).

MFC chronoamperometry - The electrical potential of the MFCs was monitored for 30 days at 25°C, 15°C, and 5°C (Fig. 5). As none of the variables were normally distributed, even after transforming the data, non-parametric tests were used for further statistical analysis.

The highest maximum potential and the fastest increase in potential was observed at 25°C, where the maximum potential obtained was 74.1 mV in MFC3 after 164 hours (Fig 5). However, this maximum was followed by a fast decline, and no increase was observed afterwards. MFC1 showed the same trend, but the maximum potential obtained was 60.7 mV. The vertical lines in figure 5 indicate when the medium was exchanged to replenish the lactate as an electron donor and as an effort to increase the electrical potential. This caused no increase in electrical output in MFC1 and MFC3, but it did increase the electrical potential in MFC2.

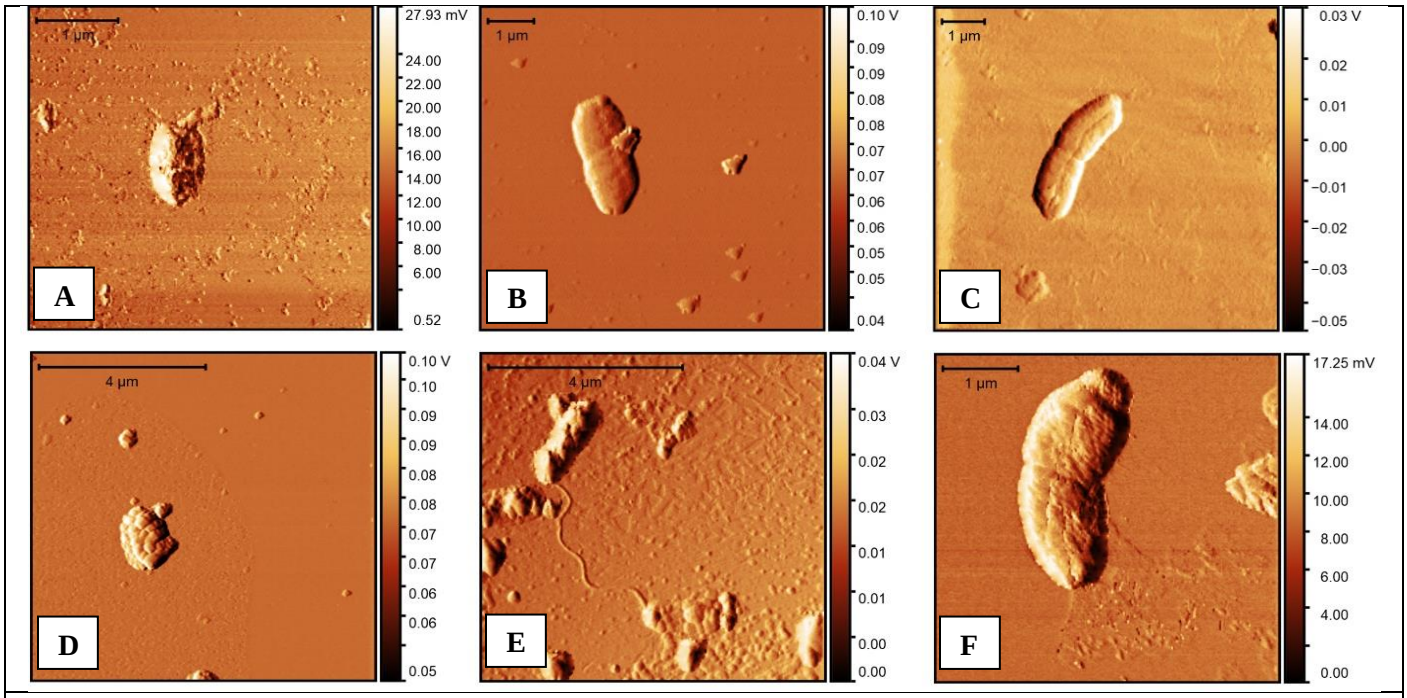

Fig. 4 – AFM images of *Shewanella loihica* sessile cells scraped off the anode after 30 days of MFC operation at 5°C (A), 15°C (B), and 25°C (C), and planktonic cells extracted from the medium after 30 days of MFC operation at 5°C (D), 15°C (E), and 25°C (F). These AFM images were obtained from amplitude modulation mode (volts).

