Biofilm Treatments with Electric Currents

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ABSTRACT

The Problem: Biofilms are a community of bacteria that cause infections which are resistant to the immune system and antimicrobial treatments, posing a significant threat for patients with implantable and indwelling medical devices.

Purpose: The purpose of this research is to effectively treat biofilms utilizing electric currents assisted by antibiotics.

Method: Evaluated the impact of direct electric current with or without vancomycin against *Staphylococcus epidermidis* biofilms.

Results: **(1)** Electric current reduced the *S. epidermidis* biofilm and **(2)** increased the effectiveness of vancomycin. **(3)** Older biofilms had an increased resistance to vancomycin treatments. **(4)** Higher electric current intensities and **(5)** longer duration treatments were more effective against biofilms.

Conclusion: Electric current increased the effectiveness of vancomycin against *S. epidermidis* biofilms.

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Symbol, Abbreviation, or Acronym	Description
A	Ampere
c-	Centi-
C	Celsius
d	Day
g	Gram
h	Hour
Hz	Hertz
I	Current
k	Kilo
L	Litre
log	Logarithmic
m-	Milli-
m	Metre
M-	Mega-
min	Minute
n-	Nano-
pH	Power of hydrogen (acidity or alkalinity of a solution)
ppm	Parts per million
R	Resistance
RPM	Revolutions per minute
s	Second
V	Voltage or Volts
µ-	Micro-
Ω	Ohm
º	Degree
%	Percent
“	Inch
/	Divide
x	Multiply
+	Add or Positive
-	Subtract or Negative
=	Equal to
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
^{2}	Squared
$_{10}$	Base of 10
$_{600}$	600 nanometres
A	Antibiotic Only Treatment (when defined in Figures and Tables)

A+EC1	Antibiotics with 22 µA DC Electric Current Treatment
A+EC2	Antibiotics with 333 µA DC Electric Current Treatment
AC	Alternating Current
ATCC	American Type Culture Collection
CDC	Centre for Disease Control
CFU	Colony Forming Unit
CI	Confidence Interval
Control	No Antibiotics and No Electric Current
DC	Direct Current
ddH_2O	Double Distilled Water
ddH_2O+NaOCl	Sodium Hypochlorite
dil.	Dilution
DTK	Dunnett-Tukey-Kramer
EC1	22 µA DC Electric Current Treatment
EC2	333 µA DC Electric Current Treatment
ITO	Indium-tin-oxide
LB	Luria-Bertani
MIC	Minimum Inhibitory Concentration
n	Sample Size
NaCl	Sodium Chloride
OD	Optical Density
PEMF	Pulsed Electromagnetic Field
S. epidermidis	*Staphylococcus epidermidis*
TET	Transcutaneous Energy Transfer
TSB	Tryptone Soy Broth
TSBg	Tryptone Soy Broth Supplemented with Glucose
TSBgg	Tryptone Soy Broth Supplemented with Glucose and Glycerol
UV	Ultraviolet

CHAPTER 1

1.0 INTRODUCTION

Bacteria have been around even before humans had the ability to observe, analyze, and understand them. They have been under constant research in the scientific community due to the infectious properties that they possess. There are two fundamental types of bacterial growth, planktonic and sessile growth [1]. Planktonic bacteria are freely floating, single cells while sessile bacteria, also known as biofilms, are groups of microorganisms that adhere to and grow on a variety of surfaces [1]. The characteristic of bacteria aggregating and forming communities was first observed and defined as a biofilm by Costerton et al. in 1978 [2] and has been a field of great interest. The cells in a biofilm have the capability of protecting themselves from the host's immune defense as well as a wide variety of antibiotics [1, 3].

Biofilms have been of major concern in a clinical setting because of their ability to cause persistent infections [4]. Approximately two thirds of bacterial infections are caused by biofilms including cystic fibrosis [5], urinary tract infections [6], and tuberculosis [7]. In relation to medical devices, biofilms pose a major threat for implanted devices such as orthopaedic [8] and cardiovascular implants [9, 10] and usually results in the removal of the device. This is due to the ineffectiveness of the patient's immune response and antibiotic treatment methods against biofilms [1, 3, 9]. There are a variety of approaches that researchers are taking to find an effective means of controlling and treating the biofilm infections, however, this thesis will focus on the idea of using electrical effects to prevent and treat the biofilm.

The research on the impact of electrical methods against biofilms focuses on the electricidal effect [11-28] and the bioelectric effect [29-43]. The electricidal effect only uses an electric current / electromagnetic field to prevent or eradicate the biofilm while the bioelectric effect uses an electric current / electromagnetic field in combination with antimicrobial agents or the host's defense mechanisms against the infection. It is also important to note that freely floating (planktonic) bacteria are also susceptible to these electric currents / electromagnetic fields alone [44-50] and in combination with antibiotics [51, 52].

In order to establish an understanding of the current state of research for this thesis, a peer-reviewed literature search concerning the prevention and treatment of biofilms using electrical effects was conducted using a variety of databases and the relevant experimental studies found are included in the literature review. The scope of the review is very broad and it will provide an overview of the instrumentation, materials, and methodology used to study the electrical effects on biofilms. The gap in knowledge will be addressed and potential applications in a healthcare setting will be explained in a general fashion. For this thesis, a set of experiments with specific objectives were implemented to observe novel results in this field of research to obtain a better understanding of the impact of electrical treatments on biofilms. The materials, methods, results, discussion, contributions, conclusions, and future work are all explained in detail throughout this thesis.

To progress electrical methods of prevention and treatment of biofilms into the clinical setting will take time, considerable financial resources, and sustained commitment from a variety of researchers with diverse backgrounds, though it is believed

that the rewards will by far outweigh the investment. The accumulation of this knowledge and the integration of these efforts can lead to the expansion of effective biofilm infection treatments, a drastic reduction of healthcare costs, and most importantly, it will improve the quality of life for a vast number of patients.

1.1 Objectives and hypotheses

The impact of a direct electric current (DC) with or without the antibiotic vancomycin on *Staphylococcus epidermidis* biofilm will be studied. The objectives of this thesis are to address the following questions:

1. Does the electric current impact the biofilm alone and/or increase the effectiveness of the antibiotic?
2. Does biofilm age impact the effectiveness of treatments?
3. What intensity of electric current tested is most effective against biofilms?
4. What duration of treatments tested is most effective against biofilms?

The overall goal is to provide data and results that can demonstrate the impact of electric current with or without vancomycin on *S. epidermidis* biofilms. It is expected that specific parameters will show a more significant impact on the development of the biofilm when comparing treatments to the control. It is also hypothesized that the electric current will increase the effectiveness of the antibiotic.

1.2 Rationale and scope

Biofilms are communities of bacteria that are resistant to both the immune system and antimicrobial treatments [3, 4]. Biofilms have been of major concern in a clinical

setting because of these properties and pose a significant threat for a variety of implantable and indwelling medical devices [4]. The removal of the device is the most effective treatment to date for biofilm infections due to the inability to treat the patient non-invasively. As such, alternative methods to prevent and treat biofilm formations are a necessity.

The parameters chosen for this thesis have not been studied in previous research. The *in vitro* impact of electric current was evaluated against a specific bacterium, *S. epidermidis*, known to be one of the most common bacteria associated with infections of medical devices [53-55]. The biofilm was formed on medical grade titanium and treated with electric current using medical grade stainless steel electrodes which are both commonly used in implantable medical devices [56]. The antibiotic utilized was vancomycin, which is used very frequently in the clinical setting [57-59].

Previous *in vitro* studies have not shown an impact of electric currents or electromagnetic fields on the effectiveness of vancomycin when compared to treatments with the antibiotic alone against *S. epidermidis* [38, 41]. Thus, this thesis provides more knowledge on the impact of variables chosen to treat a specific biofilm. This thesis used clinically relevant parameters to assess the impact of intensity of the electrical current, biofilm age, and duration of treatments. The overall purpose of this research is to prevent and treat biofilm infections with electrical currents in combination with antibiotics and host defense mechanisms.

1.3 Organization of thesis

The organization of this thesis is as follows. Chapter 1 is the introduction where the objectives, hypotheses, rationale, and scope are addressed. Chapter 2 provides an overview of all related literature concerning the use of electrical effects for the prevention and treatment of biofilms. Chapter 3 outlines the materials and methods of the experimental portion of this thesis. Chapter 4 organizes the data from the experimental process and states the results. Chapter 5 includes the discussion of results, limitations of the experiments, and novel contributions as well as incorporates the conclusions of this thesis with existing literature while addressing the potential for future work. In addition, Figure 1 provides a timeline associated with this thesis and the individuals involved.

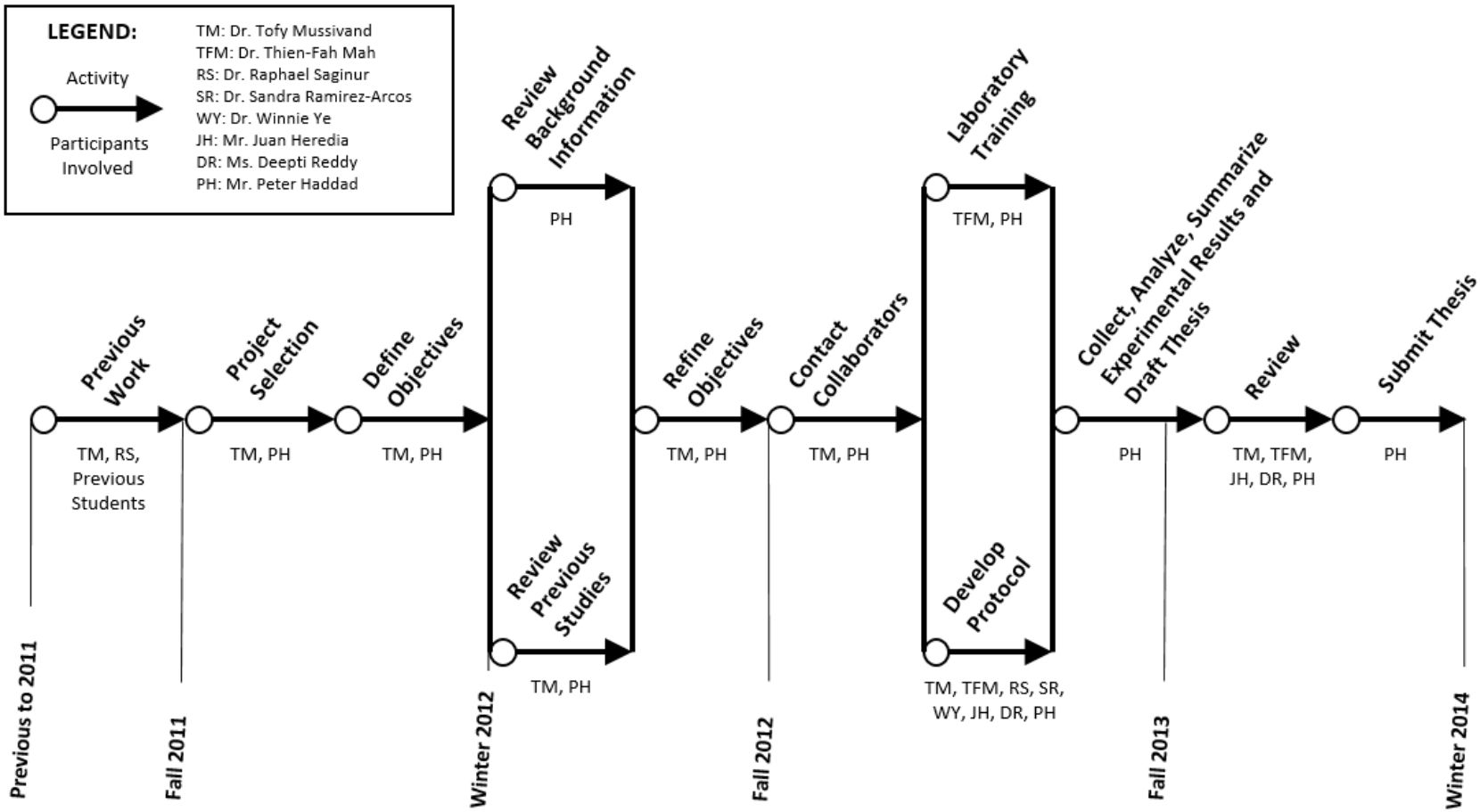

Figure 1: Timeline of thesis with individuals involved indicated.

CHAPTER 2

2.0 LITERATURE REVIEW: THE USE OF ELECTRICAL EFFECTS FOR THE PREVENTION AND TREATMENT OF BIOFILMS

Biofilms have been shown to be affected by electric currents as well as electromagnetic fields. A large portion of the research focuses on the electricidal effect (electric current / electromagnetic field only) [11-28] and the bioelectric effect (electric current / electromagnetic field with the help of antibiotics and/or the host's defense mechanisms) [29-43]. A full systematic review of the literature on the bioelectric effect has been conducted by del Pozo et al. in 2008 and proves to be a great resource [60]. A more current review by Freebairn et el. in 2013 has very effectively outlined the state of this current field of research with a focus on the electrical engineering aspects [61]. Where this review will differ from the above mentioned review articles is that it will focus on comparing and categorizing the choice of parameters and the effects of this selection on the results. The methodology of experiments will be compared to determine which approaches seem more practical and effective at yielding conclusive results. The potential applications in healthcare and patient safety will be brought forth as well.

2.1 Characteristics and complications of biofilms

There are five stages in the biofilm lifecycle; initial adherence, irreversible adherence, microcolony formation, maturation, and detachment [62]. Biofilms, whether beneficial or detrimental, are able to defend themselves against shear forces, deprivation of nutrients, pH changes, biocides, oxygen radicals, and antibiotics much better than planktonic cells [62, 63]. It has been documented that antibiotics are 500-5,000 times

more effective in killing bacteria in the planktonic state than in a biofilm of the same microorganism [3, 64]. In addition, biofilms are able to tolerate the host immune system, making them difficult to eradicate [63]. Biofilms when mature, are chronic in nature and even though their mechanisms of defense are not entirely understood, they are hypothesized to be due to a combination of slow metabolic processes, heterogeneity, and the expression of biofilm-specific resistance genes [3, 63].

Biofilms can be considered multi-cellular organisms due to the fact that they act as a cooperative community and that they utilize the surrounding environment to their benefit by making use of the nutrients that are available to them [62, 63]. They are able to adapt and modify their metabolic properties as a whole when situated in a specific location [62, 63] which gives them the ability to grow in a variety of settings. Bacterial cells are able to adhere to a multitude of surfaces. In relation to implantable medical devices, they utilize platelets and/or plasma proteins that are known to influence bacterial adhesion [65, 66]. These properties of biofilms make them a difficult target for attack which in turn causes them to be highly problematic in the clinical setting. Therefore, effective prevention and treatment methods must be researched and optimized in order to find a solution for this ever growing issue.

2.2 Experimental research on the electrical effects on biofilms

Electrical effects have been shown to be a possible route for the prevention and eradication of biofilms and the major research currently being done in this area will be addressed. The electricidal and bioelectric effects will be addressed in relation to the prevention and treatment of biofilms.

2.2.1 The electricidal effect

The electricidal effect is an approach that the scientific community is researching to prevent and treat biofilms [11-28]. Once again, this effect is based on the idea that an electrical method will inhibit growth, kill, and/or detach the biofilm. The findings in the literature are summarized in Table 1. From this table one can see that many of the parameters that are being used vary between the different research groups. Whether it is the microorganism being studied or the strength and duration of the electrical method, each variable affects the results of the experiment.

Staphylococcus epidermidis [15-17, 19, 20, 23, 28], *Staphylococcus aureus* [11-13, 15, 21, 23, 25], and *Pseudomonas aeruginosa* [12-14, 18, 22, 23, 27] were used because these are the strains of bacteria that are known to be the most prevalent in clinical cases of biofilm infections [1]. Many of the research groups developed their own experimental chambers to assess this effect on a variety of biofilms. Most groups used chambers that assessed treatment methods on biofilm after the experiments were conducted [11-19, 23, 25-28] while one group of researchers assessed the impact of treatments on biofilms in real time with the use of a microscope [22]. *In vivo* experiments demonstrated that there is a significant impact of the electricidal effect on the biofilm when longer duration treatments are utilized [20, 21, 24]. Distinctive impacts of the treatment chamber on the results are difficult to address; however, what is important is that each group of researchers utilized consistent experimental methods throughout their research.

An advantage of using an experimental set-up that observes the effects on the microorganism in real time is that it allows one to assess the exact point in time when the effect of a treatment occurs. One group was able to observe that cathodic currents caused the detachment of 80% of the biofilm with 20% still being active on the electrode [22]. Anodic currents only caused the detachment of 30% of the biofilm; however, these currents were able to kill a portion of cells in the biofilm and also increased bacterial motion which is associated with a decreased ability to bind a surface [22].

The substrate (surface) on which the biofilm was formed varied between the different research groups. The material on which the biofilm adheres to is an important factor as it can impact the results of the experiment. For example, an *in vivo* rabbit model was used by Secinti et al. in 2007 where they formed biofilms on implanted titanium electrodes that were either coated with silver or not [21]. The electric current field generated by the silver-coated titanium electrode inhibited the growth of the biofilm that adhered to the surface while the uncoated electrode did not [21].

In other studies, a variety of polarizable electrode materials were used to create a displacement current as well as an electric current field. Some electrodes that were utilized were made of stainless steel [11-13, 16, 17, 19, 20, 23], graphite [23, 25], and titanium [18, 21]. Stainless steel and titanium were utilized because many of the implantable devices currently used clinically are made up of these materials and are very susceptible to biofilm infections [8, 10, 56]. Graphite was used for its ability to be inert which would reduce the direct contact between the bacteria and electrodes [23]. With the variation of materials one can observe which electrodes produce certain effects and whether one is more effective than the other at inhibiting and killing biofilm infections.

The importance of electrode material can be highlighted when observing the impact on biofilm when using uncoated titanium compared to silver-coated titanium [21]. Similar to what was previously discussed, when coated with silver, the titanium electrodes inhibited the growth of the biofilm when polarized anodically while uncoated titanium showed no significant effects [21].

One of the major difficulties with comparing the various experiments with one another is the fact that each group uses different electric current intensities at various durations. Biofilms in this field of research are subjected to a range of electric current intensities, though most are in the 1 – 2000 μA range [11, 12, 14-17, 20, 21, 23, 24, 28]. The characteristics of the current used is important to note because the results do change. Hoon Hong et al. in 2007 showed that cathodic current caused the detachment of 80% of the biofilm whereas anodic current did not show detachment but inactivated the biofilm and increased bacterial motion. Block currents (half cathodic and half anodic DC current for a set time) detached the biofilm similar to cathodic currents and inactivated the bacteria less than anodic currents but more than cathodic currents [22]. This study was able to show the importance of the type of current and may promote future studies to utilize a current with changing polarity in order to eradicate and inhibit the biofilm formation effectively.

The time of exposure of the electric current fields ranges from minutes (min) to hours (h) and even to multiple days (d) making it even more complex when trying to compare results of various research groups. The experiments that run for days usually incorporate an *in vivo* model to make the experiment clinically relevant [20, 21, 24] while the ones that last for shorter time periods can use very high current field intensities [13].

Even though these two factors are highly variable among research groups, the results obtained in their respective experiments provide other researchers with the knowledge that this effect is showing some promising outcomes. This very broad range of exposure times and intensities illustrates how young this field of research is and it also enforces the fact that optimization of parameters is of great importance. It is difficult to make conclusions about which durations produce the most effective treatment of biofilm with *in vitro* experiments, but generally, longer treatments durations showed larger reductions of biofilms [23, 24] when compared to shorter duration treatments [14, 16, 17, 22, 27].

Table 1: Summary of the significant results with experiments testing the electricidal effect on biofilms

Biofilm Age	Method	Biofilm Substrate	Microorganism	Electric Current or Voltage	Electrode Materials	Exposure Time	Results and Conclusions	Reference
8 hours	Experimental chambers	Platinum	*Proteus mirabilis*	320 nA/mm^2 (AC, 0.25Hz)	Platinum	6 d	Few dead bacterial cells.	[26]
				75 nA/mm^2 (anodic)			Few dead bacterial cells.	
				750 nA/mm^2 (anodic)			Separation of the biofilm layers on the substrate.	
	Experimental chambers	Carbon-impregnated polyurethane	*Staphylococcus aureus* and *Staphylococcus epidermidis*	10 µA (DC)	Carbon-impregnated polyurethane	16 h	Inhibits biofilms growth around cathode. Localized production of H_2O_2 and chlorine in addition to the intrinsic activity of electric currents eradicates biofilms.	[15]
	Experimental chambers	Brain heart infusion agar	*Staphylococcus aureus*	400 µA (DC)	Silver, Gold, Platinum, and Stainless Steel	24 h	Inhibits growth on all electrodes but silver electrodes are the most effective at inhibiting bacteria growth and showed the lowest levels of toxicity.	[11]
12 hours to 72 hours	Polycarbonate treatment well	Polycarbonate	*Staphylococcus epidermidis*	2-5 mA (DC, 0.7 – 1.8 mA/cm^2)	Platinum	24 h	6.7 log reduction of biofilms.	[28]
	Experimental chambers	Indium-tin-oxide (ITO) film	*Pseudomonas aeruginosa*	15.0 µA/cm^2 (DC, negative)	Indium-tin-oxide (ITO) film	90 min	80% reduction of bacterial adhesion.	[27]
				15.0 µA/cm^2 (DC, positive)			<20% reduction of bacterial adhesion.	
				15.0 µA/cm^2 (AC)			70% reduction of bacterial adhesion with additional bactericidal effects.	

	Experimental chambers	Conventional nanotubular titanium and Anodized nanotubular titanium	*Staphylococcus aureus*	8.4 and 16.8 A/m^2 (AC, 0.4 ms pulses, 20 Hz)	Graphite	1 h / d for 2 d	After two days there was a larger reduction of biofilms with anodized titanium than with conventional titanium.	[25]
	Experimental chambers	Teflon	*Pseudomonas aeruginosa*	2000 μA (DC)	Graphite	7 d	3-6 log reduction of biofilms.	[23]
			Staphylococcus aureus			2 d		
			Staphylococcus epidermidis					
	Experimental chambers (observed in real time)	Indium-tin-oxide (ITO) coated glass	*Pseudomonas aeruginosa*	15 μA/cm^2 (cathodic, anodic, and block currents)	Indium-tin-oxide (ITO) coated glass	40 min	Cathodic current detached 80% of biofilm and anodic current inactivated biofilms and increased bacterial motion. Block currents detached (similar to cathodic currents) and inactivated (less than anodic currents but more than cathodic currents) biofilms.	[22]
	Experimental chambers	Titanium	*Pseudomonas aeruginosa*	5 V (200 Hz, 1% duty cycle)	Interdigitated titanium	6 d	Reduced area covered by the biofilm by 50%.	[18]
	Experimental chambers	Stainless steel	*Staphylococcus epidermidis*	100 μA (DC block current)	Stainless steel	150 min	Block currents of 100 μA resulted in a detachment of about 76% of adhering bacteria and were deemed less viable.	[17]
	Experimental chambers	Stainless steel	*Staphylococcus epidermidis*	60 μA (DC)	Stainless steel	6 h	37% detachment of biofilms.	[16]
				100 μA (DC)			78% detachment of biofilms.	

				60 µA (Block current, 50% duty cycle, 1 Hz)			24% detachment of biofilms.	
				100 µA (Block current, 50% duty cycle, 1 Hz)			31% detachment of biofilms.	
	Experimental chambers	Mueller-Hinton agar	*Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*	250 V (Monophasic, Pulsed Current)	Stainless Steel	2 h	Exposure of all organisms to 250 V for 2 h was enough to inhibit growth.	[13]
	Experimental chambers	Nutrient agar	*Staphylococcus aureus, Escherichia coli, Proteus vulgaris,* and *Pseudomonas aeruginosa*	400 µA (DC, 20 µA/mm^2)	Silver, Platinum, Gold, Stainless steel, and Copper	24 h	At higher currents all electrodes inhibited growth. Silver was shown to be bacteriostatic even at lower currents.	[12]
~7 days	Electrified flow cell	Platinum	*Klebsiella pneumonia, Pseudomonas fluorescens,* and *Pseudomonas aeruginosa*	50 µA (AC, 0.016 – 20 Hz, 3.1 mA/cm^2)	Platinum	6 h	The biofilm expanded by about 4% when wire was cathodic and reduced by about 74% in thickness when wire was anodic.	[14]
	Electrolytic chambers	Stainless steel	*Staphylococcus epidermidis*	6 V	Stainless steel	5 min	Killing (2.1 log reduction) and removal (4.0 log reduction) of viable cells at the anode and cathode, respectively.	[19]
In vivo	*In vivo rabbit model*	Stainless steel	*Staphylococcus epidermidis*	200 µA (DC)	Stainless steel	21 d	2-3 log reduction of biofilms.	[24]
	In vivo rabbit model	Uncoated titanium	*Staphylococcus aureus*	8 µA (DC)	Uncoated titanium	2 h / d for 3 d	No inhibition on uncoated titanium.	[21]

		Silver-coated titanium			Silver-coated titanium		Inhibition of growth on silver-coated titanium when polarized anodically.	
	In vivo goat model	Stainless steel	*Staphylococcus epidermidis*	100 μA (DC)	Stainless steel	21 d	Infection developed in 89% of the control pin sites whereas only 11% of the pin sites were infected in the electrified group.	[20]

2.2.2 The bioelectric effect

The bioelectric effect is another potential prevention and treatment option for biofilms when considering electrical effects. This effect utilizes an electrical method in combination with antibiotics or the host's defense to inhibit growth, kill, and/or detach the biofilm. It has been shown to increase the effectiveness of many commonly used antibiotics and a summary of these studies are shown in Table 2. Several researchers have demonstrated that this effect has the potential to be a promising treatment option for patients with implanted medical devices who suffer from biofilm infections [29-43]. There is still a need for more *in vivo* data, optimization of parameters, and a better understanding of the mechanisms of action involved.