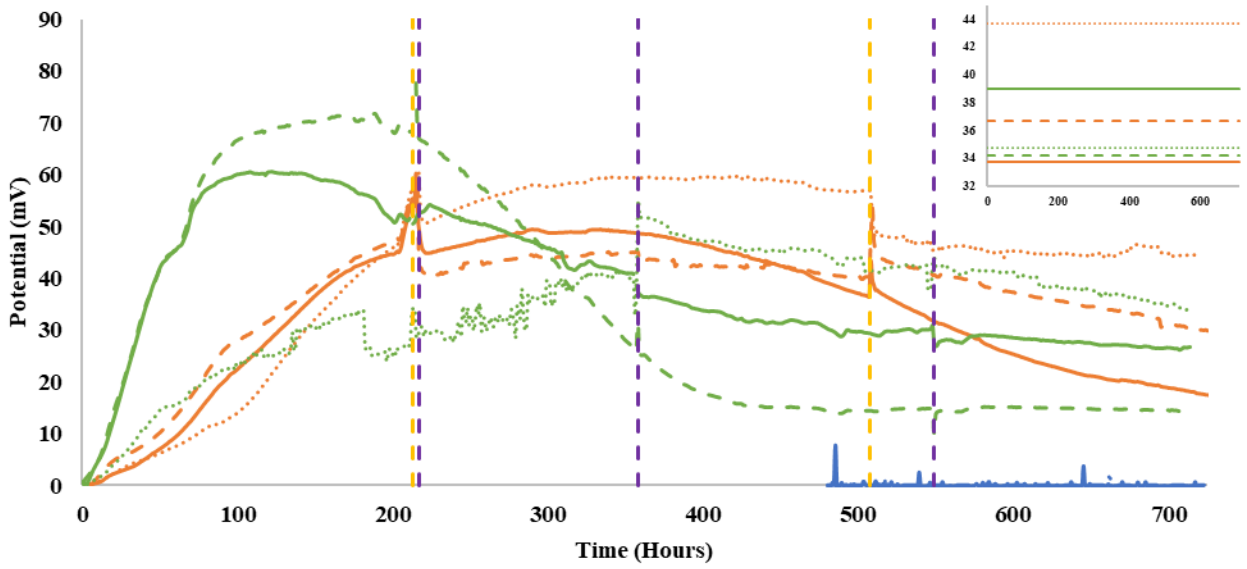

Fig. 5 – The electrical potential (millivolts) between the anode and cathode as a function of time (hours) of MFC1 (solid line), MFC2 (dotted line), and MFC3 (striped line) each inoculated with *S. loihica* and set up at 5°C (blue), 15°C (orange) and 25°C (green). Vertical striped lines indicate the points in time when old substrate was removed and new substrate was added at 15°C (yellow) as well as the MFCs at 25°C (purple). The upper right corner, displays the average electrical potential (millivolts) of the MFCs at 15°C (orange), and 25°C (green) in function of time (hours).

Within the 25°C temperature condition, all MFCs were statistically different from each other ($p<0.05$).

At 15°C, the overall increase in electrical potential occurred at a slower rate than at 25°C, however this was also the case for a decline in electrical potential (Fig. 5). The maximum potential obtained was 60.34 mV in MFC2 after 215 hours following inoculation. In comparison with 25°C, the MFCs at 15°C were more consistent and the maximum values obtained of each MFC showed less variation. Similar as at 25°C, medium exchange took place to try to increase the potential after declines. After the first exchange, the potential continued to increase. However, no increase as a result of the second medium exchange was observed. As in 25°C, all MFCs were statistically different from one another within the 15°C temperature condition ($P<0.05$).

Due to an unforeseen power cut which occurred after the first 20 days of the electrical potential monitoring of MFCs at 5°C, the data of the first 20 days was lost. However, during this period seemingly no electrical potential was produced (Fig. 3), and this did not change the last 10 days when the electrical potential obtained lied on a scale of microvolts. As a result, no medium exchange took place. Nonetheless, statistical analysis indicated that that there was a significant electrical output ($P<0.05$).

When comparing the three groups, it can be observed that all three temperature conditions differ statistically from one another ($p<0,05$). When comparing individual MFCs across the temperature condition, it can be noted that few MFCs between 15°C and 25°C have no statistically significant difference. These are MFC1 of 15°C and MFC2 at 25°C ($P = 0.8375$), and MFC3 at 15°C and MFC1 at 25°C ($P = 0.9379$).

Additionally, to obtain the average long-term electrical potential, the area under the curve was calculated for all MFCs at 15°C and 25°C, and fitted as a straight horizontal line (Fig. 5). Here it can be observed that overall MFCs at 15°C had a higher average long-term electrical output then the MFCs at 25°C. MFC2, which obtained the maximum electrical potential is overall the best performing fuel cell, and had a long-term average of 43.64 mV. This is followed by MFC1 at 25°C,

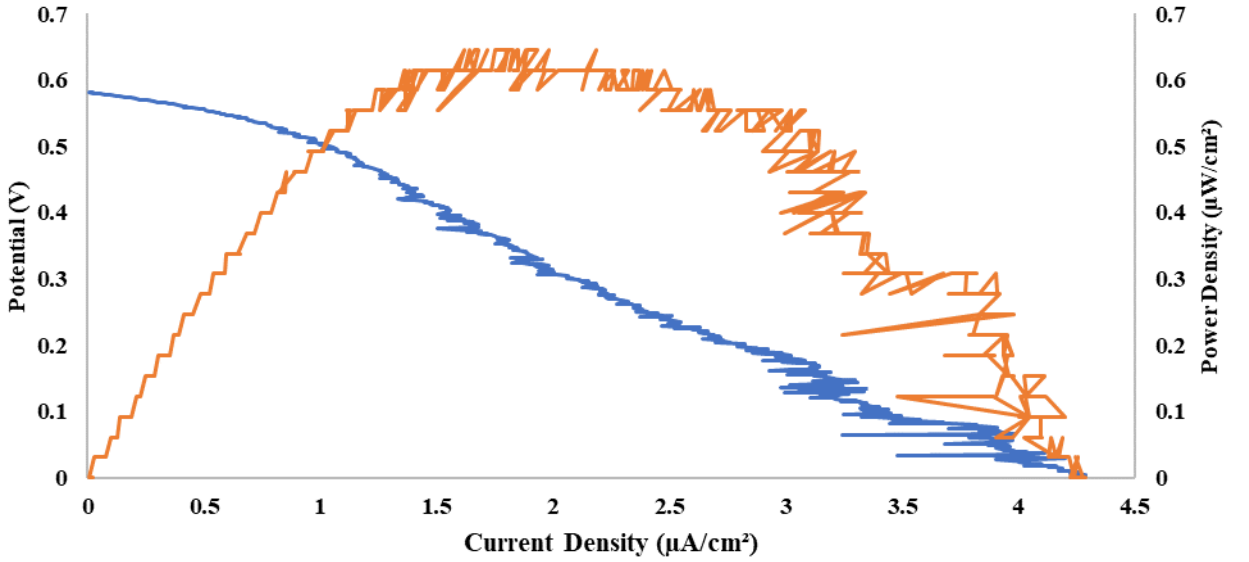

Fig. 6 – Polarisation curve (blue) and power density curve (orange), showing the electrical potential (volts) and power density (microwatts pet cm^2) as a function of the current density (microampere per cm^2) obtained from a single MFC at 25°C.

which achieved a long-term average of 38.98 mV. Interestingly, MFC3 at 25°C, which yielded the highest maximum value was outperformed by all MFCs in its temperature condition including MFC2 and MFC3 at 15°C (Fig. 5).