Similar to the past studies done on the electricidal effects on biofilms, the groups of researchers created their own experimental chambers to assess the bioelectric effect. The key difference with these sets of experiments is the addition of an antimicrobial agent to the experimental set-up. The variation of methods utilized makes it difficult to compare the findings among research groups, however, parameters that produce significant impacts on biofilms are listed in Table 2 and demonstrate the potential for this type of treatment to be used. Once again, the most common bacterial strains used were *Staphylococcus epidermidis* [38, 41], *Staphylococcus aureus* [41, 43], and *Pseudomonas aeruginosa* [29, 30, 32-36, 42, 67]. Observations in real time by de Saravia et al. in 2005 allowed for the assessment of the bioelectric effect at various stages of biofilm development. It was determined that planktonic state bacteria is the most susceptible to the bioelectric effect and that bacterial cells that are released by the biofilm are more vulnerable than cells in the sessile state [40].

The biofilm substrate and electrode materials were again highly variable among these experiments. Stainless steel was the most common choice for the substrate [30, 38, 67] and the electrode material [30, 32, 34-37, 39-41, 67]. This is because stainless steel is one of the most frequently used materials in implantable devices such as artificial hip joints [8] and many cardiovascular implants [56]. It is used for its lifelong durability and physical strength although they are vulnerable to infections and corrosion [56].

Most of the studies on the bioelectric effect are the continuation of work from observing the electricidal effect. Most of the electric currents utilized range from 1 – 6 mA [33, 35-37, 41, 43]. This is slightly higher than what was used for the electricidal effect experiments but some studies such as del Pozo et al. still test lower electric currents as well [41]. This slight increase in current intensities could be attributed to the fact that researchers are still trying to identify the mechanisms of action before they determine clinically optimal parameters.

The characteristics of the electric current are once again very important and it was shown that a variety of intensities in combination with various antibiotics affects biofilms differently [41]. For instance, when assessing treatment methods on *S. epidermidis* biofilm, daptomycin in combination with 2000 μA had a 1.20 log reduction of biofilm while erythromycin in combination with 200 μA showed a 2.04 log reduction when compared to the controls with no treatment [41]. Experiments that utilized very short treatment durations were assessing real time observations to determine the impact of treatments on different types of bacterial cells [40]. The sensitivity of the different types of *Pseudomonas fluorescens* cells to 0.4 mA / cm^2 (DC) in combination with

glutaraldehyde decreased in the following order: planktonic cells > cells released by biofilm > sessile cells [40].

The exposure time for different experiments varies from hours to days, but most studies assess the effect after 24 h [29, 30, 33, 35-37, 39, 41, 67]. Generally, treatment durations for 24 h or above [33, 35-37, 39, 41, 67] showed a larger reduction of biofilm when compared to exposure times below 24 h [32, 34, 38]. For example, *Pseudomonas aeruginosa* biofilms were more effectively treated with 24 h duration treatments [35, 36] than 12 h treatments [32] when tobramycin and low level DC currents were utilized.

Different types of antibiotics are being used in this research field where the choice of antibiotic is mostly dependent on the bacterial strain used. Tobramycin [30, 32, 33, 35, 36, 41] and gentamicin [37-39, 41] are the most frequently tested due to their frequent use clinically. Research conducted by del Pozo et al. in 2008 analyzed the effect of an electric current on the effectiveness of multiple antibiotics on three different bacterial strains [41]. This study not only varied the strength of the electric current and antibiotic used, but it also determined the best method of action for a specific species of microorganism, highlighting the fact that the effect is not generalizable [41]. This research demonstrates that treatment selection is biofilm-specific bringing forth the idea of situation-specific treatments which is undoubtedly an important aspect in healthcare delivery. Experiments that assess the effects of the choice of a specific parameter will help address whether particular materials, electrical methods utilized, and antibiotics chosen play a significant role in the effectiveness of the bioelectric effect.

Table 2: Summary of the significant results with experiments testing the bioelectric effect on biofilms [Adapted from [60] with significant additions]

Biofilm Age	Method	Biofilm Substrate	Microorganism	Electric Current or Voltage	Electrode Materials	Antimicrobial Agent	Exposure Time	Results and Conclusions	Reference
8 hours	Experimental chambers (observed in real time)	Nutrient broth (planktonic state), Stainless steel cathode (sessile state), and Platinum anode (sessile state)	*Pseudomonas fluorescens*	0.4 mA / cm^2 (DC)	Stainless steel and Platinum	Glutaraldehyde (25 – 500 ppm)	4 h	Sensitivity of bacteria to treatments decreased in the following order: planktonic cells > cells released by biofilm > sessile cells.	[40]
12 hours to 72 hours	Experimental chambers	Teflon	Methicillin-resistant *Staphylococcus aureus* (MRSA)	2000 µA (DC)	Graphite	Vancomycin (32 µg/mL)	36 h	1.59 log reduction of biofilms.	[41]
			Staphylococcus epidermidis	2000 µA (DC)		Daptomycin (4 µg/mL)		1.20 log reduction of biofilms.	
				200 µA (DC)		Erythromycin (2 µg/mL)		2.04 log reduction of biofilms.	
	Experimental chambers	Glass	*Escherichia coli*	200 mA (DC, 6 mA/cm^2)	Stainless steel	Gentamicin (5 µg/mL) and Oxytetracycline (50 µg/mL)	24 h	4-6 log reduction in biofilms.	[39]
				200 mA (radio frequency AC at 10 MHz)	No electrodes			2-3 log reduction in biofilms.	
	Experimental chambers	Polycarbonate	*Pseudomonas aeruginosa*	2 mA (DC)	Stainless steel	Tobramycin (5 µg/mL)	24 h	5-6 log reduction of biofilms.	[36]

	Experimental chambers	Polycarbonate	*Pseudomonas aeruginosa*	2 mA (DC)	Stainless steel	Tobramycin (5 µg/mL)	24 h	6 log reduction of biofilms.	[35]
	Electrical colonization cell	Dialysis membrane	*Pseudomonas aeruginosa*	9 mA/cm^2 (DC)	Stainless steel	Ciprofloxacin (5 µg/mL)	12 h	0.5-1 log reduction of biofilms.	[34]
						Polymyxin B (20 µg/mL)		0.5-1 log reduction of biofilms.	
						Piperacillin (40 µg/mL)		No reduction of biofilms.	
	Electrical colonization cell	Dialysis membrane	*Pseudomonas aeruginosa*	9 mA/cm^2 (DC) (Change in polarity after 32 s)	Stainless steel	Tobramycin (10 µg/mL)	12 h	2 log reduction of biofilms.	[32]
	Three electrode flow cell	Stainless steel	*Pseudomonas aeruginosa*	1.7 mA/cm^2 (DC) (Change in polarity after 64 s)	Stainless steel	Tobramycin (5 µg/mL)	24 h or 48 h	Electrically assisted electrophoresis was shown to have a larger effect than an electrochemical effect when eradicating biofilms.	[30]
	Electrified modified Robbins device	Stainless steel	*Pseudomonas aeruginosa*	2.1 mA/cm^2 (DC) (Change in polarity after 64 s)	Stainless steel stud with Platinum wire	Isothialazone (1.5%), Dimethyl ammonium chloride (50%), and Glutaraldehyde (25%)	24 h	3-6 log reduction of biofilms.	[67]

	Electrified modified Robbins device	Metal stud (Unknown material)	*Pseudomonas aeruginosa*	3 V (DC) (Change in polarity after 64 s)	Metal stud (Unknown material)	Glutaraldehyde (5 ppm)	24 h	Increase of effectiveness of glutaraldehyde in combination with the electric current field against biofilms.	[29]
~7 days	Microtitre trays	Stainless steel	*Staphylococcus epidermidis*	Pulsed electroma-gnetic waves (PEMF, 5 A for 380 µs at 72 Hz, 0.07 V/m)	No electrodes	Gentamicin (160 µg/mL)	12 h	1-2 log reduction of biofilms.	[38]
	Experimental chambers	Polycarbonate	*Streptococcus gordonii*	2 mA (DC, 0.4 mA/cm^2)	Stainless steel	Gentamicin (2 µg/mL)	24 h	4-5 log reduction in biofilms.	[37]
	Experimental chambers	Polycarbonate	*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*	1 mA (DC)	Platinum	Tobramycin (5 µg/mL)	24 h	6-8 log reduction of biofilms.	[33]
	Electrified modified Robbins device	Stainless steel	*Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus epidermidis*	15 - 400 µA/cm^2 (DC) (Change in polarity after 64 s)	Stainless steel and Platinum	Tobramycin (1 – 100 µg/mL)	12 h	All biofilms were killed by using electric currents in combination with the various concentrations of tobramycin.	[31]

In vivo	*In vivo* rabbit model	Titanium	*Staphylococcus aureus*	6 mA (AC)	Titanium (capacitive coupling)	Ceftriaxone (20 mg/kg of body weight)	7 days	Infection developed in 81% of the control groups whereas only 36% of the experimental group were positive for biofilm cultures.	[43]
	In vivo mouse model	*In vivo* mice pulmonary system	*Pseudomonas aeruginosa*	4 V/cm (AC, 10 MHz)	Metal electrodes insulated by ceramic material	Ceftazidime (5 mg/kg of body weight)	48 h	1-2 log reduction of biofilms.	[42]

2.3 Summary and potential for clinical applications

Observing a significant impact of the electrical effects on biofilms is the first and most important aspect of this research. The need for more conclusive *in vivo* data is also required and can be achieved through standardizing experimental methods and optimizing *in vitro* parameters. The metrics used during experimentation need to be standardized to allow for a meaningful comparative analysis of the results among different research groups. This could be achieved through workshops and/or seminars that increase the communication between researchers in this field. In addition, the introduction of a standard experimental chamber may allow for more consistent results between different experiments. The creation of this instrumentation would allow for similar experimental environments between researchers. The factors involved with electrical effects that need to be researched more in depth include the materials, electrical characteristics, antimicrobial agent type and dosage as well as the timing and length of the treatment. Conducting studies similar to del Pozo et al. [41] and Hoon Hong et al. [22] that address results that determine the most effective treatment for a specific biofilm species will lead to the creation of situation-specific treatments that will yield the best outcomes.

Another important step in determining an effective method for treating a specific condition is the understanding of the fundamental processes that occur. Possible mechanisms of action of both the electricidal and bioelectric effects are the electrochemical generation of potentiating oxidants and the electrolytic generation of oxygen [35, 68]. Other possible mechanisms include the reduction of the binding of the biofilm to antibiotics, the increase of the electrophoretic transport of antibiotics [45] and

the increase of the overall membrane permeability of the biofilm [69]. Finding conclusive evidence for the hypothesized mechanisms is vital to the progression of this field of study.

Throughout this review many principles, concepts, effects, and ideas have been brought forward concerning biofilm infections. Understanding of the importance of concepts such as biofilm adherence and resistance as well as the significance of the type of electrical method utilized are needed to fully appreciate the potential of the electricidal and bioelectric effect. Biofilms are known to be resistant to antimicrobial agents in addition to the host's immune system [1, 3]. This important fact is the origin of a significant amount of research whose goal is to find practical and effective prevention and treatment methods for biofilm infections. Biofilms are extremely relevant in healthcare due to the fact that they are known to cause most of the infections in patients and are extremely persistent [4]. They are a major concern for indwelling and implantable medical devices [8, 10].

Future applications need to address the problems in the growing number of infections seen clinically [4]. Cystic fibrosis [5], urinary tract infections [6], tuberculosis [7], orthopaedic implant infections [8], and cardiovascular infections [9, 10] are all biofilm-based infections. Electrical effects have the potential to alleviate these problems in healthcare with non-invasive treatment options. Even though there are many promising results *in vitro*, there are not enough *in vivo* studies to fully assess the potential risks for patients. It was shown that 100 μA (DC) in an *in vivo* goat model did not produce any observable side effects due to the fact that at this current strength there is no dissociation of ions which can be harmful [20]. If this treatment option were to be implemented

directly into implantable medical devices, it would be using very low level electric currents / electromagnetic fields. The patient would have a risk of microshock which would have to be taken into consideration before employing this type of treatment.

There are many areas in which further investigation is required to progress this field of study. The effectiveness of these electrical effects need to be assessed more in depth in regards to the age of the biofilm in order to address whether they can be used as a preventative measure or treatment option. Also, more *in vivo* data is necessary to assess the capabilities of the electrical effects in the human body due to the fact that they can potentially interact and enhance the host's immune response; however, observing a significant impact of the electricidal and bioelectric effect against biofilms *in vitro* is an imperative step and will allow for the further evaluation of their mechanisms of action.

CHAPTER 3

3.0 MATERIALS AND METHODS

The materials and methods will be outlined throughout this section. The microorganism, antibiotic, solutions, and materials will be stated. The experimental set-up and process will also be discussed in detail. In addition, the statistical analysis method will be described. The choice of parameters was based on published research [17, 20-22, 24, 27, 29-32, 41, 53-59, 67, 70-73] and through experimentation. The protocol was adapted from prior studies and standards [41, 74-84] and all methods were conducted using sterile techniques.

3.1 Microorganism and antibiotic

Staphylococcus epidermidis American Type Culture Collection (ATCC, Manassas, Virginia, United States of America) 35984 strain, also known as RP62A, was generously provided by Dr. Sandra Ramirez-Arcos, a Development Scientist at Canadian Blood Services, Ottawa, Ontario, Canada and Adjunct Professor at the University of Ottawa, Ottawa, Ontario, Canada. The *S. epidermidis* strain is a gram-positive bacteria, facultative anaerobe (grows in aerobic and anaerobic conditions) and ATCC 35984 is from a nosocomial infection isolated in 1982 from a catheter sepsis in Tennessee [85]. It is a hospital acquired infection known to produce polysaccharide adhesins and adhere effectively to surfaces and form biofilms [86]. The strain was stored at -80ºC.

Vancomycin V2002 Sigma (Sigma-Aldrich, Oakville, Ontario, Canada), also known as vancomycin hydrochloride from *Streptomyces orientalis*, has a potency of ≥900 µg/mg with vancomycin as the base, was purchased for use in this research. Vancomycin

is a glycopeptide antibiotic which inhibits the growth of cell walls by complex formation with peptides terminating in acetyl-D-alanyl-D-alanine [87]. Vancomycin is very commonly used in the clinical setting [58, 59, 88] and there is significant research on the effectiveness of this antibiotic for treating gram-positive bacteria such as strains of staphylococci [57, 59, 89].

3.2 Media and solutions

Tryptone Soya Broth (Oxoid Ltd, Basingstoke, Hampshire, England; Pancreatic digest of casein 17.0 g, Enzymatic digest of soya bean with papain 3.0 g, Sodium chloride 5.0 g, Di-potassium hydrogen phosphate 2.5 g, Glucose 2.5 g; 30 g per 1 L of water) medium at full strength and 1/10 strength was made in double distilled water (ddH_2O) and sterilized by autoclave for 20 min at 121ºC to make TSB and 1/10 TSB media, respectively. A stock of 50% glucose (Fisher Scientific, Fair Lawn, New Jersey, United States of America; ≥99% Dextrose or D-glucose, Anhydrous) solution was made in ddH_2O and was sterilized by vacuum filtration (Thermo Scientific, Waltham, Massachusetts, United States of America; Nalgene Rapid-Flow Sterile Filter Storage Bottles). TSB and 1/10 TSB were both supplemented with 0.5% glucose to generate TSBg and 1/10 TSBg media, respectively. A stock of 50% glycerol (Sigma-Aldrich, Oakville, Ontario, Canada; ≥99% Glycerol) was made in ddH_2O and sterilized by autoclave for 45 min at 121ºC. TSB supplemented with 0.5% glucose and 17% glycerol to generate TSBgg media. A stock of 0.9% NaCl (Wisent, St. Bruno, Quebec, Canada; ≥99% Sodium Chloride) also known as saline solution was made in ddH_2O and was sterilized by autoclave for 45 min at 121ºC. Luria-Bertani broth (Bio Basic, Markham,

Ontario, Canada; Trypton Casein Peptone 10 g, Yeast Extract 5 g, Sodium Chloride 10 g; 25 g per L of water) media supplemented with 1.5% agar A (Bio Basic, Markham, Ontario, Canada; 70% Agarose and 30% Agaropectin) was made in ddH_2O and sterilized by autoclave for 45 min at 121ºC to generate LB agar. Double distilled water alone was sterilized by autoclave for 45 min at 121ºC to make sterilized ddH_2O solution. Stock solutions of vancomycin powder dissolved in sterilized ddH_2O at specific concentrations were made and corrected for a potency of ≥900 µg/mg. Sterilized ddH_2O supplemented with 0.34-0.36% of sodium hypochlorite (Fisher Scientific, Fair Lawn, New Jersey, United States of America; 5.65-6% Sodium Hypochlorite) generated ddH_2O+NaOCl solution. Also, 95% ethanol (Commercial Alcohols, Brampton, Ontario, Canada; 95% Anhydrous Ethyl Alcohol) and 70% ethanol diluted with ddH_2O were utilized for sterilization techniques.

3.3 Minimum inhibitory concentration of vancomycin against *S. epidermidis*

The minimum inhibitory concentration (MIC) of vancomycin against *S. epidermidis* in the planktonic state was determined by adapting the two-fold series macrobroth dilution method for this research [78]. For the overnight (18 h) culture, a stock of *S. epidermidis* stored at -80ºC in TSBgg was removed from the freezer. With the use of a sterile wooden applicator stick, 5 mL of TSBg was inoculated with the strain and incubated at 37ºC overnight on a tissue culture roller set at 60 revolutions per minute (RPM). To determine the MIC, the overnight culture was diluted by 1:4,000 in fresh TSBg and exposed to a series of vancomycin concentrations. The final concentrations of vancomycin corrected for a potency of ≥900 µg/mg were 256, 128, 64, 32, 16, 8, 4, 2, 1,

and 0.5 µg/mL. A sub-culture was produced from the overnight culture by diluting it by 1:100 in fresh TSBg for a total of 100 mL in a glass flask which was then incubated at 37ºC on a shaker at 150 RPM. The sub-culture was incubated until it reached an optical density at 600 nm (OD_{600}) of ~0.500. The MIC for the sub-culture was then determined with the same process as the overnight culture. The MIC of the cultures were determined with two separate experiments with single samples in each (n = 2).

The OD_{600} of cultures was determined by measuring 1 mL of the culture in question in a cuvette and quantifying the OD_{600} of the solution with the spectrophotometer (Life Science Research Division Bio-Rad Laboratories, Mississauga, Ontario, Canada; SmartSpec Plus Spectrophotometer). Fresh TSBg in a cuvette was used as the blank for the spectrophotometer. The overnight culture was diluted in a cuvette by 1:100 with fresh TSBg and then the OD_{600} was measured with the spectrophotometer. The OD_{600} of the sub-culture was measured without diluting it. The OD_{600} of the cultures were determined as part of the two MIC experiments with single samples in each (n = 2).

To determine the number of colony forming units (CFU) per mL of the cultures, a spot titre method [79] was adapted and utilized. The dilution series of the cultures in saline solution contained the following dilutions: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000, and 1:100,000,000. 10 µL of each dilution was plated onto LB agar and incubated for 24 h at 37ºC. After incubation, the dilution that contained 3 to 30 CFUs was used to calculate the CFU/mL of the culture. CFU/mL of overnight cultures corresponding to an OD_{600} of ~0.080 (1:100 dilution in fresh TSBg) and sub-cultures corresponding to an OD_{600} of ~0.500 were determined as part of the two MIC experiments with single samples in each (n = 2).

3.4 Biofilm formation

The biofilm formation protocol was adapted from previous studies and standards [41, 74-77] for this research. Biofilms of *S. epidermidis* were formed on 24 separate grade 2 titanium coupons (Biosurface Technologies, Bozeman, Montana, United States of America; Grade 2 Titanium Disk, 1.27 cm diameter, 0.3 cm thick) which were secured in 8 polypropylene coupon rod holders (Biosurface Technologies, Bozeman, Montana, United States of America) (3 coupons per rod) and then suspended in the Centers for Disease Control and Prevention (CDC) Biofilm Reactor (Biosurface Technologies, Bozeman, Montana, United States of America). The system was also comprised of a baffled magnetic stir bar (Biosurface Technologies, Bozeman, Montana, United States of America), a magnetic stir plate (American Hospital Supply Canada, Mississauga, Ontario, Canada; Canlab Stir Plate), a retort stand and clamp, a glass flow break (Biosurface Technologies, Bozeman, Montana, United States of America), a peristaltic flow pump (Watson-Marlow Pumps Group North American Headquarters, Wilmington, Massachusetts, United States of America; 205U Watson-Marlow Peristaltic Pump), a media reservoir flask with a modified rubber stopper and glass pipettes, a waste flask, and tubing. The tubing used was 1.60 mm diameter silicone (Watson-Marlow Pumps Group North American Headquarters, Wilmington, Massachusetts, United States of America), 4.6 mm silicone (Cole-Parmer, Montreal, Quebec, Canada), and 1.65 mm diameter marprene (Watson-Marlow Pumps Group North American Headquarters, Wilmington, Massachusetts, United States of America). The experimental set-up for the biofilm formation is shown in Figure 2.

After each experiment, the media reservoir flask with the modified rubber stopper and glass pipettes, the waste flask, and the CDC Biofilm Reactor with all of the components were decontaminated by autoclave for 1 h at 121ºC, cleaned with laboratory soap (Fisher Scientific, Pittsburgh, Pennsylvania, United States of America; Sparkleen Laboratory Soap), rinsed, dried, and sterilized by autoclave for 45 min at 121ºC. The titanium coupons were decontaminated in a beaker containing ddH_2O and the final sterilization was done when the coupons were secured in the coupon holding rods inside the CDC Biofilm Reactor. The peristaltic flow pump and associated tubing was disconnected from the media reservoir flask and the CDC Biofilm Reactor and then sterilized by ddH_2O+NaOCl (5.65-6% Sodium Hypochlorite) and then rinsed with sterilized ddH_2O.

S. epidermidis was grown for 18 h and then sub-cultured to an OD_{600} of ~0.500. The sub-culture was used to inoculate a separate flask containing fresh TSBg by a 1:50 dilution for a total volume of 350 mL. It was then poured into the CDC Biofilm Reactor which contained the titanium coupons held in the coupon rods. The inoculated CDC Biofilm Reactor was then incubated at 37ºC for 1 h without any stirring or inflow of fresh media. This was called the **initial attachment phase**. After 1 h, the CDC Biofilm Reactor was placed on the stir plate which was turned on to level 6.5 (150 RPM). This was called the **batch phase** and lasted for 23 h at 37°C without any inflow of fresh media. When the batch phase was complete, the media reservoir flask containing 1/10 TSBg was connected to the peristaltic flow pump and the glass flow break was attached to the CDC Biofilm Reactor where finally the outflow spout of the reactor was connected to the waste flask. The peristaltic flow pump was turned on to 10 RPM which

corresponded to a flow rate of 1.07 mL/min and a residence time of 299.07 min (the CDC Biofilm Reactor volume below the outflow spout was measured to be approximately 320 mL). This was the beginning of the **continuous flow phase** (0 h for the biofilm age). The continuous flow phase initially lasted 96 h to confirm biofilm formation but was later reduced to 48 h with three separate sampling time points at 0 h, 24 h, and 48 h. Figure 3 depicts the biofilm formation timeline of the experiment. When the inflow media volume in the media reservoir flask was low during the continuous flow phase, it was exchanged with another flask full of fresh media. The waste flask was also exchanged with an empty flask when it could no longer hold anymore outflow media from the reactor. All openings and connections not enclosed effectively during the experiment were covered with sterile aluminum foil.

Biofilm formation of *S. epidermidis* was confirmed at the 0 h, 24 h, 48 h, 72 h, and 96 h time points of the continuous flow phase with the sampling method which will be described in the following sections. Three separate experiments done in triplicate confirmed biofilm formation at each of the time points indicated (n = 9 for each time point).

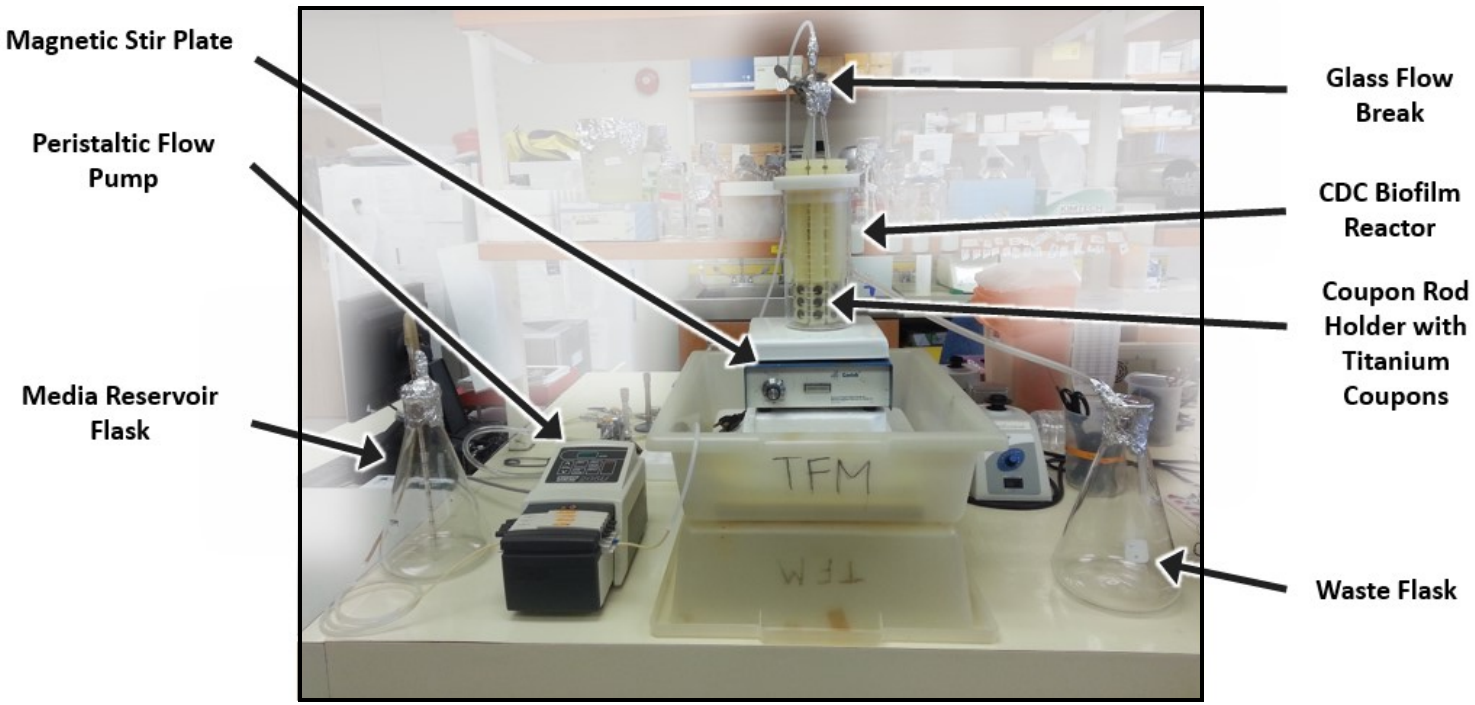

Figure 2: Experimental set-up for biofilm formation.

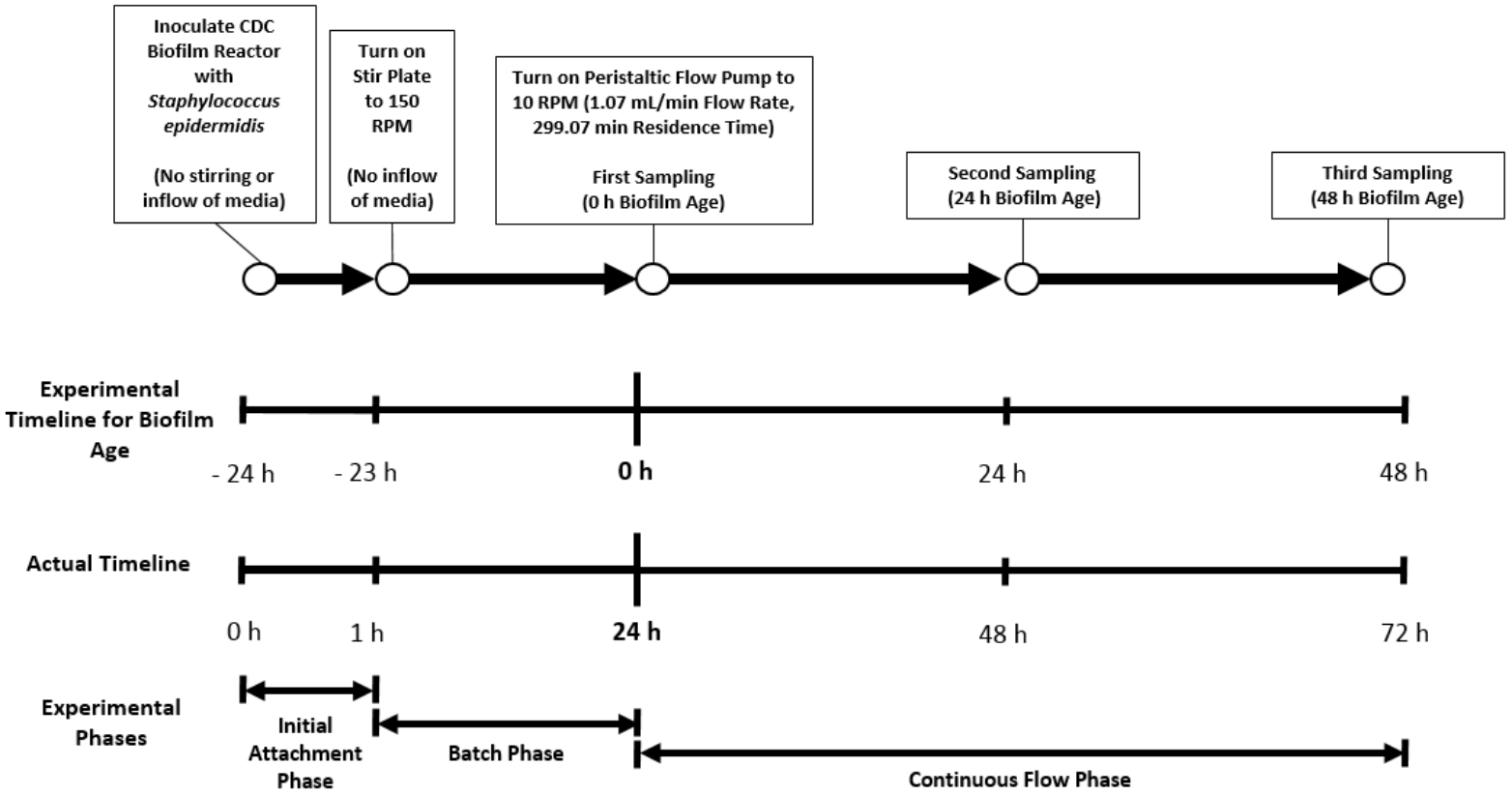

Figure 3: Biofilm formation timeline.

3.5 Electrical system set-up and biofilm treatment chamber

The treatment chamber and electrical system set-up was designed specifically for this research. The electrical system was comprised of a DC power supply or voltage source (Good Will Instrument Instek, Chino, California, United States of America; Instek GPR-1810HD Power Supply), electrical board (Digi-Key, Thief River Falls, Minnesota, United States of America; Twin Industries TW-E40-1020 Solderless BreadBoard), 12 kΩ and 180 kΩ resistors (Carleton University, Ottawa, Ontario, Canada), colour-coded and insulated copper wires (Carleton University, Ottawa, Ontario, Canada), ammeter or multi-meter (The Source, Ottawa, Ontario, Canada; Nexxtech Digital Multimeter), and grade 316 stainless steel rods (Metal Supermarkets, Ottawa, Ontario, Canada; Stainless Steel Grade 316 Round Bar, 0.5 cm diameter, 5.08 cm length). The treatment chamber was a petri plate (Bectin Dickinson Labware, Franklin Lakes, New Jersey, United States of America; Falcon Petri Dish, 3.5 cm diameter, 1 cm height) with two holes drilled into the lid (3/16" drill bit), each with a radius of approximately 0.24 cm and about 2.40 cm apart from each other (0.55 cm from the sides of the chamber) when measured from the centre of the holes. Two grade 316 stainless steel rods were used as the electrodes (anode and cathode) and were vertically inserted into the holes of the chamber. The electrodes were secured with electrical tape (The Source, Ottawa, Ontario, Canada; Electrical Tape, 2 cm width, 4 cm length) which also sealed the holes in the chamber. Electrodes were secured within the chamber such that 1 cm of the electrodes were inside the chamber while 4.08 cm remained outside. When necessary, the portion of the electrodes outside of the chamber was connected to the appropriate areas of the electrical board with copper wires.

After each experiment, the electrodes were disconnected from the system, removed from the treatment chamber, placed in separate beakers containing ddH_2O then decontaminated by autoclave for 1 h at 121ºC, cleaned with laboratory soap, rinsed, dried, and sterilized by autoclave for 45 min at 121ºC. The coupons were sterilized in the same manner as the electrodes however the final sterilization by autoclave was done when the coupons were secured in the coupon holding rods inside the CDC Biofilm Reactor. The treatment chamber was sterilized by rinsing it sequentially in two separate containers of 70% ethanol, dried under a biological laboratory hood (Canadian Cabinets Company, Ottawa, Canada; Model BM6-2A-49) for 1 h, then exposed to ultraviolet (UV) light for 1 h. The components of the electrical system and treatment chamber are shown in Figure 4.

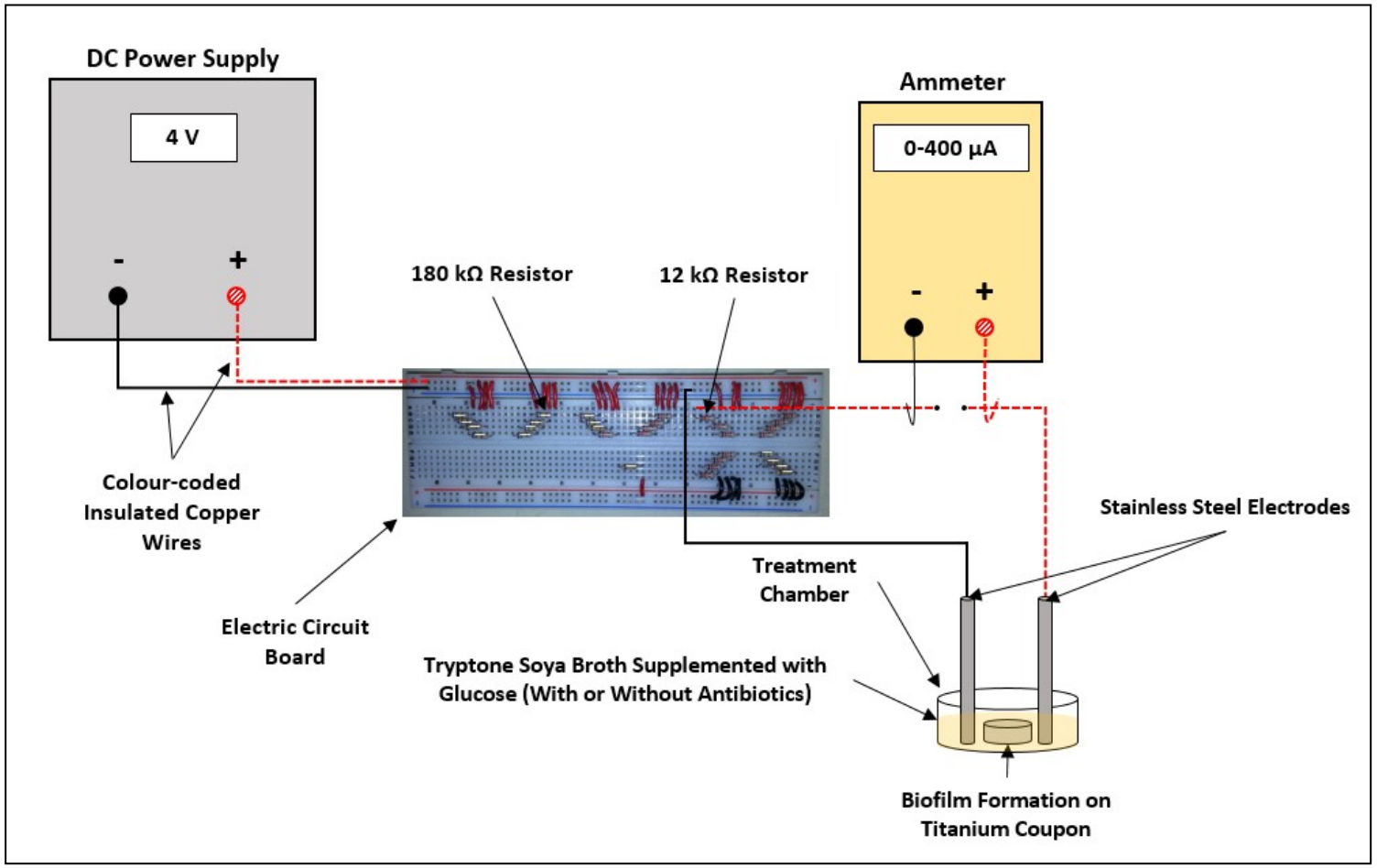

Figure 4: Electrical system and treatment chamber (Example connection is shown for the 333 µA current).

3.6 Biofilm treatment methods

The protocols for the treatment methods were adapted from previous research focusing on observing and analyzing the bioelectric effect [41, 74]. To choose the parameters of the treatment methods, extensive research evaluating the impact of parameters published in the literature was conducted as well as experimental testing in the laboratory. The factors chosen were all heavily based on the clinical relevance to implantable or indwelling medical device infections. *S. epidermidis* is known to be one of the most common bacteria associated with infections in medical devices [53-55], titanium and stainless steel are commonly used in implantable medical devices [56], and vancomycin is frequently used in the clinical setting [57-59, 88, 89]. The concentration of 16 µg/mL of vancomycin was chosen based on what is achieved maximally in the clinical setting (20-25 µg/mL) [88, 89] and experiments described later. DC electric current at 22 and 333 µA intensities were selected for testing and the duration of treatments were chosen to be 24 h and 48 h. The duration of exposure and characteristics of the electrical current were chosen based on previous studies observed impacts on bacteria and biofilm *in vitro* [17, 22, 27, 29-32, 41, 67] and *in vivo* [20, 21, 24]. The chosen parameters for this thesis are shown in Figure 5.

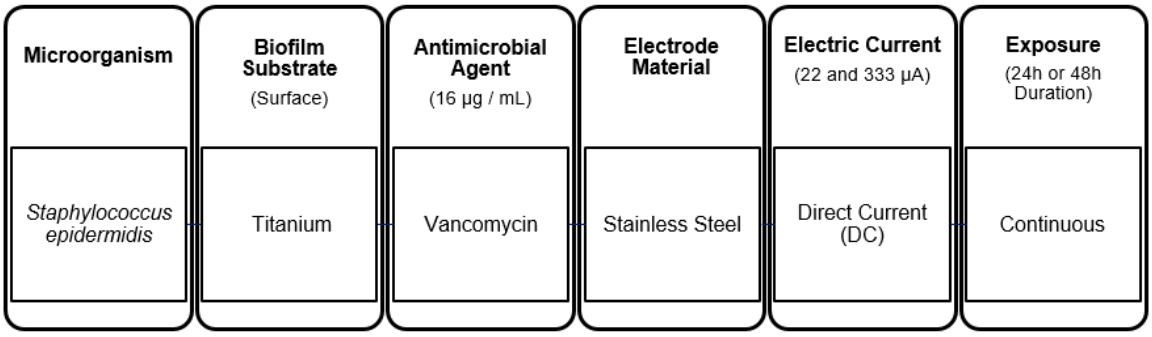

Figure 5: Selected experimental parameters.

The four major experimental treatment groups that were studied to assess the impact on *S. epidermidis* biofilm at different ages are as follows:

1. Antibiotics Treatment Group
2. Electric Current Treatment Group
3. Electric Current and Antibiotic Treatment Group
4. Control (No Electric Current, No Antibiotic) Group

Each of these major experimental treatment groups contained sub-groups in which different parameters were tested. The overview of the experimental design for assessing different treatment methods is illustrated in Figure 6.

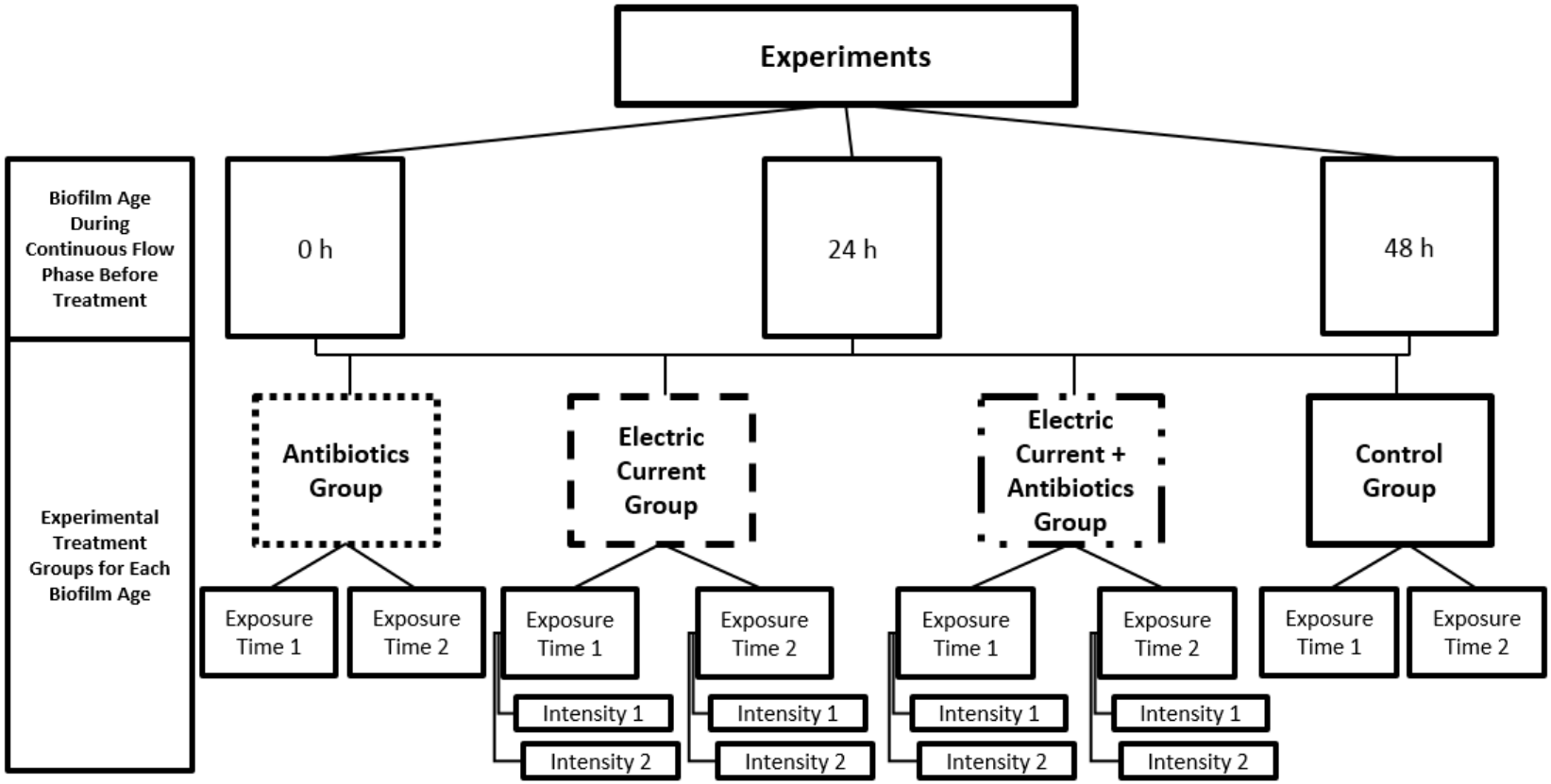

Figure 6: Overview of the experimental design.

There were 24 sampling opportunities (24 titanium coupons) with the CDC Biofilm Reactor however at times the coupons dislodged from the coupon rod holders during the sterilization process and therefore sample sizes were not always uniform. Biofilm covered titanium coupons were carefully removed from the CDC Biofilm Reactor and coupon rod holder to not disturb the surface of the coupon that was exposed to the shear forces of the baffled magnetic stir bar. It is important to note that the side of interest of the titanium coupons are the surfaces that are facing inwards and exposed to the shear forces that the stir bar generates as these forces are known to promote biofilm formation and reproducible results [74]. The coupons were rinsed in saline solution to remove planktonic cells, and then placed inside the treatment chamber that contained 5 mL of TSBg with or without antibiotics. The surface of interest (exposed to the shear forces) was on top, consequently being perpendicular to the electrodes. Copper wires were connected to each stainless steel electrode and to the electrical board and voltage source appropriately. If there was no electric current being used for the treatment, the chamber was not connected to the electrical board and voltage source. The voltage source was set at a constant 4 V to allow for DC currents of 22 μA to pass through the 180 kΩ resistor and 333 μA to pass through the 12 kΩ resistor (Voltage = Current x Resistance → V = IR). Refer to Figure 4 for a diagram of the electrical current treatment set-up. The current intensity was confirmed before and after each experiment with the multi-meter to ensure a constant current delivery to the treatment chamber. The controls were put through the exact same process and durations as the treatments, however the biofilm was not exposed to electric currents or antibiotics.

Three different experimental protocols were utilized to assess treatment methods against the formed biofilm. The sampling method for each treatment protocol will be described in the following section. The name and goal for each protocol are listed in chronological order:

1. Assess Impact of Different Antibiotic Concentrations on Biofilms:
    - Select the appropriate concentration of vancomycin to be utilized throughout the assessment of treatment methods.
2. Initial Overall Assessment of all Treatments:
    - Assess all treatment methods with selected parameters.
3. Focused Assessment of the Bioelectric Effect:
    - Evaluate the bioelectric effect and assess the impact of electric current on the effectiveness of vancomycin on biofilms with increased precision experiments to reduce variability of results.

To assess the impact of different vancomycin concentrations on *S. epidermidis* biofilms, a dilution series of the antibiotic was generated. The concentrations tested were 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL of vancomycin. Two treatment methods with the following parameters were used to primarily assess the impact of electric current with different concentrations of the antibiotic:

1. 24h Biofilm Age – 24h Duration – 22 µA Current
2. 48h Biofilm Age – 48h Duration – 333 µA Current

Two separate experiments done with single samples evaluated the impact of the concentration of vancomycin (n = 2 for each concentration).

Once the duration of treatments (24 h or 48 h), electric current intensities (22 μA or 333 μA, DC), and antibiotic concentration (16 μg/mL of vancomycin) were selected, an initial assessment of all treatment methods was conducted. To evaluate and observe an overview of all the treatment options, five separate experiments needed to be used due to the limited number (24) of titanium coupons in the CDC Biofilm Reactor. The three biofilm ages (based on the continuous flow phase) being assessed with each experiment were 0 h, 24 h, and 48 h. The following is the organization of the initial experiments:

1. Experiment #1: Electric Current (Intensity 1) Experimental Group
    i. Exposure Time 1 – Intensity 1
    ii. Exposure Time 2 – Intensity 1
2. Experiment #2: Electric Current (Intensity 2) Experimental Group
    i. Exposure Time 1 – Intensity 2
    ii. Exposure Time 2 – Intensity 2
3. Experiment #3: Antibiotics Experimental Group
    i. Exposure Time 1
    ii. Exposure Time 2
4. Experiment #4: Electric Current (Intensity 1) and Antibiotics Experimental Group
    i. Exposure Time 1 – Intensity 1
    ii. Exposure Time 2 – Intensity 1
5. Experiment #5: Electric Current (Intensity 2) and Antibiotics Experimental Group
    i. Exposure Time 1 – Intensity 2
    ii. Exposure Time 2 – Intensity 2

Each experiment ideally utilized 24 coupons and required 7 days to complete, therefore a protocol that allowed for efficient sampling was utilized and will be described in the following section. Two separate experiments, performed in duplicate, evaluated the impact of each treatment method on the specific biofilm age (n = 4 for each treatment). The controls (no electric current, no antibiotic) for the 24 h treatment duration (n = 19) and the 48 h treatment duration (n =18) were also done in duplicate when possible.