After 30 days of operation, the MFCs were uncircuited to determine the open circuit potential. Afterwards, polarisation and power density curves were made from a single MFC under each condition. The polarisation and power density curves could only be made at 25°C, because at 15°C and 5°C, the results showed signs of overshooting, and shortcutting. The open circuit potential at 25°C lied at 0.5771 V (Fig. 6). The 15°C temperature condition obtained a similar open circuit potential of 0.5176 V. However, at 5°C the open circuit potential was much lower and lied at 0.002 V.

The polarisation/power density curve at 25°C was made from MFC2 and shows a typical slow decline in the potential with increasing current density, and a steady increase and decrease in power density with increasing current density (Fig. 6). From these results, it could be deduced that at 25°C the maximum power density obtained was 0.64 μWcm^{-2}, and the corresponding short circuit current density amounted to 4.28 μAcm^{-2} (Fig. 6).

Internal resistance was measured from the maximum power point (MPP), where the current output obtained was the highest. At the MMP the external resistance equals to the internal resistance of the MFCs. The MFC at 25°C had an internal resistance of 7.07 kΩ.

Mediated electron transfer - As previously mentioned, when the electrical potential started to decline during the monitoring period, old medium was removed and new medium with electron donor was added as an effort to increase the potential. At 25°C - when the potential quickly decreased - the medium was exchanged three times: at day 9, day 14, and day 22. At 15°C the medium was exchanged only twice: at day 8 and day 20. Because no significant potential was produced at 5°C, there was no need to exchange the medium as enough electron donor was present in the medium. Because of this, the medium was removed after the 30-day measuring period.

At both 25°C and 15°C two peaks were observed. Firstly, a large peak at 440 nm, and secondly, a smaller peak at 520 nm (Fig. 7). In both conditions, it was observed that throughout the monitoring period, the relative quantity of mediators present, started to decline, where the highest intensity of fluorescence signal was obtained from the first medium exchange (Fig. 7). This also coincides with the moment in time when the electrical potential of all MFCs was higher.

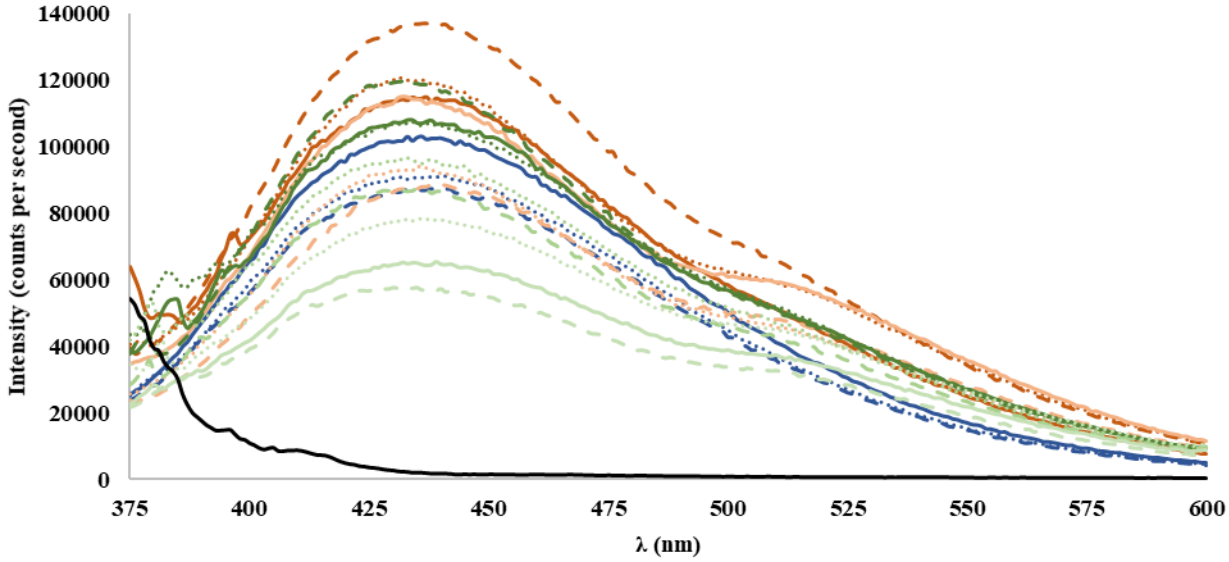

Fig. 7 – The intensity of fluorescence signal (counts per second) relative to the emission wavelength (nanometres), of medium samples of MFCs operating with *Shewanella loihica* obtained at 25°C (green), 15°C (orange), and 5°C (blue), for MFC1 (solid line), MFC2 (dotted line), and MFC3 (striped line) in comparison with a blank (black). Increasing transparency signifies later samples within the same temperature condition.

When comparing the results of the MFC voltammetry to the results obtained from the mediator quantification, it can be perceived that at 25°C, MFC3, which had the highest relative quantity at day 9, is also the MFC which yielded the highest electrical output at that point in time. At the third medium exchange, it can be seen that MFC3 had the lowest electrical potential. Coincidentally, MFC3 had the lowest relative amount of mediators present at day 22.

The highest relative amount of mediators was found at 15°C on day 8 in MFC3. Even though no electrical potential was measured at 5°C, mediators were still present in the medium, albeit in lower quantities than the MFCs from the other temperature conditions at their first medium exchange. However, the second peak that was observed at both 15°C and 25°C at 520 nm was not observed at 5°C (Fig. 7).

Biofilm formation - Biofilm thickness was determined from measuring the thickness of fluorescent signal at six different places across three electrodes within the same temperature condition (Appendix 7). The biofilm that was measured from the images was seemingly largest at 5°C (20.31 ± 7.98 µm), followed by 25°C (5 ± 1.76 µm) and 15°C (4.73 ± 4.02 µm) (Fig. 8).

When looking at the placement of the cells, it can be noted that at 25°C the cells seem more clustered (Fig. 8i) than at 15°C, where the cells are more widely spread over the electrode (Fig. 8e). Individual cells cannot be seen in abundance at 5°C compared to the other temperature conditions (Fig. 8a). However, a strong fluorescent signal is spread over the electrode (Fig. 8c).

When investigating the different layers of the samples, it could also be noted that at 25°C, some cells seemed to move in liquid, which suggest that after staining, some of the cells may have become detached from the electrode surface.

DISCUSSION

The effect of temperature on growth and morphology –When comparing the growth curves obtained at different temperatures with one another, large differences can be observed (Fig. 2). The increase in cell density not only happened faster at 25°C, but the 25°C temperature condition also obtained the overall highest cell density. Even though literature states that psychrotolerant microorganisms have an optimum growth around

15°C, the 15°C temperature condition displayed a lower cell density and slower growth rate [17].

Temperature is one of the few environmental factors that cannot be regulated by bacteria, and internal temperature is equal to that of the environment [14]. Therefore, enzymes involved in all pathways are strongly affected by this parameter. Temperatures below the optimum range for bacteria will in turn slow down cell activity and metabolism, which in turn affects the size and cell replication.

Psychrophiles and psychrotolerant bacteria are especially sensitive to higher temperatures due to their flexible proteins that are more prone to denaturation [14]. However, the results indicate that 25°C is within the temperature margins for *S. loihica* to be able to grow and still remain viable, even accelerating its growth in comparison with 15°C.

At 5°C no increase in cell density is observed, and only very minimal changes in size between different samples could be observed. This suggests that *S. loihica* growth may be abundantly inhibited or even fully inhibited at 5°C, and that this temperature is far too low for *S. loihica* to grow normally. Additionally, cells in the MFCs at 5°C are also very short in length. The average length of the cells changed minimally between the different growth phases at 5°C. However, this could be attributed to the fact that no growth phase could be

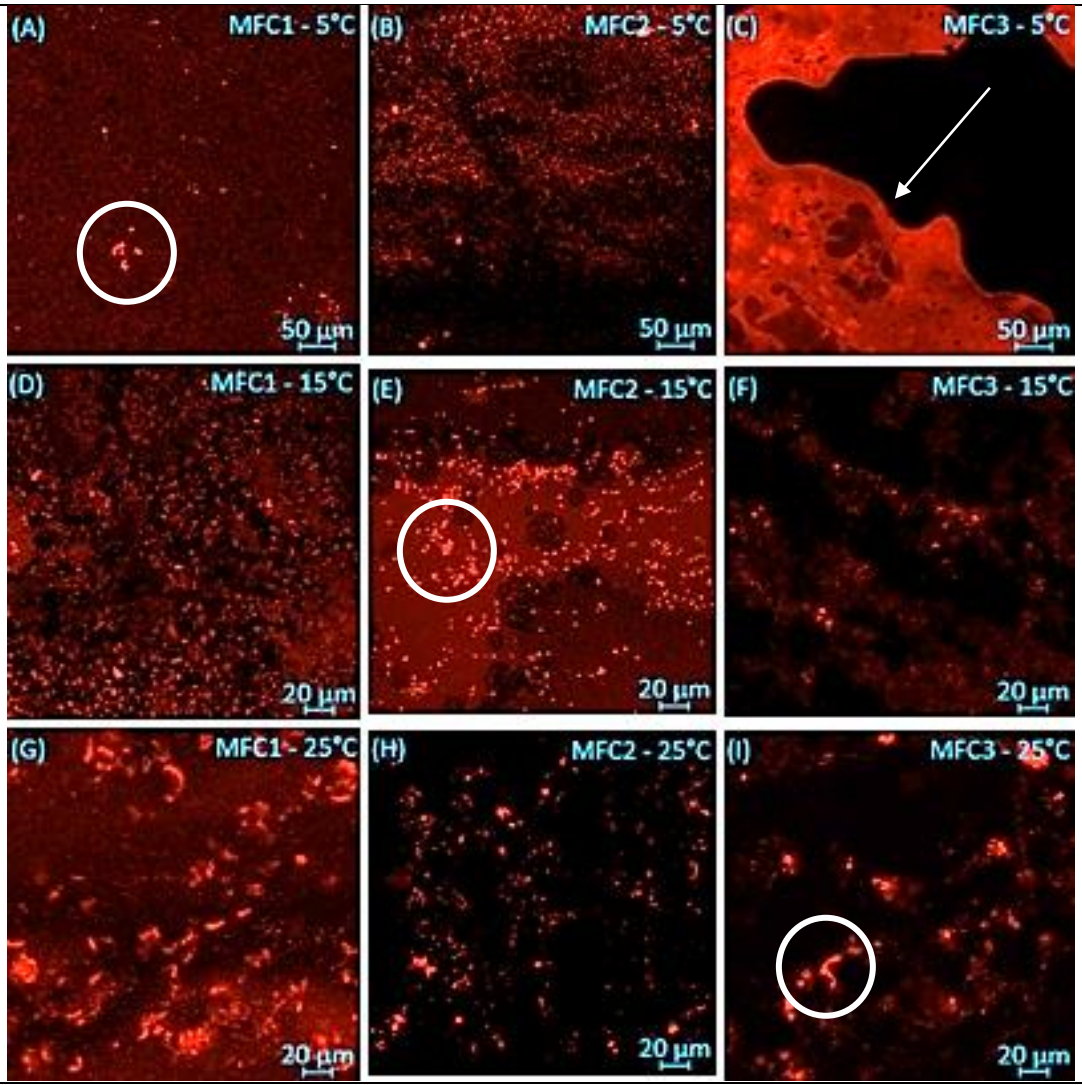

Fig. 8 – Confocal laser scanning microscopy images showing the growth of *S. loihica* on the anode for each MFC included in the study. Blue annotations indicate the MFC and its temperature condition. White annotations emphasise the presence or lack of cells/clusters.

distinguished at 5°C. At 5°C, substrate solubility is very low, which can cause lesser nutrient availability for the cells. This can also strongly affect the size of cells [30].

The reason why mostly the length of the cells changed across all temperature conditions between the different growth phases is most likely due to cell division. During binary fission, cells usually increase in cell length to form 2 distinguished cells [30]. The phenomenon of cell division could also be observed in samples in the exponential phase (Appendix 6).

It is in the exponential phase and stationary phase when the cells are abundant, that they are best used as an inoculum in MFCs to allow for a fast start-up. Because growth happens more rapidly at 25°C, it can be said that this temperature condition lies closest to the optimum culturing temperature of *S. loihica*.