To conduct the focused (final) assessment of the bioelectric treatments that demonstrated an impact on *S. epidermidis* biofilms, a protocol similar to the initial assessments was utilized however the experiment was condensed to evaluate the different biofilm ages (0 h, 24 h, and 48 h) with only 24 h treatment durations with the following parameters:

1. Final Experiment:
    i. Control (No Electric Current, No Antibiotics)
    ii. Antibiotics
    iii. Electric Current (Intensity 1) and Antibiotics
    iv. Electric Current (Intensity 2) and Antibiotics

Once again, each experiment ideally utilized 24 coupons and required 7 days to complete, however since the experimental parameters being tested have been condensed, a protocol that allowed for more precision sampling was utilized and will be described in the following section. Three separate experiments in duplicate evaluated the impact of each treatment method on the specific biofilm age (n = 6 for each treatment). The controls (no electric current, no antibiotic) for the 24 h treatment duration were also done in duplicate (n = 6).

3.7 Biofilm sampling

The biofilm sampling protocols were adapted from previous published studies and standards [41, 74-77, 79, 80]. For all experimental protocols the scraping method utilized was the same, the differences that will be explained were in relation to the plating method for the sample. When an experimental treatment was complete, the coupon was removed from the chamber, rinsed in saline solution and one surface of the coupon was scraped remove the biofilm using a sterile wooden stick. For the coupon being sampled, the biofilm was scraped off the surface of interest (the side exposed to the shear force) with one sterilized wooden stick 3 times for 15 seconds (s) and after each scraping, the tip of the stick was dipped in 3 mL of saline solution. An additional 1 mL of saline solution was used to rinse the surface of the coupon being scraped into the 3 mL sample to collect any remaining biofilm cells, thus the total volume of sample was 4 mL. This method ensured that the entire surface of interest of the coupon was sampled. The 4 mL samples were vortexed (VWR International, Mississauga, Ontario, Canada; Analog VWR Mini Vortexer) for 2 s, sonicated (Branson Ultrasonics, Danbury, Connecticut, United States of America; Bransonic Ultrasonic Cleaner B1200R-4) for 5 min, and then vortexed for another 2 s.

For the experiments that confirmed biofilm formation, evaluated the impact of the concentration of antibiotics, and initially assessed the effectiveness of all treatments, a spot titre method for counting CFU [79] was adapted. The dilution series of the samples in saline solution contained the following dilutions: undiluted, 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000. 10 μL of each dilution which contained suspensions of dislodged and disaggregated biofilms were spot plated onto LB agar and incubated for

about 24 h at 37ºC. After incubation, the dilution that contained 3 to 30 CFUs was used to calculate the CFU of bacteria per cm^2 of the titanium coupon as a logarithmic number to then analyze. The calculation was done as a previous protocol has described [75] and it is shown below:

$$\log_{10}\left(\frac{CFU}{cm^2}\right) = \log_{10}\left(\left(\frac{Volume\ Scraped\ Into}{Surface\ Area\ Scraped}\right) x \left(\frac{CFU}{Volume\ of\ Sample\ Plated}\right) x\ (Dilution\ Factor)\right)$$

$$= \log_{10}\left(\left(\frac{4\ mL}{1.27\ cm^2}\right) x \left(\frac{CFU}{Volume\ of\ Sample\ Plated}\right) x\ (Dilution\ Factor)\right)$$

The experiments that focused on evaluating the effectiveness of treatments with increased precision utilized an adapted plate spreading technique [80]. The dilution series of the samples in saline solution contained specific dilutions from the following list: undiluted, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000. 200 µL of each dilution which contained suspensions of dislodged and disaggregated biofilms were plated and spread with a glass spreader onto LB agar and incubated for about 24 h at 37ºC. After incubation, the dilution that contained 3 to 300 CFU (some contained more CFU but were still valid to analyze) was used to calculate the CFU of bacteria per cm^2 of the titanium coupon as a logarithmic number to subsequently analyze. The $\log_{10}(CFU/cm^2)$ was calculated as previously described.

3.8 Statistical analysis

The statistical analysis for this research was chosen by evaluating the objectives of the experiment, the data collected, and from a previous study in this field of research [41] as well as research on statistical approaches [81-84]. Pairwise comparisons were completed by using a one-sided Welch t-test to show whether one treatment had less bacteria than the control or another treatment. The comparisons were only done with

samples that were the same biofilm age and exposed to a treatment for the same duration (these were considered the stratified groups). To determine whether or not a treatment was more effective than the control or another treatment method, a p-value of less than 0.05 was determined to be significant. The Welch t-test assumes normality but does not assume equal variances [83]. The Levene test was conducted to show unequal variances among samples [84].

For groupwise comparisons, the Dunnett-Tukey-Kramer (DTK) Pairwise Multiple Comparison test was utilized to determine the most effective treatment when compared to the appropriate control. The output of the test provided a multiple comparison analysis with the differences between the treatments and control. A two-sided confidence interval (CI) at a 95% significance level was plotted. A significant difference between a treatment and control is when the CI does not contain zero [81]. If the upper, one-sided confidence limit is less than zero, the treatment is said to be significantly less than the control [81]. In addition, this test allowed for the visualization of the difference of means to assess the impact of biofilm age and exposure time of treatments on the effectiveness of treatments. Once again, the comparisons were only done with samples that were the same biofilm age and exposed to a treatment for the same duration (these were considered the stratified groups). The DTK test assumes normality, does not assume equal variances, and is applicable to unequal sample sizes [82].

The R Project for Statistical Computing (Institute for Statistics and Mathematics Resources, Vienna University of Economics and Business, Welthandelsplatz, Vienna, Austria) an open source software, was utilized for the statistical analysis.

CHAPTER 4

4.0 RESULTS

This section will present, analyze, and state the findings from the experiments conducted. These results will be organized in a manner that will allow for each one of the following points to be addressed:

1. Confirm repeatability of *S. epidermidis* biofilm formation in the CDC Biofilm Reactor.
2. Select electric current intensities.
    a. Based on parameters used in previous research and impact on physiology.
    b. Measure electric current with ammeter to confirm consistent current before and after treatments.
3. Select concentration of vancomycin.
    a. Based on background knowledge of the clinical setting and previous studies.
    b. Utilize experiments that test for MIC.
    c. Assess multiple concentrations of vancomycin.
4. Overall initial assessment of all treatments against *S. epidermidis* biofilms.
5. Assess the bioelectric effect with more focused and rigorous experiments to enhance precision and reduce variability.

4.1 Confirmation of *S. epidermidis* biofilm formation with the CDC Biofilm Reactor

The results to confirm the formation of *S. epidermidis* on titanium coupons using the CDC Biofilm Reactor are presented in Figure 7 and Table 3. Biofilm formation is an aspect of *in vitro* experimentation that can be highly variable due to the uncontrollable nature of biofilms. 24 h biofilm formation proved to be a very repeatable time point as the standard deviation indicates (± 0.21 $\log_{10}$(CFU/cm^2)), however, after 48 h the biofilm became unstable and extremely variable (up to ± 2.20 $\log_{10}$(CFU/cm^2)). Even though the 0 h biofilm produced variable results (± 1.91 $\log_{10}$(CFU/cm^2)), it was included in the experiments to assess the impact of treatments on early biofilm development. Therefore the 0, 24, and 48 h biofilm ages were chosen to evaluate the effectiveness of different treatment methods.

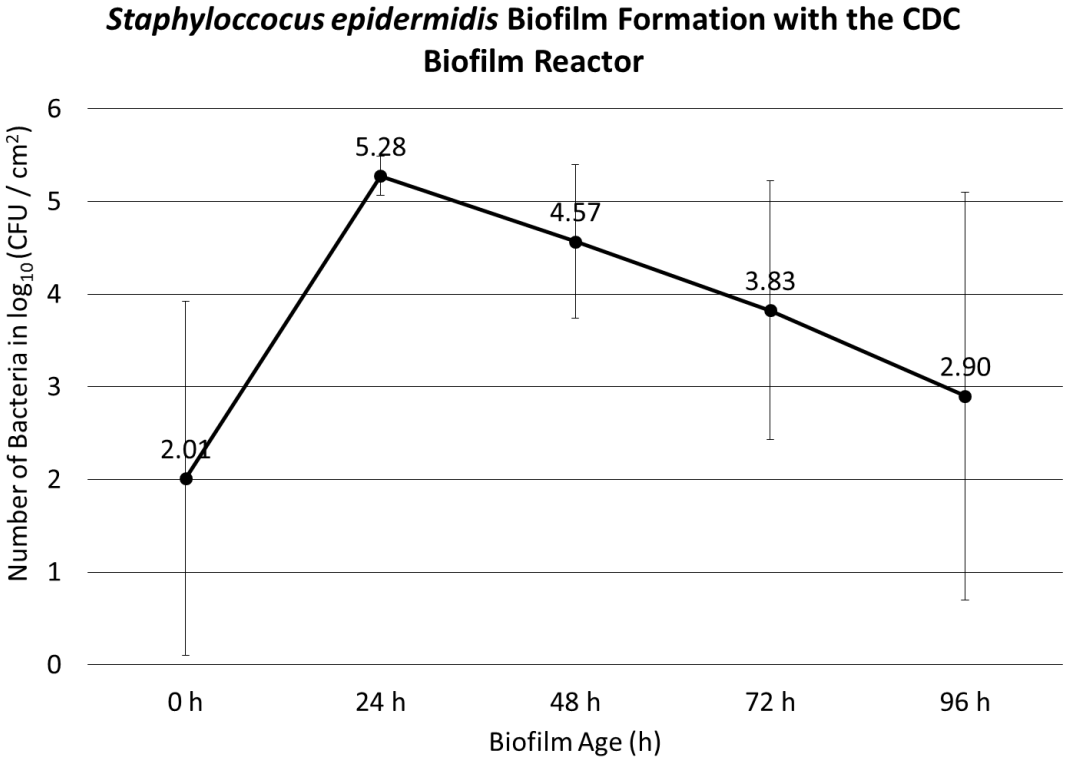

Figure 7: Assessment of *Staphylococcus epidermidis* biofilm formation on titanium coupons using the CDC Biofilm Reactor. Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. 0, 24, 48, 72, and 96 h biofilm ages were sampled (n = 9 for each age) and standard deviations are indicated (**I**).

Table 3: Mean values, standard deviations, and number of samples for confirmation of biofilm formation on titanium experiments

Biofilm Age (h)	Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in ± $\log_{10}(CFU/cm^2)$	Number of Samples
0 h	2.01	1.91	9
24 h	5.28	0.21	9
48 h	4.57	0.83	9
72 h	3.83	1.40	9
96 h	2.90	2.20	9

4.2 Confirmation of continuous electric current for treatments at set intensities

The selection of 22 and 333 μA DC currents was already rationalized in a previous section (Section 3.6). For the experimental aspects, both current intensities were confirmed with a multi-meter before and after each treatment that utilized electric currents. The 22 and 333 μA DC currents flowing through a 180 kΩ and 12 kΩ resistor, respectively, were confirmed with the use of the 4.00 V DC power supply, electrical board, and multi-meter.

4.3 Initial assessment of *S. epidermidis* resistance to vancomycin

The MIC for vancomycin of the overnight culture and sub-culture (the sub-culture was used to inoculate the CDC Biofilm Reactor) are presented in Table 4. For both the overnight culture and subculture the MIC obtained was 2 μg/mL, which is consistent with previous research conducted on this strain of *S. epidermidis* [72, 73] even though the methods were slightly different. It is also important to note that a study done which assesses the bioelectric effect of vancomycin on a different strain *S. epidermidis* obtained a MIC of 2 μg/mL, however, this research group utilized 32 μg/mL of vancomycin

against the biofilm [41] which is above the maximum of what is used in the clinical setting (20-25 µg/mL) [88, 89].

Table 4: Minimum inhibitory concentration for *Staphylococcus epidermidis* ATCC 35984

Incubation Time	Optical Density of Cultures at 600nm (OD_{600})	Colony Forming Units of Cultures (CFU/mL)	Colony Forming Units of Final Inoculum for MIC Testing (CFU/mL)	Minimum Inhibitory Concentration (µg/mL)
18 h	~0.080 (1/100 dil.)	~6×10^9	~1.5×10^6	2 µg/mL
18 h then Sub-cultured (1/100 dilution in TSBg) for 2 h 35 min	~0.500	~1×10^8	~2.5×10^4	2 µg/mL

In order to assess the effect of different concentrations of vancomycin on the *S. epidermidis* biofilm, a range of concentrations were tested in combination with specific electric currents and durations. This was done to observe and assess where one would be able to expect an impact of electric current on a specific concentration of vancomycin. The results of this experiment are presented in Figure 8 and Table 5. To determine the statistical significance of comparisons, the Welch t-test was applied to the data and the p-values are presented in Table 6. To conclude unequal variances, the Levene's test was utilized and the results are presented in Table 7. The p-values of the Levene test for the stratified groups as well as the complete data set were less than 0.01, concluding unequal variances among samples; however, the low sample size was a concern for the analysis. Also, the low sample size did not allow the data to be analyzed by the DTK test therefore it was not included for this experiment.

The objective of this experiment was to generate enough data to select an appropriate concentration of vancomycin to conduct further experiments with. This

objective was achieved and 16 μg/mL of vancomycin was selected because this concentration with 24 h Biofilm Age – 24 h Duration – 22 μA DC Electric Current treatments had a significant (p-value = 0.015) reduction of bacteria (-2.74 $\log_{10}(CFU/cm^2)$) when compared to the control of this stratified group. A significant (p-value = 0.033) reduction of bacteria (-7.01 $\log_{10}(CFU/cm^2)$) was observed when comparing the 16 μg/mL of vancomycin with 48 h Biofilm Age – 48 h Duration – 333 μA DC Electric Current treatments to the control of this stratified group. 8 μg/mL of vancomycin with the 48 h Biofilm Age – 48 h Duration – 333 μA DC Electric Current had 5.24 $\log_{10}(CFU/cm^2)$ of surviving bacteria, which was not significantly different from the control. When using 16 μg/mL of vancomycin in this treatment method, the 0 $\log_{10}(CFU/cm^2)$ of bacteria was significantly (p-value = 0.002) less than the 5.24 $\log_{10}(CFU/cm^2)$ of bacteria when using 8 μg/mL of vancomycin. The important aspect associated with the selection of 16 μg/mL of vancomycin is that it will have a greater impact on the field of research as it is below the maximum dose used clinically.

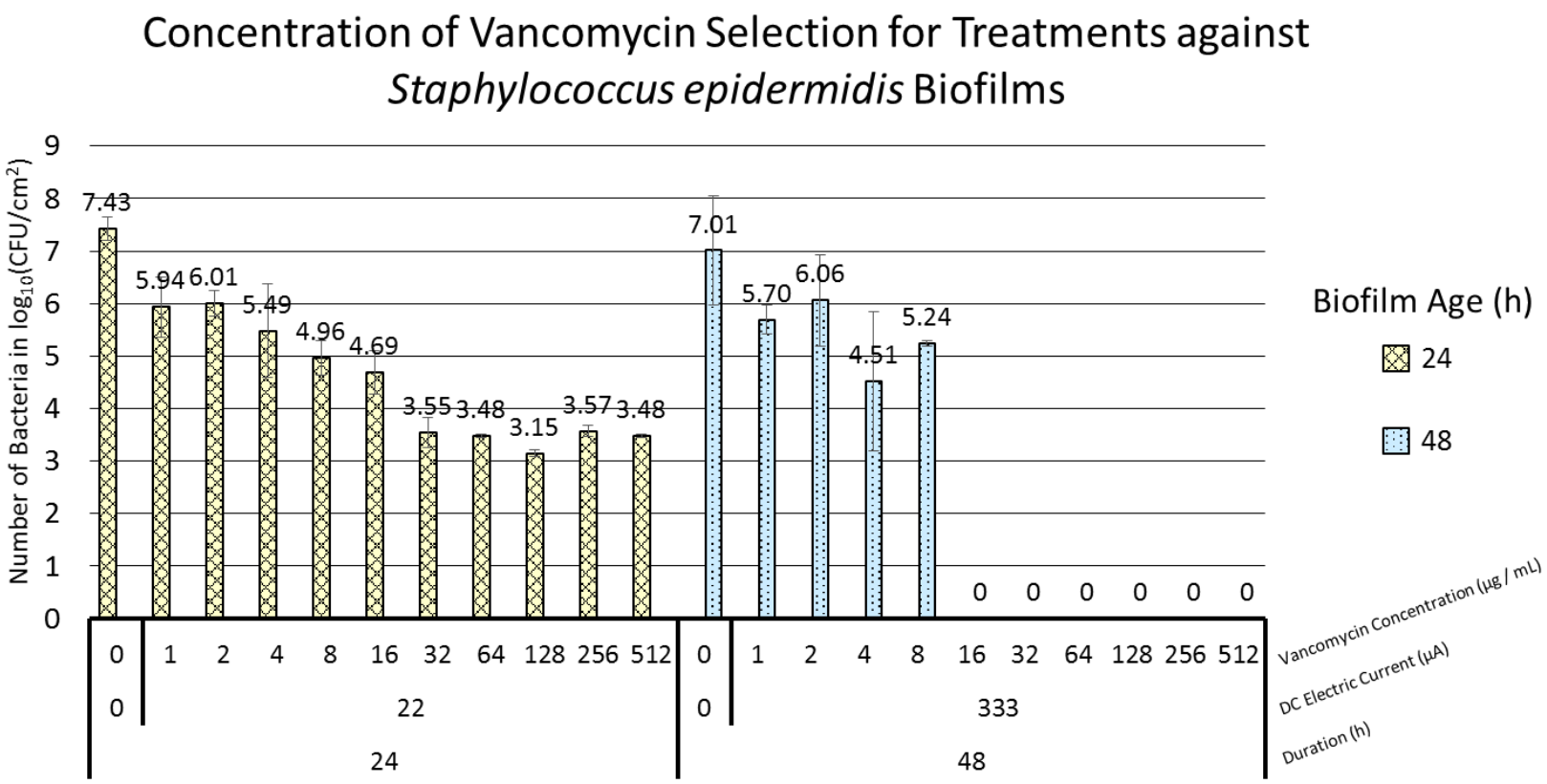

Figure 8: Assessment of 24 and 48 h duration treatment using different vancomycin concentrations in combination with 22 and 333 µA DC electric current on 24 and 48 h old *Staphyloccocus epidermidis* biofilms formed on titanium. Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. Vancomycin concentrations: 0, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg / mL (n = 2 for each). Standard deviations are indicated (I).

Table 5: Mean values, standard deviations, and number of samples for antibiotic concentration selection experiments

Treatments with Different Concentrations of Vancomycin in µg/mL					Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in ± $\log_{10}(CFU/cm^2)$	Number of Samples
	24 h Biofilm Age	24 h Duration	22 µA DC Electric Current	Control	7.43	0.21	2
				1 µg/mL	5.94	0.57	2
				2 µg/mL	6.01	0.24	2
				4 µg/mL	5.49	0.88	2
				8 µg/mL	4.96	0.33	2
				16 µg/mL	4.69	0.41	2
				32 µg/mL	3.55	0.29	2
				64 µg/mL	3.48	0.03	2
				128 µg/mL	3.15	0.07	2
				256 µg/mL	3.57	0.10	2
				512 µg/mL	3.48	0.03	2
	48 h Biofilm Age	48 h Duration	333 µA DC Electric Current	Control	7.01	1.04	2
				1 µg/mL	5.70	0.28	2
				2 µg/mL	6.06	0.86	2
				4 µg/mL	4.51	1.33	2
				8 µg/mL	5.24	0.06	2
				16 µg/mL	0.00	0.00	2
				32 µg/mL	0.00	0.00	2
				64 µg/mL	0.00	0.00	2
				128 µg/mL	0.00	0.00	2
				256 µg/mL	0.00	0.00	2
				512 µg/mL	0.00	0.00	2

LEGEND:

Control: No Antibiotic, No Current

Table 6: One-sided p-values for X less than Y comparisons using the Welch t-test for the selection of vancomycin concentration experiments

X / Y					Treatments with Different Concentrations of Vancomycin in µg/mL											
					24 h Biofilm Age											
					24 h Duration											
					22 µA DC Electric Current											
					Control	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL	128 µg/mL	256 µg/mL	512 µg/mL
Treatments with Different Concentrations of Vancomycin in µg/mL	24 h Biofilm Age	24 h Duration	22 µA DC Electric Current	Control		0.932	0.987	0.909	0.990	0.985	0.997	0.989	0.994	0.996	0.989
				1 µg/mL	0.068		0.446	0.693	0.899	0.930	0.969	0.949	0.956	0.951	0.949
				2 µg/mL	0.013	0.554		0.724	0.961	0.960	0.994	0.980	0.987	0.989	0.980
				4 µg/mL	0.091	0.307	0.276		0.723	0.797	0.914	0.904	0.918	0.901	0.904
				8 µg/mL	0.010	0.101	0.040	0.277		0.731	0.977	0.952	0.964	0.958	0.952
				16 µg/mL	0.015	0.070	0.040	0.203	0.269		0.952	0.927	0.945	0.929	0.927
				32 µg/mL	0.003	0.031	0.006	0.086	0.023	0.048		0.609	0.855	0.465	0.609
				64 µg/mL	0.011	0.051	0.020	0.096	0.048	0.073	0.391		0.973	0.201	0.500
				128 µg/mL	0.006	0.044	0.013	0.082	0.036	0.055	0.145	0.027		0.026	0.027
				256 µg/mL	0.004	0.049	0.011	0.099	0.042	0.071	0.535	0.799	0.974		0.799
				512 µg/mL	0.011	0.051	0.020	0.100	0.048	0.073	0.391	0.500	0.973	0.201	
				Control											
				1 µg/mL											
				2 µg/mL											
				4 µg/mL											
				8 µg/mL											
				16 µg/mL											
				32 µg/mL											
				64 µg/mL											
				128 µg/mL											
				256 µg/mL											
				512 µg/mL											
					48 h Biofilm Age											
					48 h Duration											
					333 µA DC Electric Current											
					Control	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL	128 µg/mL	256 µg/mL	512 µg/mL
	48 h Biofilm Age	48 h Duration	333 µA DC Electric Current	Control		0.845	0.786	0.911	0.875	0.967	0.967	0.967	0.967	0.967	0.967
				1 µg/mL	0.155		0.328	0.790	0.875	0.989	0.989	0.989	0.989	0.989	0.989
				2 µg/mL	0.214	0.672		0.841	0.800	0.968	0.968	0.968	0.968	0.968	0.968
				4 µg/mL	0.089	0.210	0.160		0.291	0.935	0.935	0.935	0.935	0.935	0.935
				8 µg/mL	0.125	0.125	0.202	0.709		0.998	0.998	0.998	0.998	0.998	0.998
				16 µg/mL	0.033	0.011	0.032	0.065	0.002		N/A	N/A	N/A	N/A	N/A
				32 µg/mL	0.033	0.011	0.032	0.065	0.002	N/A		N/A	N/A	N/A	N/A
				64 µg/mL	0.033	0.011	0.032	0.065	0.002	N/A	N/A		N/A	N/A	N/A
				128 µg/mL	0.033	0.011	0.032	0.065	0.002	N/A	N/A	N/A		N/A	N/A
				256 µg/mL	0.033	0.011	0.032	0.065	0.002	N/A	N/A	N/A	N/A		N/A
				512 µg/mL	0.033	0.011	0.032	0.065	0.002	N/A	N/A	N/A	N/A	N/A	

LEGEND:

Control: No Antibiotic, No Current

p-value ≤ 0.05

Table 7: Levene's test p-values for the selection of vancomycin concentration experiments

Levene's Test (unreliable due to low sample size)	
Stratified Group	**P-value**
24 h Biofilm Age – 24 h Duration	< 0.01
48 h Biofilm Age – 48 h Duration	< 0.01
_____	
Complete Data Set	< 0.01

4.4 Initial assessment of all treatment methods

Once the selection of 16 µg/mL of vancomycin was established, the subsequent experiments utilized this concentration to assess the variety of treatment methods. All of the data for the initial assessment of all treatment methods was analyzed as a complete data set but to simplify this section of the results, the full data set was broken down and organized into the following sections:

1. Effect of vancomycin
2. Effect of DC electric current
3. Effect of combination of vancomycin and DC electric current

Each of the sections listed above will analyze the impact of the duration of treatments, biofilm age, and the intensity of electric current (if utilized in the treatment method). These results were analyzed with the Levene test, Welch t-test, and DTK test as a complete data set with stratified groups that only allow the comparison of samples with the same biofilm age and treatment duration. Once again, the results have been separated into smaller sections for easier interpretation of the results. The overall analysis of the initial assessment is included as a separate section.

4.4.1 Effect of vancomycin on *S. epidermidis* biofilms

To determine whether a vancomycin treatment was significantly more effective than controls, the $\log_{10}(CFU/cm^2)$ values of bacteria for the initial vancomycin treatments and controls with the standard deviations are presented in Figure 9 and Table 8. To conclude statistical significance of pairwise comparisons, the one-sided p-values of the Welch t-test associated with Figure 9 and Table 8 are presented in Table 9. To observe trends of biofilm resistance and indications of increased effectiveness for specific treatments, the differences between vancomycin treatments and controls were analyzed and presented in Figure 10. To determine statistical significance of groupwise comparisons, the two-sided DTK test was applied and Table 9 presents the results which are associated with Figure 10.