Even though flagella were expected to be observed abundantly in the samples, especially the planktonic cells extracted from the medium as flagella enhance cell motility, only a few cells across all samples exhibited flagella. This could be because of the sample preparation [31]. Samples were also vortexed very shortly to resuspend the pellet to ensure proper washing. However, vortexing sample may also contribute to the detachment of the flagellum from the cell body [32]. However, it should also be noted that flagella are not permanent structures and may be ejected by the cells [33]. This can take place in the stationary phase or during biofilm formation, as a means to conserve energy, or as a result of nutrient depletion. After loss, flagella may be reassembled [34]. Another surface structure observed were fimbriae. Fimbriae found in all gram-negative bacterial species, are long polymeric protein structures on the cell surface used to adhere to surfaces [35]. As expected, these were observed in sessile cells, and not in planktonic cells.

When comparing the results obtained regarding growth with the potential output of the MFCs, it can be seen that the potential increases overall at a slower rate at 15°C than at 25°C. This is most likely due to the delayed growth of the bacteria at lower temperatures. This could also be the reason that there was limited electrical output at 5°C. The cells may possibly have been able to generate a higher electrical potential if there was a higher cell count. However, because of the low temperature coupled with a measuring period of 30 days, the cell count was not able to increase enough in this time window.

The effect of temperature on the electrical performance – Even though the highest maximum electrical potential of 74.1 mV was recorded at 25°C, it was observed that at 15°C the average long-term electrical output was higher in MFC2. This could be explained by higher coulombic efficiency associated with decreasing temperatures [17]. Coulombic efficiency describes the capability of building up charge in a system. This tells us more about the battery life, where a high coulombic efficiency indicated a longer battery life. This was observed at 15°C.

As previously mentioned, when the electron donor started to deplete, old medium was removed and new medium with a higher concentration was added. The removal of old medium also caused the removal of redox mediators present in the medium. To avoid the removal of too much mediators, a higher concentration of lactate was used, to allow for the exchange of little volume. However, the minimal removal of mediators that may have occurred, could have caused a slight decrease of the electron transfer and thus lower the electrical output of the MFCs [11]. However, at 15°C when the medium was first removed, the potential continued to increase, so this may differ between temperature conditions due to different diffusion rate at different temperatures.

In a study by Newton *et. al.* where the electrical output of *S. loihica* was compared with that of *S. oneidensis*, it is noticeable that *S. loihica* had a higher electrical output than obtained in this study [36]. Here, lactate was added to the fuel cells without the removal of old medium. Thus, no removal of mediators took place. However, in this study, it was a necessity to remove medium, for the analysis of redox mediators.

Several other reasons as to why the electrical output was lower, is perhaps because the MFCs established in this study may not have been fully airtight. This was further indicated by pellicle formation in the first weeks after set-up and MFC operation. Pellicles are biofilms that are formed on the air water interface. It is a phenomenon observed in facultative anaerobes [37]. However, this pellicle vanished afterwards. Moreover, the medium had an orange like coloration, which according to Gao *et*

al. happens when the bacteria are exposed to oxic conditions [22].

Even though there was statistically significant electrical potential formed at 5°C, it should be noted that non-parametric tests were used. Non-parametric tests tend to have lower power than parametric tests. This means that there is a high probability of committing type II errors [38].

In an MFC, the open circuit voltage is the potential difference between the anode and the cathode. When no external load is connected to the circuit, no current is generated. This also coincides with the maximum potential that can be obtained from them. In the MFCs, the rate at which the exoelectrogens perform EET will determine the build-up of the potential difference and negative charge at the anode. At 5°C, this took place at a much lower rate, causing the open circuit potential to lie much lower. Additionally, as was observed from the growth curves at 5°C, growth was highly affected by such cold temperatures. The density of cells also affects the open circuit voltage [39].

As with the potential output measure, the results from the MFC at 25°C and 15°C are very similar. In literature, normal open circuit voltage between 0.5 V and 0.6 V have been reported [40] [41]. This means that the open circuit voltage obtained at 15°C and 25°C lie within this margin, and that *S. loihica* was able to increase the charge on the anode adequately at both temperatures.

The non-conforming figures and the reduction in current density with increasing power density at 5°C and 15°C are indications of overshooting. This occurs when exoelectrogens are not able to meet increasing electron demands at a higher resistance [42].

Overshooting should be avoided as it implies an underperformance of the system. There are several causes for this phenomenon. Firstly, this can occur when microbial community in the anode chamber is immature, and not sufficiently expanded. Secondly, another cause of overshooting is a high sample rate during the acquisition of the polarisation and power density curve, which may be too fast for the rate of EET in the bacterial community. Thirdly, this can be explained by insufficient electron donor present in the medium. Lastly, this can be caused by the presence of toxic substances in the anode chamber [42] .

At 5°C, a low cell density and growth was observed. Therefore, overshooting is most likely caused by the lack of a developed microbial population at the anode. This can further be noticed by minimal biofilm formation which occurred at 5°C (Fig. 8a). Because the power density curve also overshot at 15°C, but the confocal laser scanning microscopy showed that biofilm formation was similar as in the 25°C temperature condition, it could be assumed that the process of EET did not occur at a high sufficient rate to keep up with the high sample rate. In other words, the metabolism at 15°C is slowed down noticeably enough to affect the acquisition of polarisation and power density curves. This claim is further solidified by the results obtained from the optical density analysis that demonstrated that growth was slowed down significantly at 15°C in comparison with the 25°C temperature condition (Fig. 2b). This was also seen at the electrical output at 15°C, where it took more time for the maximum electrical potential to be reached (Fig. 5), both indicating a decreased metabolic rate at 15°.

At 25°C, there were no indications of overshooting, which indicate a microbial community that is able to keep up with the electron demand at higher resistance.