By evaluating the data in Figure 9 and Table 8 as well as the results from the Welch t-test in Table 9, a significant (p-value < 0.025) decrease in number of bacteria (ranges between -2.99 to -7.47 $\log_{10}(CFU/cm^2)$) was observed in all cases when 16 μg/mL of vancomycin was used against *S. epidermidis* biofilms when compared to the controls. Interestingly, the 48 h treatments of vancomycin for the 48 h biofilm age produced quite variable results which means decreased precision of the values obtained.

The results of the DTK test presented in Table 10 indicate that the number of bacteria that survived the vancomycin treatments is significantly (95% confidence intervals, upper limit below 0) less than the controls for every duration and biofilm age except for the 48 h biofilm age stratified groups (ranges between -2.99 to -7.47 $\log_{10}(CFU/cm^2)$). The results presented in Figure 10 indicate a trend that longer (48 h duration) treatments of vancomycin are more effective at controlling and reducing the

bacteria than shorter (24 h duration) treatments. The data presented in Figure 10 also suggest that the longer the biofilm ages, the more resistant it is to vancomycin treatments for the 24 h duration. For the 48 h duration treatments, this trend is seen to a certain extent however the 24 h biofilm age was observed to be the least resistant.

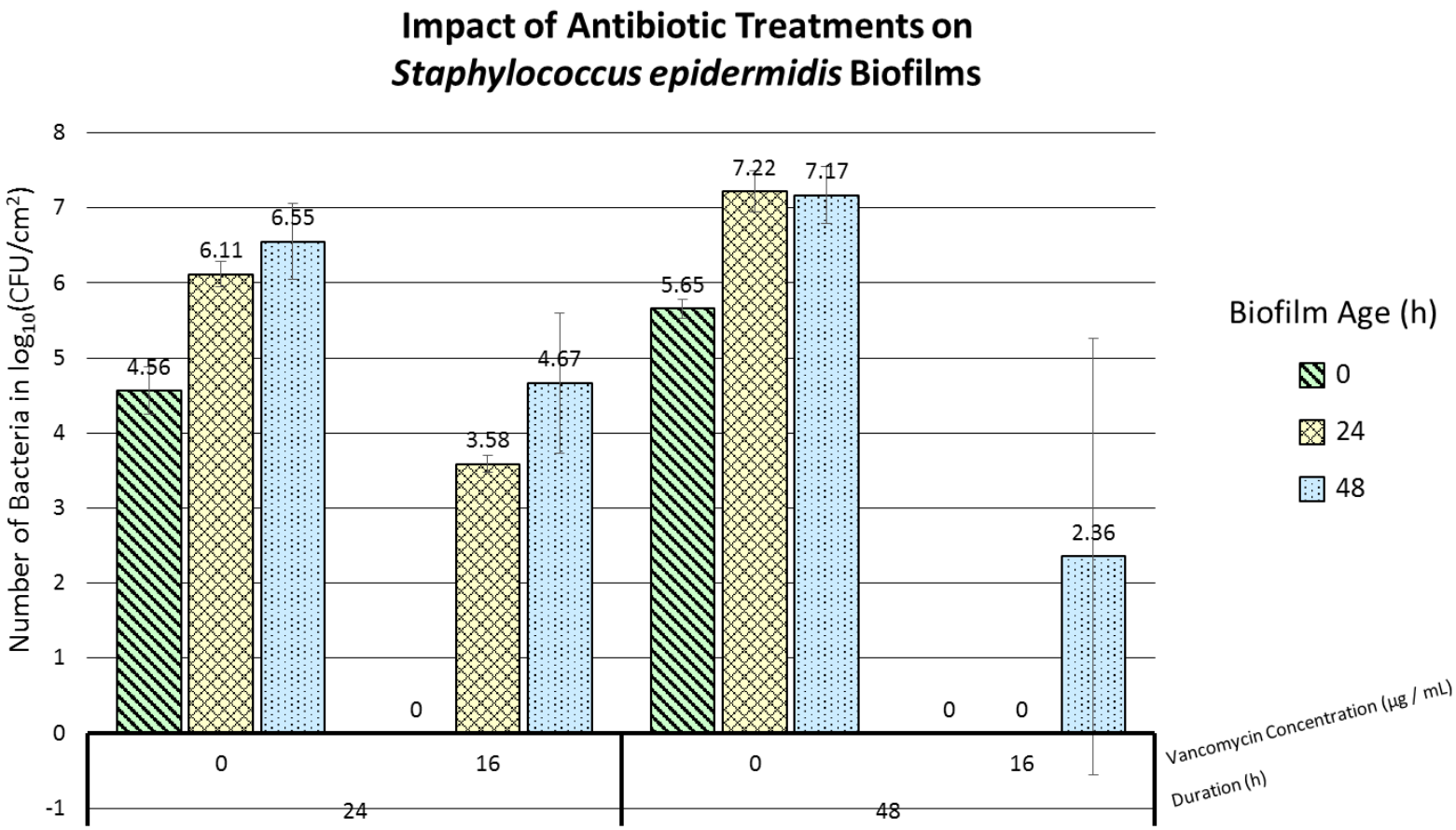

Figure 9: Assessment of treatments using vancomycin (0 or 16 µg / mL), different duration times (24h or 48h), and various *Staphyloccocus epidermidis* biofilm age (0, 24, or 48h). Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For all controls (0 µg / mL of vancomycin) and treatments n = 4. Standard deviations are indicated (I).

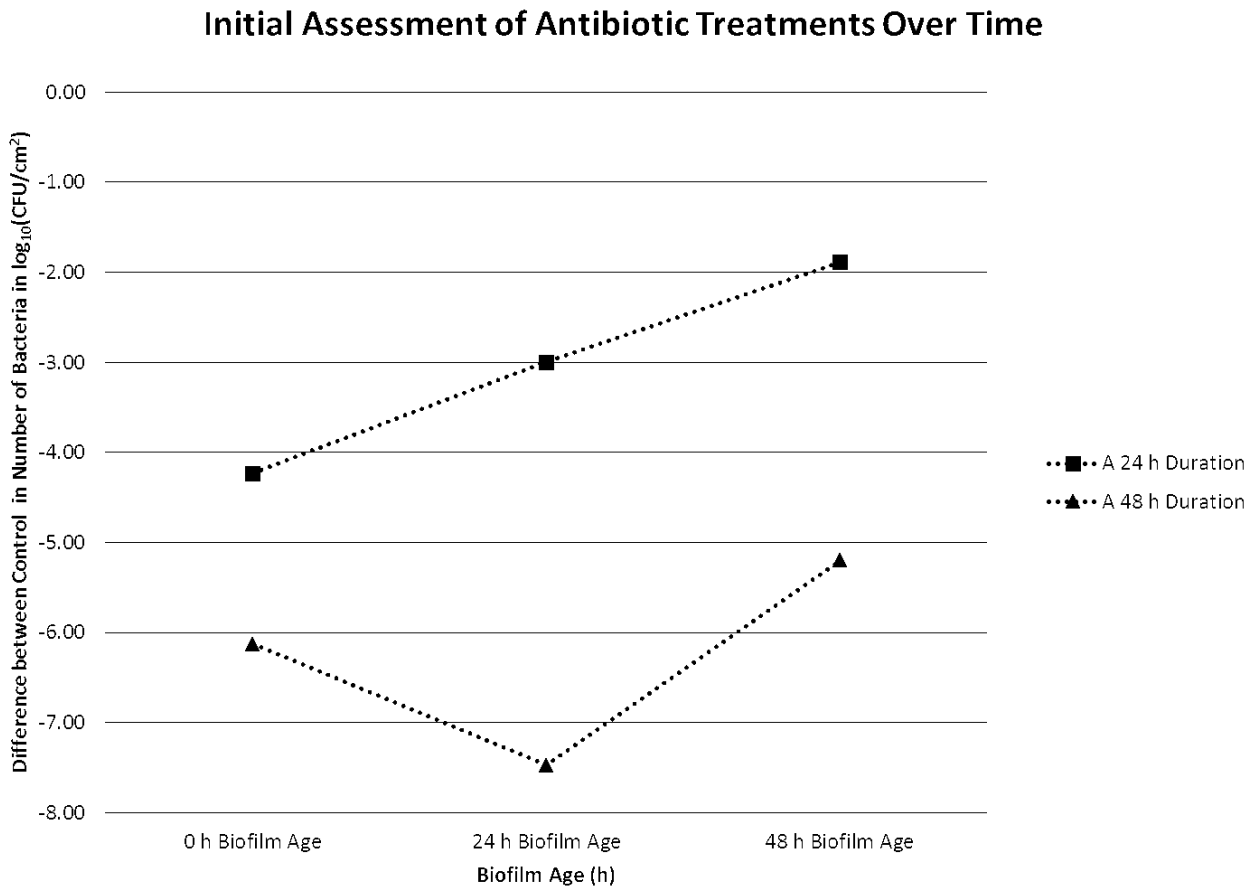

Figure 10: Mean differences between controls and vancomycin (16 µg / mL) treatments for 24 h duration treatments (A 24 h Duration) or 48 h duration treatments (A 48 h Duration). Values at different *Staphyloccocus epidermidis* biofilm ages are indicated (0, 24, or 48 h). Differences in number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For all controls (0 µg / mL of vancomycin) and vancomycin treatments n = 4.

Table 8: Mean values, standard deviations, and number of samples for vancomycin experiments

Treatments				Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in $\pm \log_{10}(CFU/cm^2)$	Number of Samples
0 h Biofilm Age	24 h Duration	Control		4.23	1.13	4
		A		0.00	0.00	4
	48 h Duration	Control		6.13	0.49	4
		A		0.00	0.00	4
24 h Biofilm Age	24 h Duration	Control		6.58	0.43	4
		A		3.58	0.11	4
	48 h Duration	Control		7.47	0.47	4
		A		0.00	0.00	4
48 h Biofilm Age	24 h Duration	Control		6.56	0.47	4
		A		4.67	0.94	4
	48 h Duration	Control		7.56	0.37	4
		A		2.36	2.91	4

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

Table 9: One-sided p-values for X less than Y comparisons using the Welch t-test for vancomycin experiments

X \ Y				Treatments											
				0 h Biofilm Age				24 h Biofilm Age				48 h Biofilm Age			
				24 h Duration		48 h Duration		24 h Duration		48 h Duration		24 h Duration		48 h Duration	
				Control	A	Control	A	Control	A	Control	A	Control	A	Control	A
Treatments	0 h Biofilm Age	24 h Duration	Control		1.000										
			A	<0.01											
		48 h Duration	Control				1.000								
			A			<0.01									
	24 h Biofilm Age	24 h Duration	Control						1.000						
			A					<0.01							
		48 h Duration	Control								1.000				
			A							<0.01					
	48 h Biofilm Age	24 h Duration	Control										0.988		
			A									0.012			
		48 h Duration	Control												0.981
			A											0.019	

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

p-value ≤ 0.05

Table 10: Two-sided results for X - Y differences using the DTK test for vancomycin experiments

X / Y				Mean Differences from Controls in Number of Bacteria in $log_{10}(CFU/cm^2)$						95% Confidence Intervals	
				0 h Biofilm Age		24 h Biofilm Age		48 h Biofilm Age			
				24 h Duration	48 h Duration	24 h Duration	48 h Duration	24 h Duration	48 h Duration		
				Control	Control	Control	Control	Control	Control	Lower Limit	Upper Limit
Treatments	0 h Biofilm Age	24 h Duration	A	-4.23						-5.06	-3.41
		48 h Duration	A		-6.13					-6.50	-5.75
	24 h Biofilm Age	24 h Duration	A			-2.99				-3.43	-2.56
		48 h Duration	A				-7.47			-7.83	-7.12
	48 h Biofilm Age	24 h Duration	A					-1.89		-4.55	0.78
		48 h Duration	A						-5.20	-13.47	3.07

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

95% Confidence Intervals that are Statistically Significant

4.4.2 Effect of electric current on *S. epidermidis* biofilms

To determine whether an electric current treatment was significantly more effective than other electric current treatments or controls, the $\log_{10}(CFU/cm^2)$ values of bacteria for the initial electric current treatments and controls with the standard deviations are presented in Figure 11 and Table 11. To conclude statistical significance of pairwise comparisons, the one-sided p-values of the Welch t-test associated with Figure 11 and Table 11 are presented in Table 12. To observe trends of biofilm resistance and indications of increased effectiveness for specific treatments, the differences between vancomycin treatments and controls were analyzed and presented in Figure 12. To determine statistical significance of groupwise comparisons, the two-sided DTK test was applied to the data and Table 13 presents the results which are associated with Figure 12.

By evaluating the data in Figure 11 and Table 11 as well as the results from the Welch t-test in Table 12, a significant (p-values < 0.05) decrease in the number of bacteria was observed in a variety of cases when the 22 and 333 μA of DC electric current was used against *S. epidermidis* biofilms when compared to the controls. For instance, the number of bacteria that survived the 333 μA treatments was significantly (p-values < 0.05) less than the control for all stratified groups (ranges between -0.58 to -1.99 $\log_{10}(CFU/cm^2)$) except for the 0 h Biofilm Age – 24 h Duration group. The number of bacteria that survived the 22 μA treatments was significantly (p-values < 0.05) less than the control in the 0 h Biofilm Age – 48 h Duration (-1.39 $\log_{10}(CFU/cm^2)$) and 24 h Biofilm Age – 24 h Duration (-0.35 $\log_{10}(CFU/cm^2)$) stratified groups. The number of bacteria that survived the 333 μA treatments was observed to be significantly (p-values <

0.05) less than the 22 μA treatments in the 24 h Biofilm Age – 24 h Duration, 24 h Biofilm Age – 24 h Duration, and 48 h Biofilm Age – 48 h Duration stratified groups.

The results of the DTK test presented in Table 13 indicate that the 333 μA treatments significantly (95% confidence intervals, upper limit below 0) reduce the number of bacteria when compared to the controls for the 24 h biofilm age stratified groups (ranges between -0.58 to -1.47 $\log_{10}(CFU/cm^2)$). The number of bacteria that survived 22 μA treatments are significantly less than (95% confidence intervals, upper limit below 0) the controls for the 0 h Biofilm Age – 48 h Duration stratified group (-1.39 $\log_{10}(CFU/cm^2)$). The results presented in Figure 12 indicate a trend that the 333 μA treatments for 48 h durations are more effective at controlling and reducing the bacteria than the other electric current treatments. It is difficult to conclusively determine which intensity of electric current is more effective for the 24 h duration treatments, however, the 333 μA treatments produced the only significant (95% confidence intervals, upper limit below 0) finding when the results were compared to the control with a 24 h biofilm age. The trends observed when comparing different current intensities in a specific stratified group are not conclusive findings as the confidence intervals do overlap. The data presented in Figure 12 suggest that the resistance to both electric current treatments stays relatively constant as the biofilm develops over time for the 24 h duration treatments. Trends observed for the 48 h duration electric current treatments suggest that the 24 h biofilm age was the most resistant, followed by the 48 h biofilm age, and finally the 0 h biofilm age.

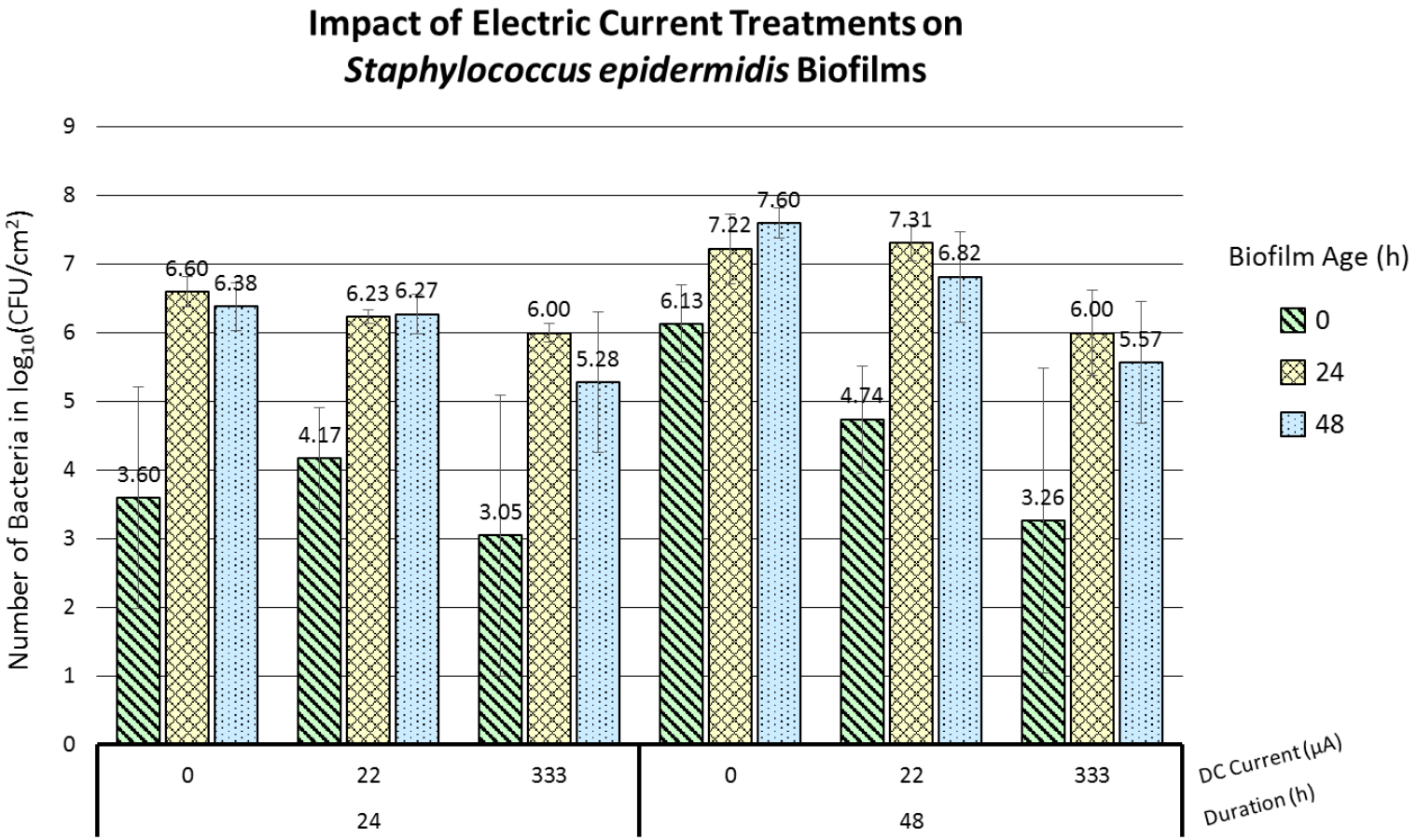

Figure 11: Assessment of all treatments using various combinations DC current (0, 22, or 333 µA), durations (24 h or 48 h), and *Staphyloccocus epidermidis* biofilm age (0, 24, or 48 h). Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For controls (0 µA DC current) n = 6-8, for all treatments n = 4. Standard deviations are indicated (I).

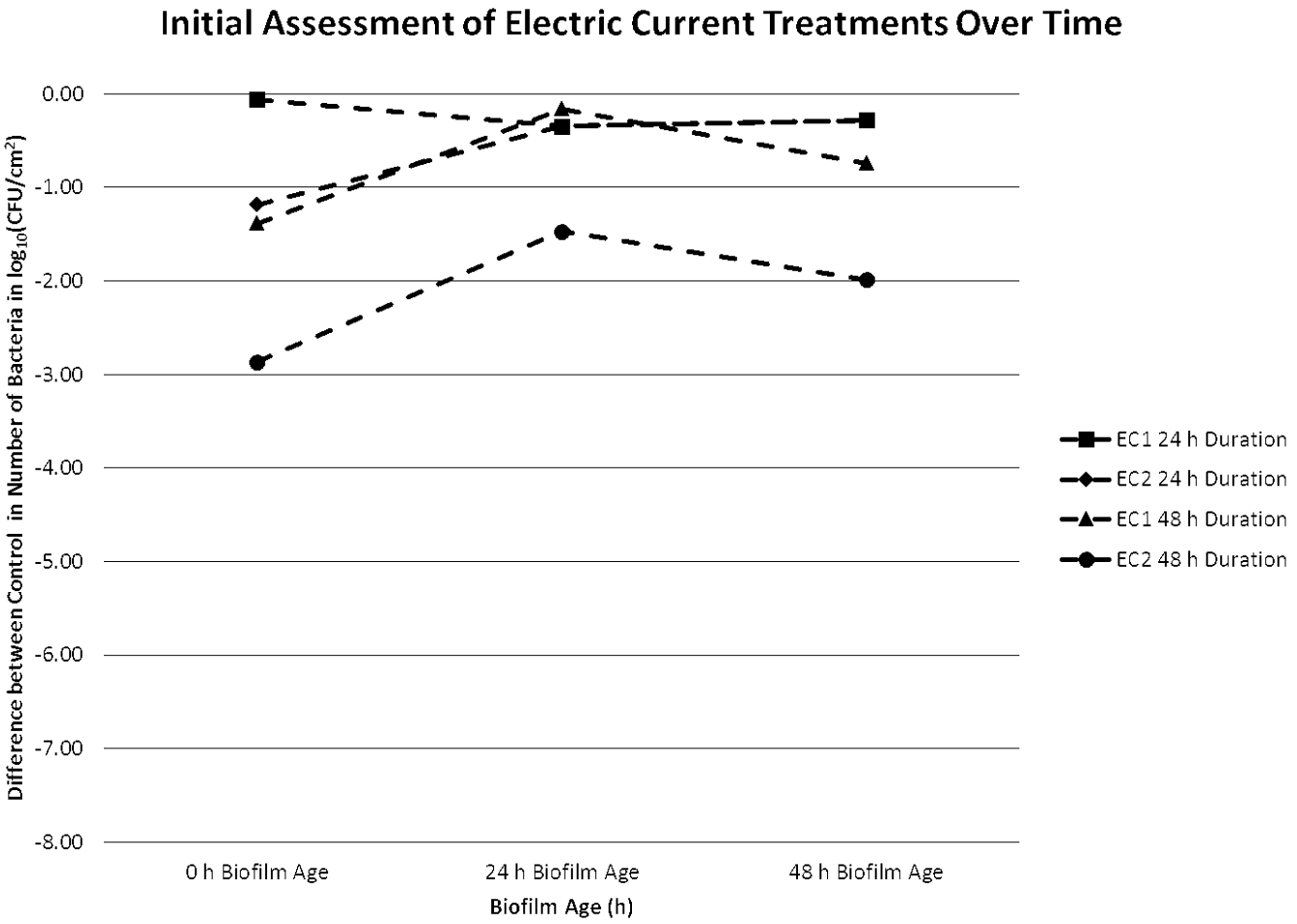

Figure 12: Mean differences between controls and 22 µA (EC1) or 333 µA (EC2) treatments and controls for 24 h duration treatments (24 h Duration) or 48 h duration treatments (48 h Duration). Values at different *Staphyloccocus epidermidis* biofilm ages are indicated (0, 24, or 48 h). Differences in number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For controls (0 µA DC current) n = 6-8, for all treatments n = 4.

Table 11: Mean values, standard deviations, and number of samples for electric current experiments

Treatments				Mean Number of Bacteria in $log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in ± $log_{10}(CFU/cm^2)$	Number of Samples
	0 h Biofilm Age	24 h Duration	Control	4.23	1.13	7
			EC1	4.17	0.74	4
			EC2	3.05	2.05	4
		48 h Duration	Control	6.13	0.49	6
			EC1	4.74	0.78	4
			EC2	3.26	2.22	4
	24 h Biofilm Age	24 h Duration	Control	6.58	0.43	8
			EC1	6.23	0.10	4
			EC2	6.00	0.13	4
		48 h Duration	Control	7.47	0.47	7
			EC1	7.31	0.27	4
			EC2	6.00	0.62	4
	48 h Biofilm Age	24 h Duration	Control	6.56	0.47	8
			EC1	6.27	0.29	4
			EC2	5.28	1.02	4
		48 h Duration	Control	7.56	0.37	6
			EC1	6.82	0.66	4
			EC2	5.57	0.89	4

LEGEND:

Control: No Antibiotic, No Current

EC1: 22 μA DC Electric Current Alone

EC2: 333 μA DC Electric Current Alone

Table 12: One-sided p-values for X less than Y comparisons using the Welch t-test for electric current experiments

X	Y	Treatments	Control	EC1	EC2	Control	EC1	EC2	Control	EC1	EC2	Control	EC1	EC2	Control	EC1	EC2	Control	EC1	EC2
			0 h Biofilm Age						24 h Biofilm Age						48 h Biofilm Age					
			24 h Duration			48 h Duration			24 h Duration			48 h Duration			24 h Duration			48 h Duration		
Treatments	0 h Biofilm Age	24 h Duration	Control		0.553	0.832														
			EC1	0.447		0.817														
			EC2	0.168	0.183															
		48 h Duration	Control					0.984	0.959											
			EC1				**0.016**		0.859											
			EC2				**0.041**	0.141												
	24 h Biofilm Age	24 h Duration	Control								0.997	1.000								
			EC1							**<0.01**		0.983								
			EC2							**<0.01**	**0.017**									
		48 h Duration	Control											0.808	0.994					
			EC1										0.192		0.992					
			EC2										**<0.01**	**<0.01**						
	48 h Biofilm Age	24 h Duration	Control														0.920	0.958		
			EC1													0.080		0.927		
			EC2													**0.042**	0.073			
		48 h Duration	Control																	0.947	0.991
			EC1															0.053		0.966
			EC2															**<0.01**	**0.034**	

LEGEND:

Control: No Antibiotic, No Current

EC1: 22 µA DC Electric Current Alone

EC2: 333 µA DC Electric Current Alone

p-value ≤ 0.05

Table 13: Two-sided results for X - Y differences using the DTK test for electric current experiments

X / Y				Mean Difference from Controls in Number of Bacteria in $log_{10}(CFU/cm^2)$						95% Confidence Intervals	
				0 h Biofilm Age		24 h Biofilm Age		48 h Biofilm Age			
				24 h Duration	48 h Duration	24 h Duration	48 h Duration	24 h Duration	48 h Duration		
				Control	Control	Control	Control	Control	Control	Lower Limit	Upper Limit
Treatments	0 h Biofilm Age	24 h Duration	EC1	-0.06						-1.51	1.38
			EC2	-1.18						-4.54	2.17
		48 h Duration	EC1		**-1.39**					-2.69	-0.08
			EC2		-2.86					-6.43	0.71
	24 h Biofilm Age	24 h Duration	EC1			-0.35				-0.77	0.07
			EC2			**-0.58**				-1.03	-0.13
		48 h Duration	EC1				-0.16			-0.72	0.39
			EC2				**-1.47**			-2.52	-0.42
	48 h Biofilm Age	24 h Duration	EC1					-0.28		-1.28	0.72
			EC2					-1.28		-4.17	1.62
		48 h Duration	EC1						-0.74	-2.67	1.19
			EC2						-1.99	-4.55	0.57

LEGEND:

Control: No Antibiotic, No Current

EC1: 22 µA DC Electric Current Alone

EC2: 333 µA DC Electric Current Alone

95% Confidence Intervals that are Statistically Significant

4.4.3 Effect of electric current in combination with antibiotics on *S. epidermidis* biofilms

To determine whether an electric current in combination with vancomycin treatment was significantly more effective than other combination treatment or control, the $\log_{10}(CFU/cm^2)$ values of bacteria for initial antibiotic in combination with electric current treatments and controls with the standard deviations are presented in Figure 13 and Table 14. To conclude statistical significance of pairwise comparisons, the one-sided p-values of the Welch t-test associated with Figure 13 and Table 14 are presented in Table 15. To observe trends of biofilm resistance and indications of increased effectiveness for specific treatments, the differences between vancomycin treatments and controls were analyzed and presented in Figure 14. To determine statistical significance of groupwise comparisons, the two-sided DTK test was applied and Table 16 presents the results which are associated with Figure 14.

By evaluating the data in Figure 13 and Table 14 as well as the results from the Welch t-test in Table 15, a significant (p-values < 0.05) decrease in number of bacteria (ranges between -1.99 to -7.47 $\log_{10}(CFU/cm^2)$) was observed in all cases when 16 μg/mL of vancomycin in combination with the 22 or 333 μA of DC electric current was used against *S. epidermidis* biofilms when compared to controls. In addition, the number of bacteria that survived the combination treatments with 333 μA was significantly (p-values < 0.05) less than the number of bacteria that survived the combination treatments with 22 μA in the 48 h Biofilm Age – 24 h Duration stratified group (-2.08 $\log_{10}(CFU/cm^2)$).

The results of the DTK test presented in Table 16 indicate that the number of bacteria in all combination treatments are significantly (95% confidence intervals, upper limit below 0) less than the controls (ranges between -1.99 to -7.47 $\log_{10}(CFU/cm^2)$) except for the combination treatment with the 333 μA in the 48 h Biofilm Age – 24 h Duration stratified group and the combination treatment with 22 μA in the 48 h Biofilm Age – 48 h Duration stratified group. The trends presented in Figure 14 suggest that combination treatments with 333 μA for 48 h durations are more effective at controlling and reducing the bacteria than the other combination treatments. Results from Figure 14 also suggest that longer (48 h) duration treatments are more effective than shorter (24 h) treatments when comparing treatments to controls. Trends also show that the 333 μA in combination with 16 μg/mL of vancomycin treatments are more effective than the 22 μA combination treatments. The trends observed when comparing different current intensities in a specific stratified group are not conclusive findings as the confidence intervals do overlap. The data presented in Figure 14 also suggest that the resistance of the biofilm to 24 h duration treatments increases over time with the 22 μA combination treatments and stays constant with the 333 μA combination treatments. The results in Figure 14 also suggest that for the 48 duration treatments, the resistance of the biofilm over time stays constant with the 22 μA combination treatments. For the 333 μA combination 48 h treatments, 24 h old biofilms are the least resistant, whereas the 0 h old biofilms are the most resistant. It is again important to note that the combination treatments for a 48 h duration produced quite variable results which means decreased precision of the values obtained.

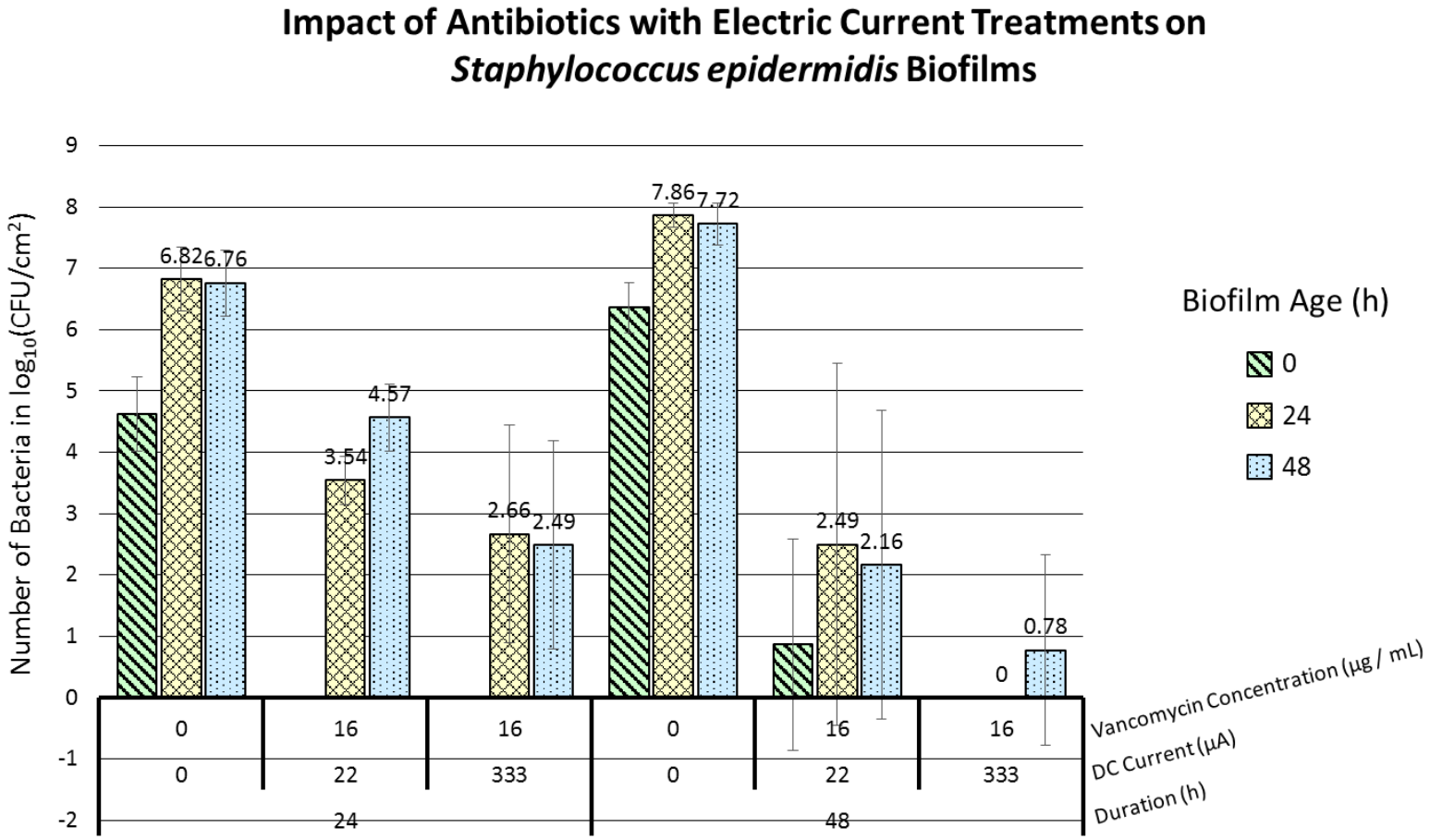

Figure 13: Assessment of treatments using various combinations of vancomycin (0 or 16 µg / mL), DC current (0, 22, or 333 µA), durations (24 h or 48 h), and *Staphyloccocus epidermidis* biofilm age (0, 24, or 48 h). Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For controls (0 µg / mL of vancomycin and 0 µA DC current) n = 7-8, for all treatments n = 4. Standard deviations are indicated (I).

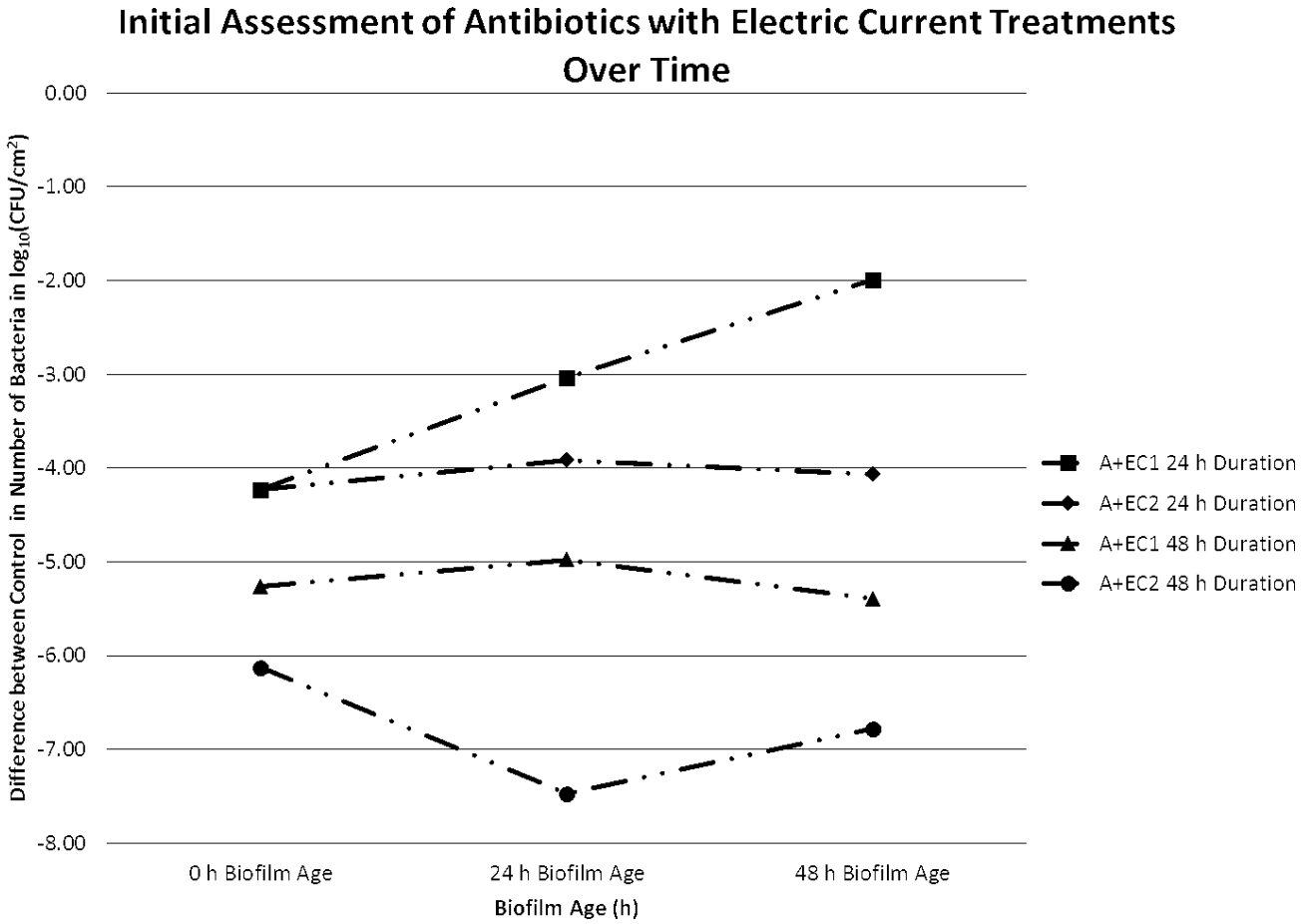

Figure 14: Mean differences between controls and vancomycin (16 µg / mL) with 22 µA (A+EC1) or vancomycin (16 µg / mL) with 333 µA (A+EC2) for 24 h duration treatments (24 h Duration) or 48 h duration treatments (48 h Duration). Values at different *Staphyloccocus epidermidis* biofilm ages are indicated (0, 24, or 48 h). Differences in number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For controls (0 µg / mL of vancomycin and 0 µA DC current) n = 7-8. for all treatments n = 4.

Table 14: Mean values, standard deviations, and number of samples for antibiotics with electric current experiments

Treatments				Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in ± $\log_{10}(CFU/cm^2)$	Number of Samples
	0 h Biofilm Age	24 h Duration	Control	4.23	1.13	8
			A+EC1	0.00	0.00	4
			A+EC2	0.00	0.00	4
		48 h Duration	Control	6.13	0.49	8
			A+EC1	0.86	1.73	4
			A+EC2	0.00	0.00	4
	24 h Biofilm Age	24 h Duration	Control	6.58	0.43	7
			A+EC1	3.54	0.39	4
			A+EC2	2.66	1.78	4
		48 h Duration	Control	7.47	0.47	7
			A+EC1	2.49	2.95	4
			A+EC2	0.00	0.00	4
	48 h Biofilm Age	24 h Duration	Control	6.56	0.47	7
			A+EC1	4.57	0.55	4
			A+EC2	2.49	1.70	4
		48 h Duration	Control	7.56	0.37	8
			A+EC1	2.16	2.51	4
			A+EC2	0.78	1.55	4

LEGEND:

Control: No Antibiotic, No Current

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

Table 15: One-sided p-values for X less than Y comparisons using the Welch t-test for antibiotics with electric current experiments

X \ Y				Treatments																	
				0 h Biofilm Age						24 h Biofilm Age						48 h Biofilm Age					
				24 h Duration			48 h Duration			24 h Duration			48 h Duration			24 h Duration			48 h Duration		
				Control	A+EC1	A+EC2	Control	A+EC1	A+EC2	Control	A+EC1	A+EC2	Control	A+EC1	A+EC2	Control	A+EC1	A+EC2	Control	A+EC1	A+EC2
Treatments	0 h Biofilm Age	24 h Duration	Control		1.000	1.000															
			A+EC1	<0.01		N/A															
			A+EC2	<0.01	N/A																
		48 h Duration	Control					0.996	1.000												
			A+EC1				<0.01		0.804												
			A+EC2				<0.01	0.196													
	24 h Biofilm Age	24 h Duration	Control								1.000	0.990									
			A+EC1							<0.01		0.800									
			A+EC2							0.010	0.200										
		48 h Duration	Control											0.979	1.000						
			A+EC1										0.021		0.905						
			A+EC2										<0.01	0.095							
	48 h Biofilm Age	24 h Duration	Control														0.999	0.992			
			A+EC1													<0.01		0.956			
			A+EC2													<0.01	0.044				
		48 h Duration	Control																	0.989	0.999
			A+EC1																0.011		0.805
			A+EC2																<0.01	0.195	

LEGEND:

Control: No Antibiotic, No Current

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

p-value ≤ 0.05

Table 16: Two-sided results for X – Y differences using the DTK test for antibiotics with electric current experiments

X / Y				Mean Difference from Controls in Number of Bacteria in $\log_{10}(CFU/cm^2)$						95% Confidence Intervals	
				0 h Biofilm Age		24 h Biofilm Age		48 h Biofilm Age			
				24 h Duration	48 h Duration	24 h Duration	48 h Duration	24 h Duration	48 h Duration		
				Control	Control	Control	Control	Control	Control	Lower Limit	Upper Limit
Treatments	0 h Biofilm Age	24 h Duration	A+EC1	**4.23**						-5.06	-3.41
			A+EC2	**4.23**						-5.06	-3.41
		48 h Duration	A+EC1		**5.26**					-8.05	-2.48
			A+EC2		**6.13**					-6.50	-5.75
	24 h Biofilm Age	24 h Duration	A+EC1			**3.04**				-3.86	-2.21
			A+EC2			**3.91**				-7.30	-0.52
		48 h Duration	A+EC1				**4.98**			-9.71	-0.25
			A+EC2				**7.47**			-7.83	-7.12
	48 h Biofilm Age	24 h Duration	A+EC1					**1.99**		-3.63	-0.36
			A+EC2					-4.06		-8.82	0.69
		48 h Duration	A+EC1						-5.39	-12.54	1.75
			A+EC2						**6.78**	-11.21	-2.36

LEGEND:

Control: No Antibiotic, No Current

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

95% Confidence Intervals that are Statistically Significant

4.4.4 Overall initial analysis of all treatment methods

To determine whether treatments were significantly more effective than other treatments or controls, the $\log_{10}(CFU/cm^2)$ values of bacteria exposed to all initial treatments and controls with the standard deviations are presented in Figure 15 and Table 17. To conclude statistical significance of pairwise comparisons, the one-sided p-values of the Welch t-test associated with Figure 15 and Table 17 are presented in Table 18. To observe trends of biofilm resistance and indications of increased effectiveness for specific treatments, the differences between vancomycin treatments and controls were analyzed and presented in Figure 16. To determine statistical significance of groupwise comparisons, the two-sided DTK test was applied to the data associated with Figure 16 and the results are presented in Table 19. To evaluate whether the samples have unequal variances, the Levene test was applied to the data and the results are indicated in Table 20.

The results for the Levene test presented in Table 20 indicate that the p-value for this complete data set was less than 0.01, concluding unequal variances among samples. For the stratified groups, the Levene test resulted in p-values less than 0.01 for all but one group. The only stratified group that resulted in a p-value greater than 0.01 was the 0 h Biofilm Age – 24 h Duration samples. Important and significant (p-values < 0.05) decreases in number of bacteria surviving for all initial treatments when compared to the respective controls were stated in previous sections (Sections 4.4.1 to 4.4.3). This section will focus on differences observed between different treatments within a specific stratified group (specific biofilm age and treatment duration) that have not already been

addressed by evaluating the data in Figure 15 and Table 17 as well as the results from the Welch t-test in Table 18.

Within each stratified group, utilizing vancomycin and both combination treatments always resulted in a significant ($p < 0.05$) reduction of bacteria (more effective at treating the biofilm) when compared to all of the 22 μA treatments (ranges -0.28 between to -7.31 $\log_{10}(CFU/cm^2)$). For all stratified groups, number of bacteria that survived the 333 μA combination treatments was significantly ($p < 0.05$) less than the 333 μA treatments (ranges between -3.34 to -6.00 $\log_{10}(CFU/cm^2)$). For all stratified groups except for 0 h Biofilm Age – 48 h Duration and 48 h Biofilm Age – 24 h Duration, the number of bacteria that survived the 22 μA combination treatments was significantly ($p < 0.05$) less than the 333 μA treatments (ranges between -2.46 to -3.51 $\log_{10}(CFU/cm^2)$). Also, for all stratified groups except for 48 h Biofilm Age – 24 h Duration, the number of bacteria that survived the vancomycin treatments was significantly ($p < 0.05$) less than the 333 μA treatments (ranges between -2.24 to -6.00 $\log_{10}(CFU/cm^2)$). It was only in the 48 h Biofilm Age – 24 h Duration stratified group that the effectiveness of the antibiotic was increased by an electric current where the number of bacteria that survived the 333 μA combination treatments was significantly (p-value < 0.05) less than the 16 μg/mL vancomycin treatments (-2.18 $\log_{10}(CFU/cm^2)$).

To demonstrate trends observed with different treatment durations as well as the increase or decrease of resistance to treatments which was dependent on biofilm age, the data presented in Figure 16 and the results from the DTK test presented in Table 19 have been utilized. The DTK analysis of the full data set allows for the visualization of the difference of means of all treatment methods from their respective controls. This will

permit an analysis of trends as well as statistical significance to select the treatment observed to be the most effective within a stratified group. Through the analysis of Figure 16, one can make observations of the trends seen with different treatment durations and biofilm ages on resistance when comparing the differences between the treatments with the appropriate controls. Electric current treatments were the least effective at treating the biofilms (both 24 and 48 h durations at all biofilm ages) while the 48 h duration antibiotic and all combination treatments were the most effective (for all biofilm ages). When observing all of the 24 h duration treatments, the vancomycin and 22 μA combination treatments follow the same resistance trend (increasing with increasing biofilm age), while biofilm resistance to the 333 μA combination treatments was relatively constant as biofilm age increased, suggesting that a bioelectric effect was present.

For each stratified group the most effective treatment was selected from Table 19 based first on whether it was significantly different from the appropriate control (95% confidence interval, upper limit less than 0) and then by the largest difference. Table 21 lists the treatment methods selected as most significant for each stratified group. It is important to note that the selections of the most effective treatments are not conclusive findings as the confidence intervals of the different treatments do overlap, however, it does allow for one to assess effectiveness of treatments to a certain extent. The 333 μA combination treatment method was the most frequently identified as the best treatment when comparing the treatments to their respective controls. Since the combination treatments for a 48 h duration produced variable results with specific parameters, the next section states results from a separate experiment that more precisely evaluated the 24 h duration treatments comparing antibiotic treatments and combination treatments.

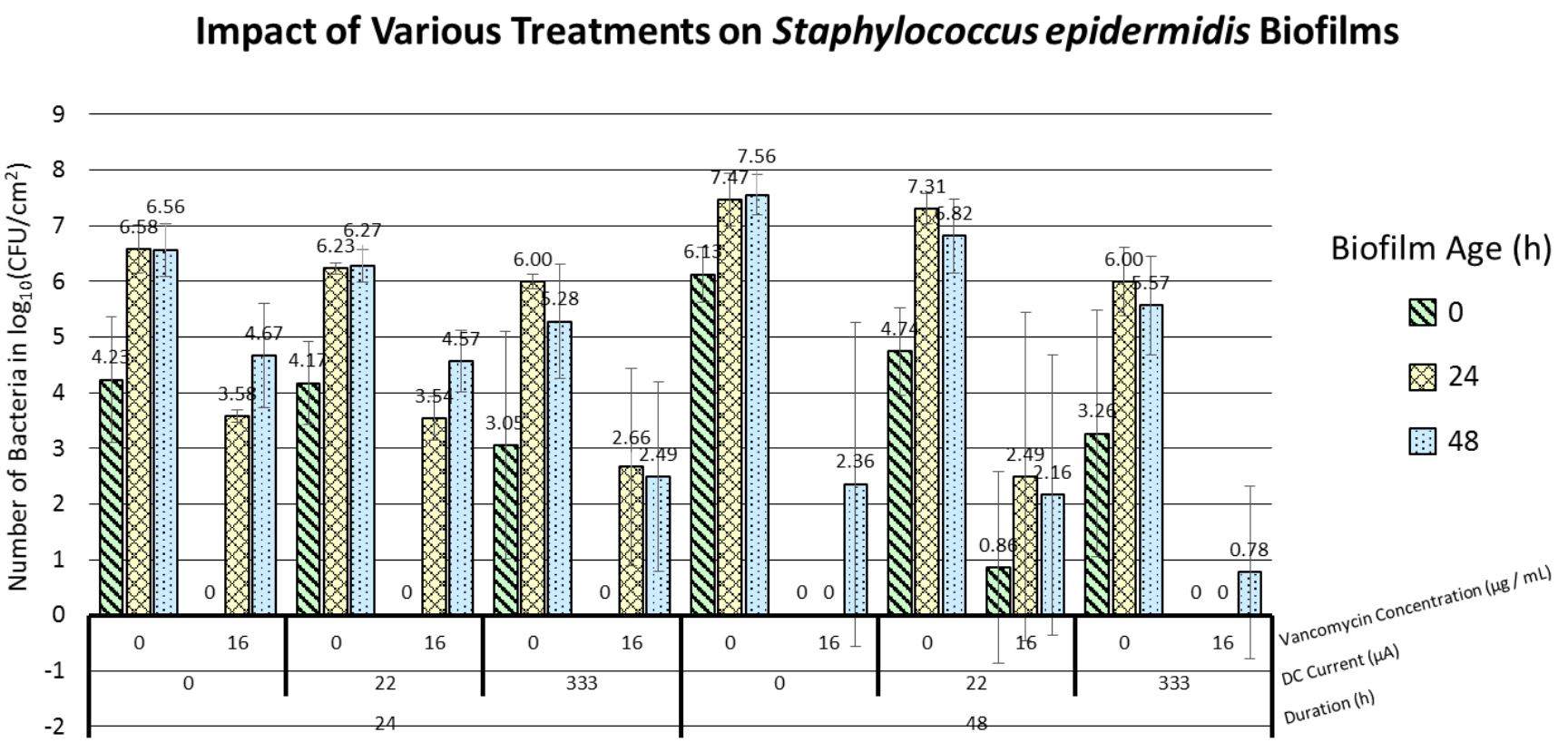

Figure 15: Assessment of all treatments using various combinations of vancomycin (0 or 16 µg / mL), DC current (0, 22, or 333 µA), durations (24h or 48h), and *Staphyloccocus epidermidis* biofilm age (0, 24, or 48 h). Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For 24 h duration controls (0 µg / mL of vancomycin and 0 µA DC current) n = 19, for 48 h duration controls (0 µg / mL of vancomycin and 0 µA DC current) n = 18, and for all treatments n = 4. Standard deviations are indicated (I).