The internal resistance is an important parameter, as according to the maximum power theory, an MFC that is equipped with an external resistance equal to that of the internal resistance, will obtain the maximum power output [43]. Moreover, the internal resistance tells us more about the overall power output, where higher internal resistance predicts lower power output [43]. Internal resistance is affected by many parameters in MFCs. For example the spacing between electrodes, or the type of bacterial species and their metabolism used [43]. Moreover, increased biofilm formation is also associated with a lower internal resistance [42]. The internal resistance of the MFC at 25°C is comparable to that found in literature [43] [44].

The effect of temperature on mediated EET – As previously mentioned, two peaks were observed from the results obtained from fluorescence spectroscopy. The first peak at 440 nm, coincides with the fluorescence emission spectra of quinone derivatives. The second small peak observed at 520 nm, coincides with the fluorescence emission spectra of flavin derivatives. The presence of these quinone derivatives and flavin derivatives further

confirm that *S. loihica* is capable of long distance mediated EET.

Redox mediators have a large contribution to the electrical output of MFCs [45]. This is also perceived at 25°C, where the highest relative amount of mediators was found in MFC3, which is also the MFC with the highest electrical potential relative value. This suggests that there is a direct relation with the relative amount of mediators and electrical potential. MFC3 also had the lowest electrical output at day 22, and also the lowest relative amount of mediators. Because of this observation, and an overall decrease in the electrical output paired with a decrease in relative amount of mediators, this claim is further strengthened.

At 15°C the MFC with the highest relative amount of mediators does not coincide with the highest electrical output at different points in time. However, the overall highest relative amount of quinone derivatives and flavin derivatives was found at 15°C (day 8) in MFC2 and MFC3. Coincidentally, these MFCs had the highest average long-term electrical output. Nonetheless, at 15°C the solubility in aqueous media is lower than at 25°C, which can affect the diffusion of mediators in the medium. Despite that, this could also indicate the importance of direct EET, and biofilm formation at lower temperatures.

Even though the levels of quinone derivatives are comparable at 25°C (day 9) and 5°C, the reason could be that they are synthesised. However, because of low solubility at lower temperatures in aqueous media, no transportation of electrons took place. No peak around 520 nm was observed at 5°C. This indicates that flavin derivatives are absent and may disclose that flavin derivatives are not synthesised at lower temperature. The synthesis of redox mediators has never been studied for different temperatures. These results suggest differential synthesis of redox mediators at different temperatures.

The effect of temperature on biofilm formation – In all anode samples, it could be observed that cells were present on the anode, suggesting that at all three temperatures, sessile cells grow on the anode. Similar thicknesses of biofilm between 25°C and 15°C were observed. Seemingly, the thickest formation was unexpectedly at 5°C. However, because of the negligible electrical output at 5°C, this is most likely due to an inferior sample preparation. When the samples were prepared, the electrodes were submerged in acridine orange dye, and afterwards put in a washing buffer. Even though extra precaution was taken, and an extra washing step was implemented, the washing buffer present on the sample to maintain a hydrated biofilm could still have some loaded dye. Between the electrode and the top glass slide there is also 170 µm of negative space. If this is the case, fluorescent signal of dye loaded washing buffer could interfere with the fluorescent signal originating from biofilm cells.

5°C showed low anode coverage in comparison with 25°C and 15°C and only a small amount of cells were observed on the anode (Fig. 8a). At 15°C and 25°C, individual cells could be seen in abundance. When taking into account the growth of the species at different temperatures, it is expected that 5°C did not have high anodic cell coverage.

Notably, at 15°C, the anode with the highest coverage and biofilm thickness within its temperature condition was obtained from MFC2. This was also the MFC with the highest average long-term electrical output (Fig 8e). This implies the importance of biofilm formation on the electrical output of MFCs.

CONCLUSION

This study was first in determining the effect of temperature on pure cultures of PTEs, to establish whether they can be used for low temperature MFC operation. The results revealed the first indications that PTE species *S. loihica* can be used to expand the operational temperature window of MFCs towards lower temperatures. Similarities in electrical potential were found at 25°C and 15°C, with the highest achieved maximum electrical potential obtained at 25°C, but the highest long-term average potential obtained at 15°C. Additionally, at 15°C, the highest relative amount of mediators was found after the first sample removal, and the presence of quinone and flavin derivatives was detected. At 5°C, no flavin derivatives were recorded, suggesting the lack of synthesis when the environment is below optimum temperatures. Other similarities between the 25°C and 15°C temperature conditions were found in biofilm formation since both temperature

conditions yielded similar thicknesses. Differences were more noticeably in the placing of the cells, where cells were more clustered at 25°C, and more spread at 15°C. The largest difference between 25°C and 15°C was observed during growth. Growth was strongly affected by decreasing temperatures, almost doubling the culture time when lowering the temperature from 25°C to 15°C. However, when cultured in Hungate tubes, cells were slightly longer at 15°C in comparison with 25°C, and shortest at 5°C.

The results confirm the hypothesis that decreasing temperatures to an extent allows for a higher electrical output from MFCs using *S. loihica*. It was also noticeable that at 5°C MFCs could not function properly, indicating that this temperature is too low. This further confirms that *S. loihica* has limited adaptations to cold and is indeed psychrotolerant.

To determine if the psychrotolerant characteristics of *S. loihica* overcompensate for low temperature MFC operation in comparison with mesophiles, further comparison with *S. oneidensis* needs to be carried out. Additionally, to work towards increasing the operational temperature window of MFCs in addition to an increased power output, future studies should focus on the characterisation and use of extremophilic exoelectrogens in defined mixed cultures.

Using different forms of sustainable energy is a necessity in order to reduce the burden on the environment. This study aids in this by extending the working temperature of MFCs. An increased temperature window of operation allows for more possible MFCs applications. Utilising green energy where necessary eliminates unnecessary contamination, ultimately benefitting all.

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Author contributions – Both Thessa Van Limbergen and I conceived and designed the project, and I performed experiments and data analysis, under guidance of Thessa Van Limbergen, and the whole X-LAB research group. I wrote the paper, which was closely reviewed and edited by Thessa Van Limbergen and Prof. dr. Jean Manca.