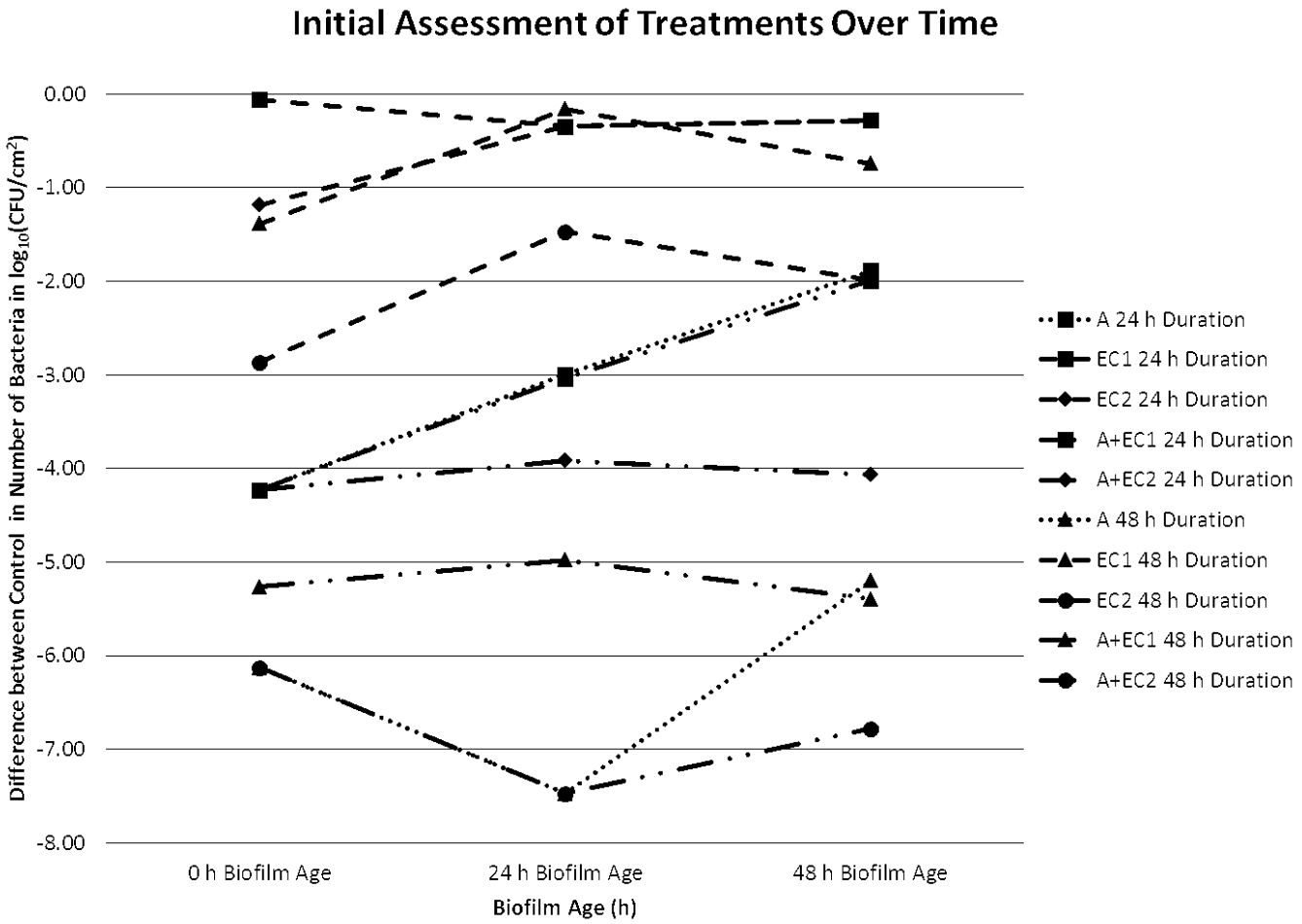

Figure 16: Mean differences between controls and 16 µg / mL of vancomycin (A), 22 µA (EC1), 333 µA (EC2), vancomycin (16 µg / mL) with 22 µA (A+EC1), or vancomycin (16 µg / mL) with 333 µA (A+EC2) for 24 h duration treatments (24 h Duration) or 48 h duration treatments (48 h Duration). Values at different *Staphyloccocus epidermidis* biofilm ages are indicated (0, 24, or 48 h). Differences in number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For 24 h duration controls (0 µg / mL of vancomycin and 0 µA DC current) n = 19, for 48 h duration controls (0 µg / mL of vancomycin and 0 µA DC current) n = 18, and for all treatments n = 4.

Table 17: Mean values, standard deviations, and number of samples for all initial experiments

Treatments				Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in $\pm \log_{10}(CFU/cm^2)$	Number of Samples
0 h Biofilm Age	24 h Duration		Control	4.23	1.13	19
			A	0.00	0.00	4
			EC1	4.17	0.74	4
			EC2	3.05	2.05	4
			A+EC1	0.00	0.00	4
			A+EC2	0.00	0.00	4
	48 h Duration		Control	6.13	0.49	18
			A	0.00	0.00	4
			EC1	4.74	0.78	4
			EC2	3.26	2.22	4
			A+EC1	0.86	1.73	4
			A+EC2	0.00	0.00	4
24 h Biofilm Age	24 h Duration		Control	6.58	0.43	19
			A	3.58	0.11	4
			EC1	6.23	0.10	4
			EC2	6.00	0.13	4
			A+EC1	3.54	0.39	4
			A+EC2	2.66	1.78	4
	48 h Duration		Control	7.47	0.47	18
			A	0.00	0.00	4
			EC1	7.31	0.27	4
			EC2	6.00	0.62	4
			A+EC1	2.49	2.95	4
			A+EC2	0.00	0.00	4
48 h Biofilm Age	24 h Duration		Control	6.56	0.47	19
			A	4.67	0.94	4
			EC1	6.27	0.29	4
			EC2	5.28	1.02	4
			A+EC1	4.57	0.55	4
			A+EC2	2.49	1.70	4
	48 h Duration		Control	7.56	0.37	18
			A	2.36	2.91	4
			EC1	6.82	0.66	4
			EC2	5.57	0.89	4
			A+EC1	2.16	2.51	4
			A+EC2	0.78	1.55	4

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

EC1: 22 µA DC Electric Current Alone

EC2: 333 µA DC Electric Current Alone

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

Table 18: One-sided p-values for X less than Y comparisons using the Welch t-test for all initial experiments

X	Y	Treatments	0 h Biofilm Age												24 h Biofilm Age												48 h Biofilm Age											
			24 h Duration						48 h Duration						24 h Duration						48 h Duration						24 h Duration						48 h Duration					
			Control	A	EC1	EC2	A+EC1	A+EC2	Control	A	EC1	EC2	A+EC1	A+EC2	Control	A	EC1	EC2	A+EC1	A+EC2	Control	A	EC1	EC2	A+EC1	A+EC2	Control	A	EC1	EC2	A+EC1	A+EC2	Control	A	EC1	EC2	A+EC1	A+EC2
0 h Biofilm Age	24 h Duration	Control		1.000	0.553	0.832	1.000	1.000																													
		A	<0.01		<0.01	0.029	N/A	N/A																													
		EC1	0.447	0.999		0.817	0.999	0.999																													
		EC2	0.168	0.971	0.183		0.971	0.971																													
		A+EC1	<0.01	N/A	<0.01	0.029		N/A																													
		A+EC2	<0.01	N/A	<0.01	0.029	N/A																														
	48 h Duration	Control								1.000	0.984	0.959	0.996	1.000																							
		A							<0.01		<0.01	0.030	0.196	N/A																							
		EC1							0.016	0.999		0.859	0.993	0.994																							
		EC2							0.041	0.970	0.141		0.929	0.970																							
		A+EC1							<0.01	0.804	<0.01	0.071		0.804																							
		A+EC2							<0.01	N/A	<0.01	0.030	0.196																								
	24 h Duration	Control														1.000	0.997	1.000	1.000	0.990																	
		A													<0.01		<0.01	<0.01	0.579	0.812																	
		EC1													<0.01	1.000		0.983	1.000	0.986																	
		EC2													<0.01	1.000	0.017		1.000	0.984																	
		A+EC1													<0.01	0.421	<0.01	<0.01		0.800																	
		A+EC2													0.010	0.188	0.014	0.016	0.200																		
	48 h Duration	Control																				1.000	0.808	0.994	0.979	1.000											
		A																			<0.01		<0.01	<0.01	0.095	N/A											
		EC1																			0.192	1.000		0.992	0.997	1.000											
		EC2																			<0.01	1.000	<0.01		0.952	1.000											
		A+EC1																			0.021	0.905	0.023	0.048		0.905											
		A+EC2																			<0.01	N/A	<0.01	<0.01	0.095												
24 h Biofilm Age	24 h Duration	Control																										0.988	0.920	0.958	0.999	0.992						
		A																									0.012		0.018	0.205	0.571	0.961						
		EC1																									0.080	0.982		0.927	0.998	0.990						
		EC2																									0.042	0.795	0.073		0.862	0.981						
		A+EC1																									<0.01	0.429	<0.01	0.138		0.956						
		A+EC2																									<0.01	0.039	<0.01	0.019	0.044						
	48 h Duration	Control																															0.981	0.947	0.991	0.989	0.999
		A																															<0.019		0.026	0.056	0.539	0.807
		EC1																															0.053	0.974		0.966	0.985	0.999
		EC2																															<0.01	0.944	0.034		0.966	0.998
		A+EC1																															0.011	0.461	0.015	0.034		0.805
		A+EC2																															<0.01	0.193	<0.01	<0.01	0.195	

LEGEND:

Control: No Antibiotic, No Current
A: 16 µg/mL of Vancomycin
EC1: 22 µA DC Electric Current Alone
EC2: 333 µA DC Electric Current Alone
A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current
A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current
p-value ≤ 0.05

Table 19: Two-sided results for X - Y differences using the DTK test for all initial experiments

X			Y	Mean Difference from Controls in Number of Bacteria in $\log_{10}$(CFU/cm^2)						95% Confidence Intervals	
				0 h Biofilm Age		24 h Biofilm Age		48 h Biofilm Age			
				24 h Duration	48 h Duration	24 h Duration	48 h Duration	24 h Duration	48 h Duration		
				Control	Control	Control	Control	Control	Control	Lower Limit	Upper Limit
Treatments	0 h Biofilm Age	24 h Duration	A	**-4.23**						-5.06	-3.41
			EC1	-0.06						-1.51	1.38
			EC2	-1.18						-4.54	2.17
			A+EC1	**-4.23**						-5.06	-3.41
			A+EC2	**-4.23**						-5.06	-3.41
		48 h Duration	A		**-6.13**					-6.50	-5.75
			EC1		**-1.39**					-2.69	-0.08
			EC2		-2.86					-6.43	0.71
			A+EC1		**-5.26**					-8.05	-2.48
			A+EC2		**-6.13**					-6.50	-5.75
	24 h Biofilm Age	24 h Duration	A			**-2.99**				-3.43	-2.56
			EC1			-0.35				-0.77	0.07
			EC2			**-0.58**				-1.03	-0.13
			A+EC1			**-3.04**				-3.86	-2.21
			A+EC2			**-3.91**				-7.30	-0.52
		48 h Duration	A				**-7.47**			-7.83	-7.12
			EC1				-0.16			-0.72	0.39
			EC2				**-1.47**			-2.52	-0.42
			A+EC1				**-4.98**			-9.71	-0.25
			A+EC2				**-7.47**			-7.83	-7.12
	48 h Biofilm Age	24 h Duration	A					-1.89		-4.55	0.78
			EC1					-0.28		-1.28	0.72
			EC2					-1.28		-4.17	1.62
			A+EC1					**-1.99**		-3.63	-0.36
			A+EC2					-4.06		-8.82	0.69
		48 h Duration	A						-5.20	-13.47	3.07
			EC1						-0.74	-2.67	1.19
			EC2						-1.99	-4.55	0.57
			A+EC1						-5.39	-12.54	1.75
			A+EC2						**-6.78**	-11.21	-2.36

LEGEND:

Control: No Antibiotic, No Current
A: 16 µg/mL of Vancomycin
EC1: 22 µA DC Electric Current Alone
EC2: 333 µA DC Electric Current Alone
A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current
A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current
95% Confidence Intervals that are Statistically Significant

Table 20: Levene's test p-values for all initial experiments

Levene's Test	
Stratified Group	**P-value**
0 h Biofilm Age – 24 h Duration	0.10
24 h Biofilm Age – 24 h Duration	< 0.01
48 h Biofilm Age – 24 h Duration	< 0.01
0 h Biofilm Age – 48 h Duration	< 0.01
24 h Biofilm Age – 48 h Duration	< 0.01
48 h Biofilm Age – 48 h Duration	< 0.01
———————————	
Complete Data Set	< 0.01

Table 21: Selection of most effective treatment for the initial experiments within each stratified group based on significant DTK test results with 95% confidence intervals

Stratified Group	Most Effective Treatment(s)	Mean Difference from Controls in Number of Bacteria in $\log_{10}(CFU/cm^2)$
0 h Biofilm Age – 24 h Duration	A	-4.23
	A+EC1	-4.23
	A+EC2	-4.23
0 h Biofilm Age – 48 h Duration	A	-6.13
	A+EC2	-6.13
24 h Biofilm Age – 24 h Duration	A+EC2	-3.91
24 h Biofilm Age – 48 h Duration	A	-7.47
	A+EC2	-7.47
48 h Biofilm Age – 24 h Duration	A+EC1	-1.99
48 h Biofilm Age – 48 h Duration	A+EC2	-6.78

LEGEND:

A: 16 µg/mL of Vancomycin

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

4.5 Increased precision assessment of the bioelectric effect

After evaluating the results from the initial assessment of treatments experiments, a more precise method of counting the CFU was utilized to confirm the presence a bioelectric effect when using vancomycin in combination with electric currents against *S. epidermidis* biofilms. This method utilized larger volumes when plating viable cells isolated from the biofilm sample.

To assess whether treatments were significantly more effective than other treatments or controls, the $\log_{10}(CFU/cm^2)$ values of bacteria for all treatments and controls with the standard deviations are presented in Figure 17 and Table 22. To conclude statistical significance of pairwise comparisons, the one-sided p-values of the Welch t-test associated with Figure 17 and Table 22 are presented in Table 23. To observe trends of biofilm resistance and indications of increased effectiveness for specific treatments, Figure 18 shows the differences between all treatments and controls. To determine statistical significance of groupwise comparisons, the two-sided DTK test was applied to the data associated with Figure 18 and is presented in Table 24. To evaluate whether the samples have unequal variances, the Levene test was applied to the data and the results are indicated in Table 25.

The results for the Levene test presented in Table 25 indicate that the p-value for this complete data set was less than 0.01, concluding unequal variances among samples. For the stratified groups, the Levene test resulted in p-values less than 0.01 for the 24 h Biofilm Age – 24 h Duration stratified group. For the 0 h Biofilm Age – 24 h Duration and 48 h Biofilm Age – 24 h Duration groups, the p-value for the Levene test was greater than 0.01. The Welch t-test results listed in Table 23 allowed for the assessment of the

effectiveness of treatments when comparing with the appropriate controls and with other treatments within a specific stratified group.

For the increased precision experiments, the number of bacteria that survived for all treatments was significantly (p-values < 0.01) less than the controls in all stratified groups (ranges between -1.60 to -3.55 $\log_{10}(CFU/cm^2)$). In the 24 h Biofilm Age – 24 h Duration stratified group, the 333 μA combination treatment was significantly (p-value = 0.012) more effective at reducing the biofilm by -1.12 $\log_{10}(CFU/cm^2)$ when compared to the vancomycin treatments. This was the most significant impact of the bioelectric effect observed and is the main focus of Figure 19 which indicates the number of bacteria in $\log_{10}(CFU/cm^2)$. In addition, the most significant impact of an electric current on the effectiveness of vancomycin in terms of number of surviving bacteria is presented in Figure 20. The number of bacteria that survived the 22 μA combination treatment was significantly (p-value = 0.04) less than the control in the 48 h Biofilm Age – 24 h Duration stratified group. Additional differences among treatments were observed at a p-value < 0.10 which include the number of bacteria that survived the 333 μA combination treatments was less than the vancomycin treatments within the 0 h Biofilm Age – 24 h Duration (-0.45 $\log_{10}(CFU/cm^2)$) and 48 h Biofilm Age – 24 h Duration (-0.71 $\log_{10}(CFU/cm^2)$) stratified groups. The number of bacteria that survived the 333 μA combination treatment was less than the 22 μA combination treatment with a p-value < 0.10 in the 24 h Biofilm Age – 24 h Duration stratified group (-1.06 $\log_{10}(CFU/cm^2)$).

To demonstrate trends observed with the increase or decrease of resistance to treatments which was dependent on biofilm age, the data presented in Figure 18 and the results from the DTK test presented in Table 24 have been utilized. The biofilm

resistance patterns over time of the vancomycin and the 333 μA combination treatments were similar as the trends were suggesting that the 48 h biofilm age was most resistant and the 24 h biofilm age was the least resistant to the 24 h duration treatments when compared to the controls. The biofilm resistance over time to the 22 μA combination treatment was relatively constant with a slight increase in resistance at the 24 h biofilm age.

For each stratified group the most effective treatment was selected from Table 24 based first on whether it was significantly different from the appropriate control (95% confidence interval, upper limit less than 0) and then by the largest difference. Table 26 lists the treatment methods selected as the most significant for each stratified group. It is important to note that the selections of the most effective treatments are not conclusive findings as the confidence intervals of the different treatments do overlap, however, it does allow for one to assess effectiveness of treatments to a certain extent. The 333 μA combination treatment method was identified as the best treatment method the most frequently when comparing the treatments to their respective controls.

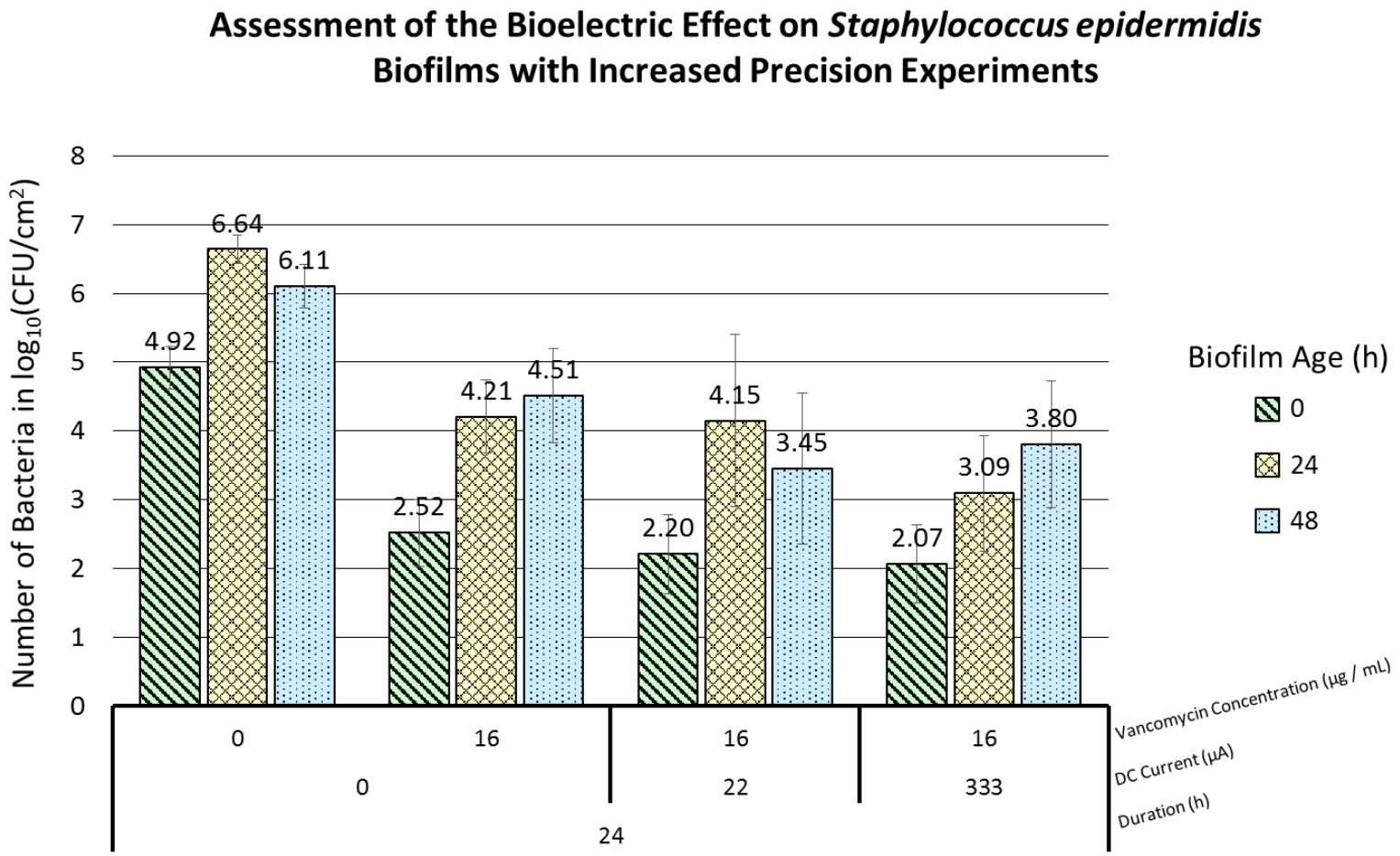

Figure 17: Assessment of bioelectric effect treatments with a more precise experiment using various combinations of vancomycin (0 or 16 µg / mL), DC current (0, 22, or 333 µA), and *Staphyloccocus epidermidis* biofilm age (0, 24, or 48 h). Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For all controls (0 µg / mL of vancomycin and 0 µA DC current) and treatments n = 6. Standard deviations are indicated (I).

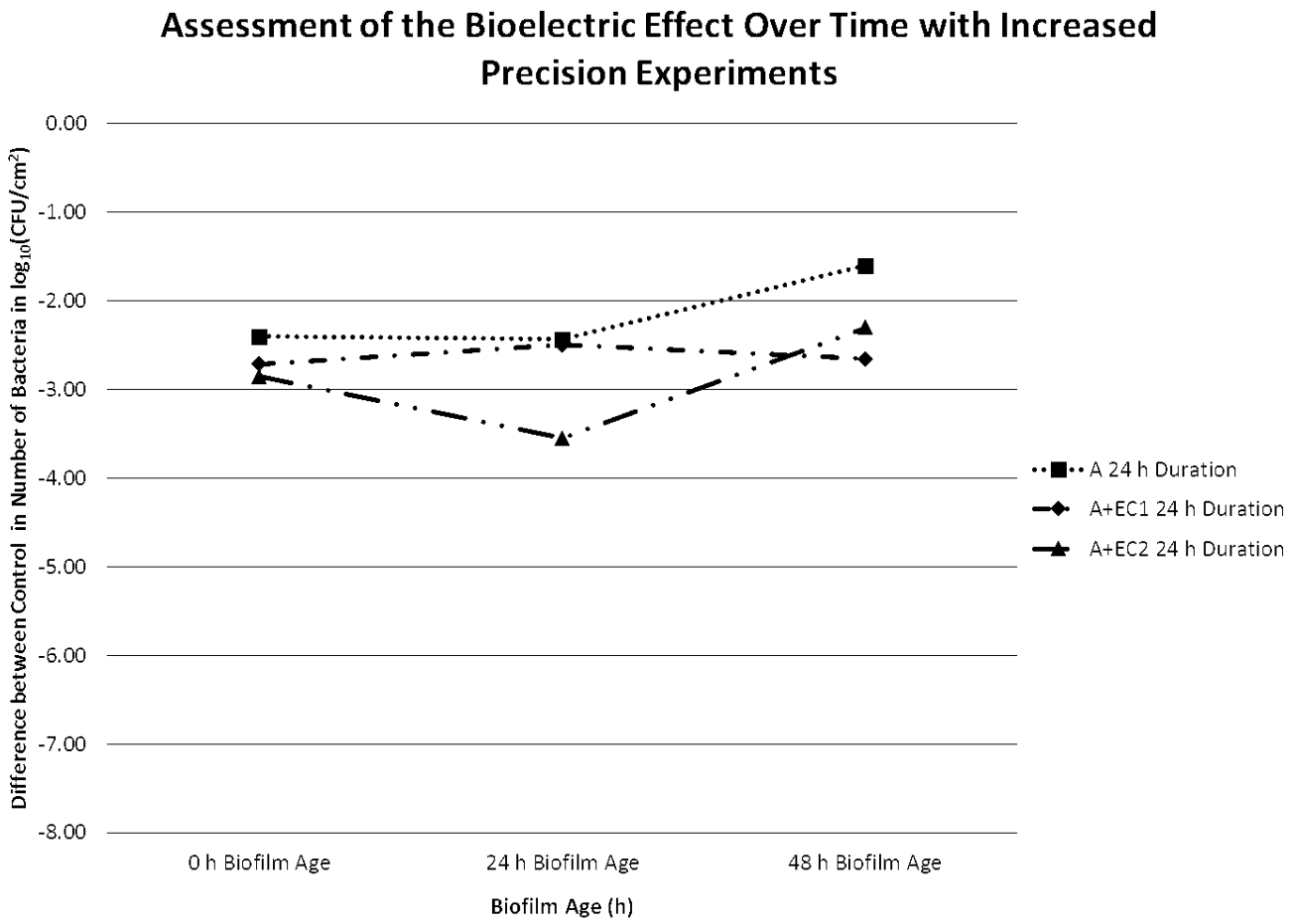

Figure 18: Mean differences for increased precision experiments between controls and 16 µg / mL of vancomycin (A), vancomycin (16 µg / mL) with 22 µA (A+EC1), or vancomycin (16 µg / mL) with 333 µA (A+EC2) for 24 h duration treatments (24 h Duration) or 48 h duration treatments (48 h Duration). Values at different *Staphyloccocus epidermidis* biofilm ages are indicated (0, 24, or 48 h). Differences in number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For duration controls (0 µg / mL of vancomycin and 0 µA DC current) and treatments n = 6.

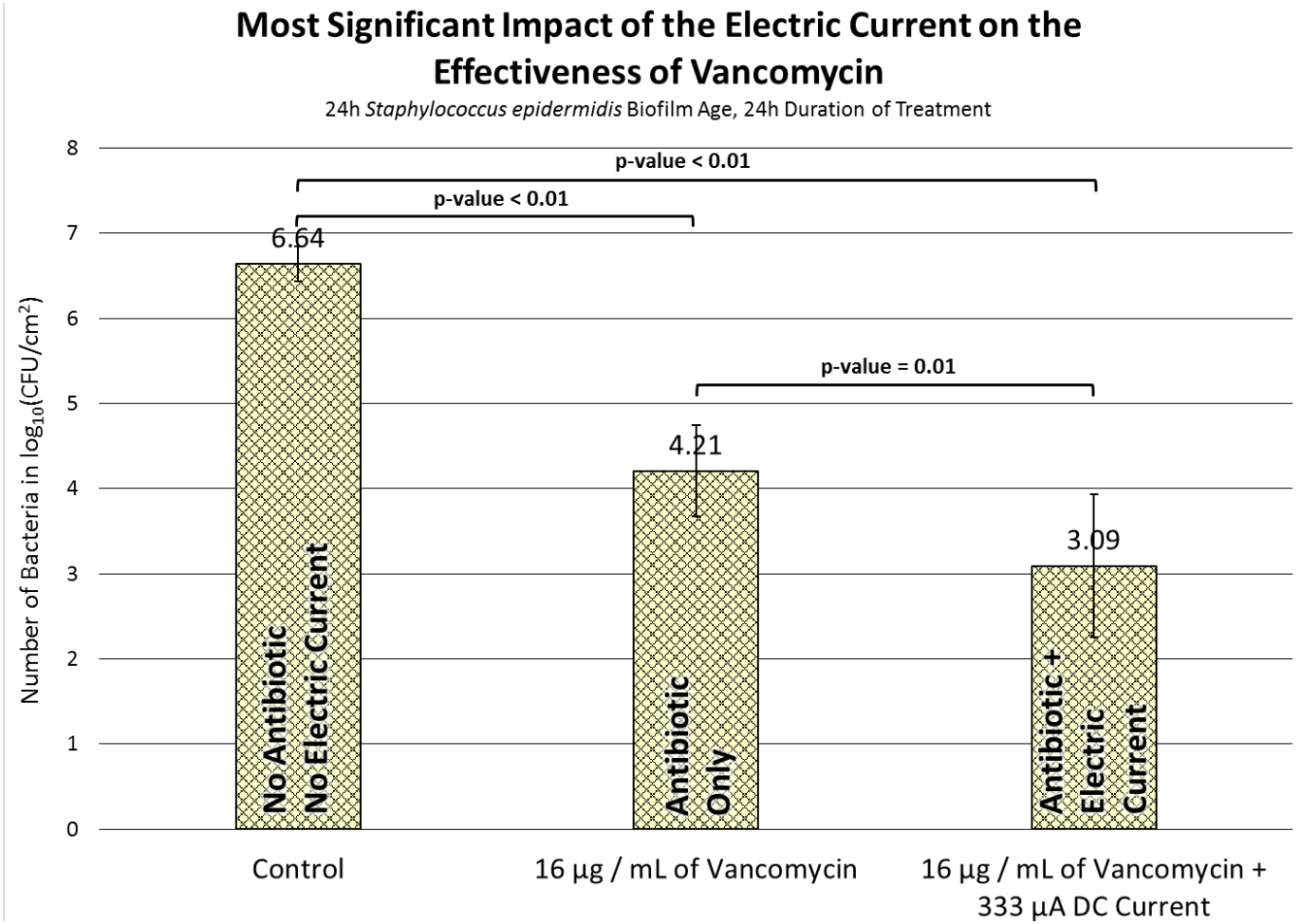

Figure 19: Assessment of the most significant impact of the bioelectric effect with 333 uA in combination with 16 µg / mL of vancomycin. Mean number of colony forming units (CFU) of bacteria per cm^2 are shown as a logarithmic number. For control (no antibiotic and no electric current) and treatments n = 6. Standard deviations (I) and Welch t-test one-sided p-values are indicated.

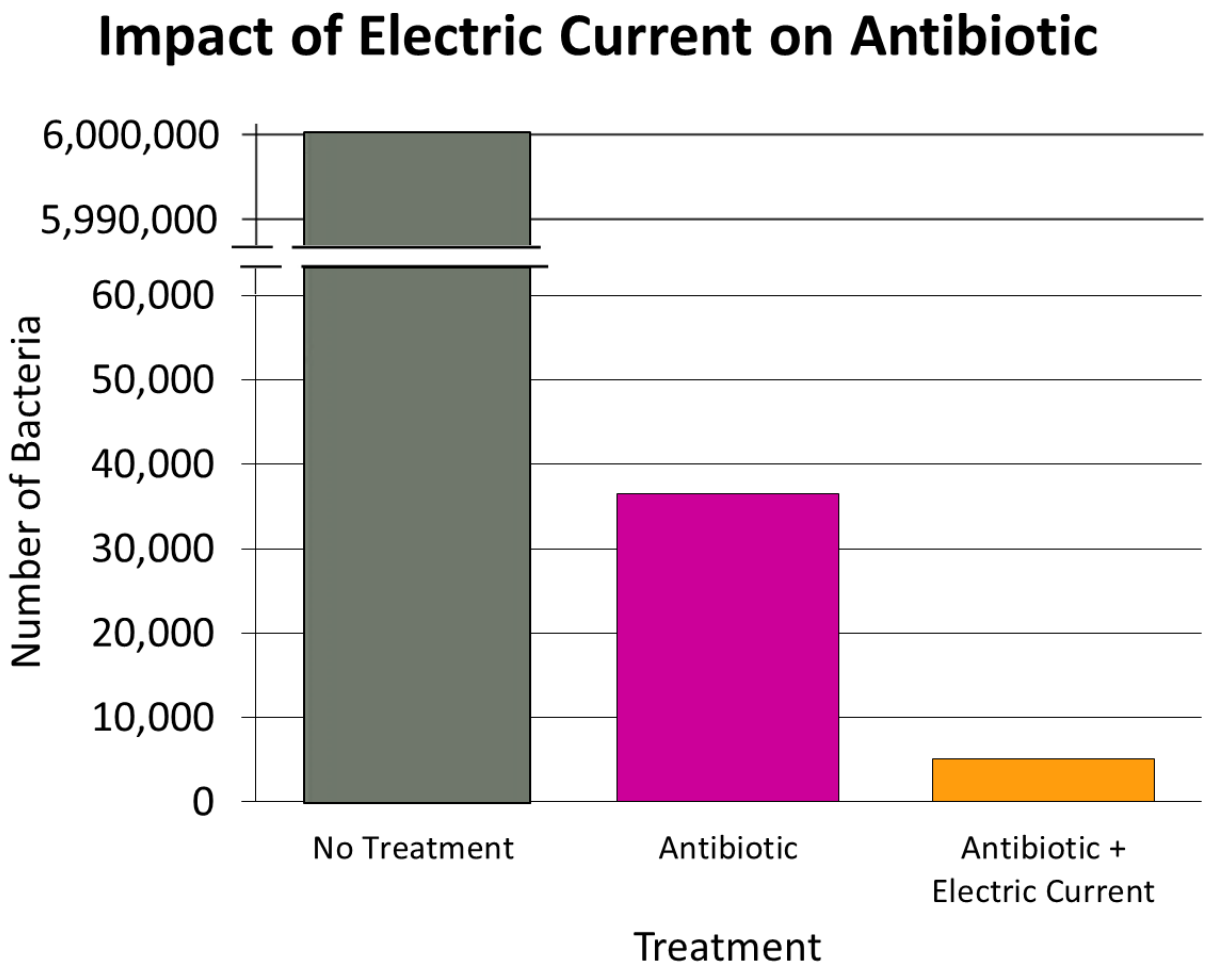

Figure 20: Assessment of the most significant impact of the bioelectric effect on a 24 h *Staphylococcus epidermidis* biofilm age with a 24 h treatment duration. No treatment (no antibiotic and no electric current), antibiotic (16 µg / mL of vancomycin), and antibiotic + electric current (333 uA in combination with 16 µg / mL of vancomycin). Mean number of surviving bacteria are shown. For no treatment, antibiotic, and antibiotic + electric current n = 6.

Table 22: Mean values, standard deviations, and number of samples for increased precision experiments

Treatments				Mean Number of Bacteria in $\log_{10}(CFU/cm^2)$	Standard Deviation of Mean Number of Bacteria in ± $\log_{10}(CFU/cm^2)$	Number of Samples
	0 h Biofilm Age	24 h Duration	Control	4.92	0.31	6
			A	2.52	0.48	6
			A+EC1	2.20	0.57	6
			A+EC2	2.07	0.56	6
	24 h Biofilm Age	24 h Duration	Control	6.64	0.21	6
			A	4.21	0.53	6
			A+EC1	4.15	1.25	6
			A+EC2	3.09	0.84	6
	48 h Biofilm Age	24 h Duration	Control	6.11	0.31	6
			A	4.51	0.69	6
			A+EC1	3.45	1.10	6
			A+EC2	3.80	0.92	6

LEGEND:
Control: No Antibiotic, No Current
A: 16 µg/mL of Vancomycin
A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current
A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

Table 23: One-sided p-values for X less than Y comparisons using the Welch t-test for increased precision experiments

X \ Y				Treatments											
				0 h Biofilm Age				24 h Biofilm Age				48 h Biofilm Age			
				24 h Duration				24 h Duration				24 h Duration			
				Control	A	A+EC1	A+EC2	Control	A	A+EC1	A+EC2	Control	A	A+EC1	A+EC2
Treatments	0 h Biofilm Age	24 h Duration	Control		1.000	1.000	1.000								
			A	<0.01		0.833	0.915								
			A+EC1	<0.01	0.167		0.659								
			A+EC2	<0.01	0.085	0.341									
	24 h Biofilm Age	24 h Duration	Control						1.000	0.998	1.000				
			A					<0.01		0.543	0.988				
			A+EC1					<0.01	0.457		0.939				
			A+EC2					<0.01	0.012	0.061					
	48 h Biofilm Age	24 h Duration	Control										0.999	0.999	1.000
			A									<0.01		0.960	0.917
			A+EC1									<0.01	0.040		0.280
			A+EC2									<0.01	0.083	0.720	

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

p-value ≤ 0.05

Table 24: Two-sided results for X - Y differences using the DTK test for increased precision experiments

X / Y				Mean Difference from Controls in Number of Bacteria in $\log_{10}(CFU/cm^2)$			95% Confidence Intervals	
				0 h Biofilm Age	24 h Biofilm Age	48 h Biofilm Age		
				24 h Duration	24 h Duration	24 h Duration		
				Control	Control	Control	Lower Limit	Upper Limit
Treatments	0 h Biofilm Age	24 h Duration	A	-2.40			-3.268291	-1.5358876
			A+EC1	-2.71			-3.698101	-1.7293842
			A+EC2	-2.85			-3.822659	-1.8823298
	24 h Biofilm Age	24 h Duration	A		-2.43		-3.298915	-1.5682617
			A+EC1		-2.50		-4.408134	-0.5827676
			A+EC2		-3.55		-4.85182	-2.2457865
	48 h Biofilm Age	24 h Duration	A			-1.60	-2.736752	-0.4632069
			A+EC1			-2.65	-4.3778	-0.9311227
			A+EC2			-2.30	-3.762661	-0.8418518

LEGEND:

Control: No Antibiotic, No Current

A: 16 µg/mL of Vancomycin

A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current

A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

95% Confidence Intervals that are Statistically Significant

Table 25: Levene's test p-values for increased precision experiments

Levene's Test	
Stratified Group	**P-value**
0 h Biofilm Age – 24 h Duration	0.73
24 h Biofilm Age – 24 h Duration	< 0.01
48 h Biofilm Age – 24 h Duration	0.24
_____	
Complete Data Set	< 0.01

Table 26: Selection of most effective treatment for the increased precision experiments within each stratified group based on significant DTK test results with 95% confidence intervals

Stratified Group	Most Effective Treatment(s)	Mean Difference from Controls in Number of Bacteria in $\log_{10}(CFU/cm^2)$
0 h Biofilm Age – 24 h Duration	A+EC2	-2.85
24 h Biofilm Age – 24 h Duration	A+EC2	-3.55
48 h Biofilm Age – 24 h Duration	A+EC1	-2.65

LEGEND:

Control: No Antibiotic, No Current
A+EC1: 16 µg/mL of Vancomycin + 22 µA DC Electric Current
A+EC2: 16 µg/mL of Vancomycin + 333 µA DC Electric Current

CHAPTER 5

5.0 DISCUSSION, CONTRIBUTIONS, CONCLUSIONS, AND FUTURE WORK

This thesis provides an overview of past experimental research concerning electrical effects on biofilms, describes the methodology of the conducted experiments, as well as states the novel results observed. Potential methods for the prevention and treatment of biofilm infections include the use of the electricidal effect and the bioelectric effect. The electricidal effect has been shown to reduce the biofilm *in vitro* [11-19, 22, 23, 25-28] and *in vivo* [20, 21, 24] while the bioelectric effect can increase the effectiveness of antimicrobial agents against biofilms [29-43]. A summary of the literature concerning electrical effects on biofilms was presented throughout this thesis and the relevance that this potential prevention and treatment method has for healthcare was also addressed. The experiments described in this thesis demonstrate that the bioelectric effect is more effective than conventional antibiotic treatments against biofilms.

5.1 Discussion

On its own, electric current was observed to impact *S. epidermidis* biofilms as well as increase the effectiveness of vancomycin by controlling and reducing the number of biofilm cells, supporting the concept of the bioelectric effect. With a 24 h old biofilm and a 24 h treatment duration, the 333 μA DC electric current in combination with 16 μg/mL of vancomycin had the most significant bioelectric effect when compared to the vancomycin treatment. Generally, biofilms that developed for longer periods of time had an increased resistance to vancomycin treatment methods, a result observed in previous

studies [32]. Importantly, the resistance of biofilm decreased when electric current in combination with vancomycin was utilized. Overall, the higher constant electric current intensity of 333 μA was more effective against biofilms than the lower intensity of 22 μA. Longer duration treatments were observed to be more effective, however some of the results were quite variable.

By assessing different parameters applied to the experiments for this thesis, the bioelectric effect observed was significant where previous studies observed no significant difference between the combination of an electrical method with vancomycin compared to vancomycin alone against a strain of *S. epidermidis* [38, 41]. Pickering et al. in 2003 utilized a pulsed electromagnetic field (PEMF, 5 A for 380 μs at 72 Hz with a 0.07 V/m at the experimental zone) and up to 256 times the MIC of vancomycin (MIC was 1.25 μg/mL) [38]. Five day old biofilm was formed on stainless steel (Grade 316L) pegs and then treated for a total of 12 h [38]. In 2008, del Pozo et al. assessed the effectiveness of 20, 200, and 2000 μA DC electric currents with 32 μg/mL of vancomycin for a 24 h treatment duration [41]. Graphite electrodes were utilized and the 36 h old biofilm was formed on Teflon coupons [41].

Key differences between the experiments conducted for this thesis and the ones mentioned above are the fact that Pickering et al. in 2003 tested an electromagnetic field and formed the biofilm on the stainless steel [38] while the biofilm was formed on titanium for this thesis. The use of graphite electrodes for treatments, Teflon coupons for biofilm formation, and 32 μg/mL of vancomycin by del Pozo et al. in 2008 [41] were important differences to note when comparing the parameters chosen for this thesis. It

was noted by this group of researchers that stainless steel electrodes did produce a more significant bioelectric effect amongst different studies [41].

This thesis assessed different current intensities, biofilm ages, and durations of treatment within a set of constant parameters such as the antibiotic concentration, electrode material, biofilm substrate material, and the treatment apparatus set-up. Throughout this thesis, one can observe the importance of the chosen parameters when evaluating the concept of the bioelectric effect because a significant increase in the effectiveness of vancomycin was not observed when in combination with electric current in the majority of cases. However, the important point is that for specific parameters, the bioelectric effect does exist when utilizing specific electric currents in combination with vancomycin against various ages of *S. epidermidis* biofilms. This effect was observed with the use of vancomycin (16 μg/mL) below the concentration that is used maximally in the clinical setting (20-25 μg/mL) [88, 89].

In 2008 del Pozo et al. used graphite electrodes because the stainless steel electrodes were observed to corrode at the chosen electric current intensities [41]. This slight corrosion was observed at the higher current intensity of 333 μA in the experiments conducted for this thesis and may be due the release of iron ions (Fe^{2+} and Fe^{3+}). Despite this observation, stainless steel was still utilized throughout the experiments due to the relevance to implantable medical devices. The effects of electric currents observed may be due to electrolysis [35], however, the mechanisms of action needs to be more rigorously addressed in future research studies.

In the experiments conducted for this thesis, corrosion was not observed on the surfaces of the titanium coupons suggesting that future studies could utilize titanium

electrodes in place of stainless steel due to the relevance of titanium for implantable medical devices as well as their characteristics of being non-corrosive [90]. In addition, due to the use of a DC current, ions in the treatment chamber media would build up on the electrode acting as the cathode after 48 h treatment durations. This may account for the variability observed in the longer duration exposure times. The variability of the longer treatment times could also be attributed to the fact that the treatment chamber utilized for this thesis was a static environment. Therefore, a treatment chamber which uses a dynamic in-flow of fresh media instead may better assess long term treatment options as it better mimics an *in vivo* or human model. The variability may also be due to the relatively low sample sizes which was due to the multitude of parameters being assessed. Future research studies should build on the results from the research presented in this thesis to focus the experiments on the further optimization of parameters where a known bioelectric effect exists. This would allow for larger sample sizes to be tested.

When assessing the bioelectric effect, some studies have utilized media that lacks NaCl to eliminate the possible electrolytic effect of chlorine [14, 16, 17, 22, 30, 32-36, 67]. NaCl is, however, an important factor to include in an *in vitro* experimental design in order to effectively assess whether a treatment can be used in the clinical setting as NaCl is abundant *in vivo* and physiologically relevant [28]. Therefore, NaCl was included in both the growth media and treatment media for these experiments. Future experiments are warranted to further assess the bioelectric effect and could entail the analysis of a variety of additional parameters in order to optimize a preventative or treatment protocol that is safe and effective. For instance, experiments could also include platelets and/or plasma proteins since they are known to influence bacterial adhesion [66]. Not including

these factors in the growth media for these experiments could have attributed to the unstable biofilm formation after 48 h in the continuous phase.

5.2 Contributions

The results of the experiments presented in this thesis shed new light on the bioelectric effect when considering treating *S. epidermidis* biofilm infections with vancomycin in combination with low level electric currents. By assessing various parameters, a selection of the most apparent and significant increase of vancomycin effectiveness was established. The choice of parameters was shown to have an important role in observing the bioelectric effect when comparing the results of this thesis with previous studies which observed no significant difference between vancomycin treatments and combination treatments of vancomycin with an electrical method against a strain of *S. epidermidis* [38, 41]. Demonstrating a significant bioelectric effect when compared to antibiotic treatments against *S. epidermidis* is an important finding as it validates that there is potential for optimizing parameters to effectively treat specific strains that display resistance to antibiotic and other electrical treatments.

5.3 Conclusions

The major conclusion of this thesis is that electric current increased the effectiveness of a frequently used antibiotic against a very common biofilm known to cause infections with indwelling and implanted medical devices. Electric current alone slightly reduced the bacteria in biofilms. Older biofilms had an increased resistance to

vancomycin treatments. Higher electric current intensities and longer duration treatments were more effective against biofilms.

The intention of this thesis was to expand the breadth of knowledge and extend the horizon of promising and potential research in this area into ground-breaking, novel ideas that can lead to more effective treatments for a large population of patients suffering from a variety of health conditions. One can use the principles of biology, microbiology, electrical engineering, biomechanics, medicine, and physiology to formulate new treatment methods and devices which have the potential to take the concepts discussed in this thesis and apply them to real situations enhancing the quality of life for a multitude of patients in need for such treatments.

5.4 Future Work

In relation to indwelling and implantable medical devices, one can focus the research on optimizing bioelectric effect treatments for *Staphylococcus epidermidis* and *Staphylococcus aureus* strains. These are the two most prevalent strains that cause infections with medical devices [53-55]. A better understanding of the mechanisms of action of these electrical effects is necessary to optimize the effectiveness of these treatment methods as well as to assess the pathophysiological effects. In addition, *in vitro* and *in vivo* studies that can assess the impact of a variety of clinically relevant parameters should be utilized in order to progress this research into clinical trials. *In vivo* studies have indicated significant electrical effects on biofilms without the use of antibiotics [20, 21, 24], therefore, evaluating the impact of electric current and/or electromagnetic fields on the response of the host immune system to infections is an area of research that is

unexplored and could be highly rewarding if pursued. If conclusive, these findings could potentially reduce the need for antibiotics which are constantly being overused in the clinical setting.

A potential way to implement this electrical method of prevention and treatment for biofilm infections in implanted medical devices is by transcutaneously (across the skin) inducing an electric current on a specific target through induction. Such a device does exist and is called a transcutaneous energy transfer (TET) system [91-96]. Its primary use is to transfer energy across the skin of a patient to power an implanted medical device [91-96]. The TET system is being utilized as an effective means of energy transfer [91-96], but there is also an opportunity for this device to be miniaturized and applied to biofilm infections. The characteristic that sets this prospective treatment apart from others is that it can be done without invasive interventions. Therefore, more research on electric current / electromagnetic effects is not only appropriate but is essential due to the possibility of establishing a new era of novel, effective, and low-cost treatments for a variety of patients.

The electricidal and bioelectric effects were addressed as potential preventative and treatment measures for biofilm infections, and current experiments support the concept of the bioelectric effect. The overall purpose of this research is to prevent and treat biofilm infections with electrical currents and/or electromagnetic fields in combination with antibiotics and host defense mechanisms. Recommendations to further progress the research concerning electrical effects on biofilms were proposed. It is important to realize that there is always space for improvement in healthcare and that the

best way to initiate progress is the realization that there is still so much that is yet to be discovered.

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