Appendix 1:

Luria Bertani broth ingredients:

- Tryptone (10g)
- NaCl (10g)
- Yeast extract (5g)

Appendix 2:

AB minimal medium ingredients:

- NH_4Cl (2g)
- Na_2HPO_4 (7.31g)
- KH_2PO_4 (7.85g)
- NaCl (3g)
- Na_2SO_4 (0.011g)
- $MgCl_2.6H_20$ (0.2g)
- CaCl (3.625g)
- $FeCl_3.6H_2O$ (0.2g)
- Lactate (20mM)

Appendix 3:

Isotonic wash buffer ingredients:

- Morpholinepropanesulfonic acid (4.1895 g)
- $NaH_2PO_4.H_2O$ (0.6003 g)
- KCl (0.1g)
- NaCl (5g)
- $MgSO_4.7H_2O$ (0.3007g)
- $CaCl_2.2H_2O$ (0.0103g)

Appendix 4:

Buffer solution ingredients:

- NH_4Cl (0.31g)
- KCl (0.13g)
- $NaH_2PO_4.H_2O$ (2.69g)
- Na_2HPO_4 (4.33g)

Appendix 5:

Table 1 – Size of *S. loihica* according to temperature during different growth phases, and forms in MFCs.

25°C					15°C					5°C				
Lag														
Length (µm)	**Width (µm)**	**Thickness (µm)**	**Flagellum**	**Fimbriae**	**Length (µm)**	**Width (µm)**	**Thickness (µm)**	**Flagellum**	**Fimbriae**	**Length (µm)**	**Width (µm)**	**Thickness (µm)**	**Flagellum**	**Fimbriae**
2.83	1.08	0.09	no	no	1.26	0.84	0.17	no	no	1.14	0.63	0.21	no	no
1.92	1.38	0.3	no	no	1.3	1.01	0.31	no	no	1.42	0.89	0.2	no	no
2.9	1.35	0.24	no	no	2.75	1.98	0.2	no	no	1.2	0.69	0.155	no	no
2.17	1.14	0.025	no	no	1.93	1.75	0.3	no	no	1.03	0.88	0.19	no	no
1.63	1.15	0.27	no	no	1.73	0.73	0.152	no	no	1.29	0.89	0.23	no	no
2.29 ± 0.25	1.22 ± 0.01	0.19 ± 0.01	/	/	1.79 ± 0.29	1.26 ± 0.26	0.23 ± 0.004	/	/	1.22 ± 0.02	0.8 ± 0.01	0.197 ± 0.001	/	/
Exponential														
1.5	0.82	0.2	no	no	3.63	1.45	0.133	yes	no	1.64	0.97	0.19	no	no
3.33	1.3	0.075	no	yes	2.9	1.72	0.12	no	no	2.1	1.49	0.062	no	no
2.67	1.01	0.09	no	no	2.77	1.56	0.102	no	no	1.58	1.07	0.14	no	no
3.17	1.04	0.104	no	no	2.41	1.51	0.065	no	no	1.95	1.09	0.12	no	yes
3.62	1	0.097	no	no	3.46	1.09	0.135	no	no	2.52	1.12	0.13	no	no
2.86 ± 0.56	1.03 ± 0.02	0.11 ± 0.002	/	/	3.03 ± 0.2	1.47 ± 0.04	0.11 ± 0.001	/	/	1.96 ± 0.12	1.15 ± 0.03	0.13 ± 0.002	/	/
Stationary														
2.12	1.47	0.08	no	no	4.32	1.24	0.15	no	no	2.29	1.09	0.18	no	no
2.35	1.2	0.1	no	no	3.66	1.48	0.17	no	no	1.91	0.7	0.41	no	no
2.54	1.28	0.1	no	no	2.8	1.06	0.16	no	no	1.93	1.54	0.24	no	no
1.83	1.08	0.075	no	no	1.94	1.22	0.14	no	no	2.97	1.55	0.35	no	no
2.81	1.64	0.25	no	no	1.83	0.86	0.1	no	no	2.16	0.61	0.14	no	no
2.33 ± 0.11	1.33 ± 0.04	0.12 ± 0.004	/	/	2.91 ± 0.93	1.17 ± 0.04	0.14 ± 0.001	/	/	2.25 ± 0.15	1.1 ± 0.16	0.26 ± 0.01	/	/
Death														
1.76	1.34	0.23	no	no	1.84	0.88	0.11	no	no	1.88	1.1	0.22	no	no
1.64	1.27	0.15	no	no	2.26	1.19	0.115	no	yes	2.78	1.19	0.1	no	no
2.35	0.97	0.12	no	no	2.37	1.19	0.113	no	yes	1.22	0.74	0.14	no	no
2.09	1.07	0.1	no	no	2.27	1.08	0.12	no	yes	1.32	1.16	0.12	no	no
1.36	0.67	0.18	no	no	1.76	0.93	0.14	no	yes	1.19	1.09	0.22	no	no
1.84 ± 0.12	1.06 ± 0.06	0.16 ± 0.002	/	/	2.1 ± 0.06	1.05 ± 0.02	0.12 ± 0.0001	/	/	1.68 ± 0.37	1.06 ± 0.03	0.16 ± 0.003	/	/
Sessile cells														
2.18	1.14	0.13	no	no	1.94	1.16	0.11	no	no	1.29	0.73	0.15	no	no
1.94	1.02	0.14	no	no	2.66	1.08	0.10	no	no	1.70	0.61	0.12	no	no
3.43	1.05	0.14	no	no	2.75	0.89	0.16	no	no	1.43	0.76	0.18	no	no
2.46	0.91	0.09	no	no	2.52	0.94	0.13	no	no	1.41	0.85	0.13	no	no
1.71	0.81	0.14	yes	no	1.21	0.67	0.15	no	no	2.09	1.27	0.19	no	no
2.36	1.04	0.15	no	no	1.90	0.74	0.07	no	no	0.97	0.60	0.08	no	no
2.84	0.79	0.13	no	yes	1.40	1.11	0.17	no	no	1.15	0.64	0.18	no	no
2.74	0.79	0.12	no	no	1.19	0.89	0.21	no	no	1.09	0.64	0.14	no	no
2.55	0.90	0.07	no	no	1.82	1.17	0.30	no	no	1.52	0.99	0.19	no	no
2.47 ± 0.48	0.94 ± 0.12	0.12 ± 0.02	/	/	1.93 ± 0.57	0.96 ± 0.17	0.15 ± 0.06	/	/	1.41 ± 0.32	0.79 ± 0.21	0.15 ± 0.04	/	/
Planktonic cells														
2.05	0.97	0.18	no	no	2.78	0.85	0.10	no	no	1.73	1.22	0.13	no	no
2.98	1.03	0.12	no	no	1.59	1.03	0.09	no	no	1.49	1.03	0.12	no	no
2.22	0.94	0.09	no	no	2.15	0.82	0.09	no	no	1.42	0.80	0.12	no	no
3.06	1.16	0.09	no	no	2.08	0.69	0.14	yes	no	1.49	0.59	0.15	no	no
1.80	0.77	0.10	no	no	1.79	0.80	0.08	no	no	1.05	0.75	0.16	no	no
1.29	0.70	0.08	no	no	2.11	0.85	0.08	no	no	1.37	1.02	0.17	no	no
3.38	1.07	0.27	no	no	1.69	0.77	0.06	no	no	1.58	0.78	0.22	no	no
3.02	0.85	0.12	no	no	1.52	0.99	0.09	no	no	1.20	0.81	0.17	no	no
2.12	0.81	0.11	no	no	2.67	1.03	0.08	yes	no	1.55	0.95	0.20	no	no
2.44 ± 0.66	0.92 ± 0.14	0.13 ± 0.06	/	/	2.04 ± 0.42	0.87 ± 0.11	0.09 ± 0.02	/	/	1.43 ± 0.19	0.88 ± 0.18	0.16 ± 0.03	/	/

The length, width, and thickness (micrometres) of *Shewanella loihica* in function of temperature during the lag, exponential, stationary, and death phase and of planktonic cells extracted from medium and sessile cells from the anode.

Appendix 6:

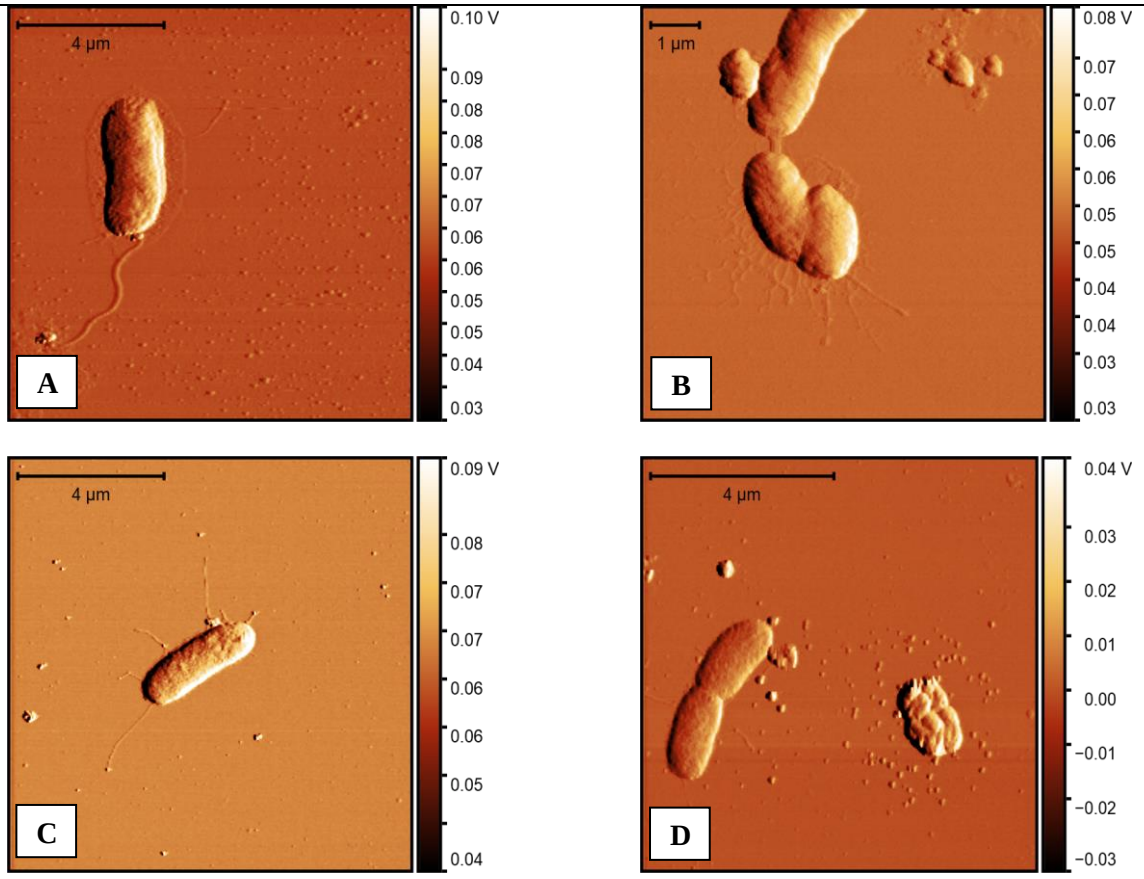

Fig. 9 – AFM images of *S. loihica* cells in the exponential phase exhibiting a flagellum (A), in the death phase exhibiting Fimbriae (B), in the exponential phase exhibiting fimbriae (C), and during cell division in the exponential phase (D). Image (A) and (B) were obtained at 15°C, and image (C) and (D) at 25°C. These AFM images were obtained from amplitude modulation mode (volts).

Appendix 7:

Table 2 – Biofilm thickness of *S. loihica* on the anode according to different temperatures.

Biofilm Thickness (µm)		
25°C	15°C	5°C
8.6	2.66	12.4
5	1.53	24.8
3.6	13.2	35.8
3.4	5.55	13.6
5.4	1.8	17.6
4	3.6	17.7
5.00 ± 1.76	4.72 ± 4.02	20.32 ± 7.98

The thickness of anodic biofilm of *S. loihica* (micrometres) in function of temperature.