ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CONTROLLING BACTERIAL BIOFILMS BY APPLYING EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS (ELF-EMFS)

Ph.D. THESIS

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ÇOK DÜŞÜK FREKANSLI ELEKTROMANYETİK ALAN (ÇDF-EMA) UYGULANARAK BAKTERİYEL BİYOFİLMLERİN KONTROLÜ

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Hasan KAHRAMAN, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 521082056, successfully defended the thesis entitled "CONTROLLING BACTERIAL BIOFILMS BY APPLYING EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS (ELF-EMFS)", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my beloved parents and my dear wife,

FOREWORD

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ABBREVIATIONS

agr : Accessory gene regulator
AHL : N-acyl-L-homoserine lactones
atlE : Gene responsible for biofilm development in *S. epidermidis*
DNA : Deoxyribonucleic Acid
ELF-EMF : Extremely Low Frequency Electromagnetic Fields
EMF : Electromagnetic Fields
EPM : Extracellular Polymeric Matrix
EPS : Extracellular Polymeric Substances (or Structures)
Gram+ : Bacteria that give a positive result in the Gram stain test
Gram- : Bacteria that do not retain the crystal violet stain
LAB : Lactic Acid Bacteria
OD : Optical Density
P. aeruginosa : *Pseudomonas aeruginosa* strain PAO1 (ATCC 15692)
PCR : Polymerase Chain Reaction
PIA : Polysaccharide Intercellular Adhesion
QS : Quorum Sensing
rpoS : Gene responsible for biofilm development in *P. aeruginosa*
S. epidermidis : *Staphylococcus epidermidis* strain (ATCC 35984)
TSB : Trypticase Soy Broth
UV : Ultraviolet

SYMBOLS

CFU/ml	**:** Colony Forming Unit per Milliliter
CO_2	**:** Carbon dioxide
h, hr	**:** Hour
Hz	**:** Hertz
MHz	**:** Megahertz
ml	**:** Milliliter
µl	**:** Microliter
mm	**:** Millimeter
mT	**:** Millitesla
nm	**:** Nanometre
V/m	**:** Volt per meter
°C	**:** Degree Celsius

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CONTROLLING BACTERIAL BIOFILMS BY APPLYING EXTREMELY LOW FREQUENCY ELECTROMAGNETIC FIELDS (ELF-EMFS)

SUMMARY

Bacteria are often studied as individual organisms in planktonic form. However, many applications have shown that they grow in communities known as biofilms which work symbiotically to enhance their ability to survive and obtain nutrients. Bacteria produce exopolymeric matrix which enhance them to attach and form microcolonies on surfaces. Extracellular polymeric structures which form the matrix are mainly composed of polymeric sugars, proteins, nucleic acids and lipids.

Due to their structural differences and metabolic pathways, biofilm bacteria are extremely resistant to conventional antimicrobial agents. Biofilm infections cause so many deaths all around the world and additionally high medical expenses as well. Use of medical devices and implants are the main sources of biofilm formation on human body.

The aim of this study is to investigate the effects of exposure to Extremely Low Frequency Electromagnetic Fields (ELF-EMF) both on biofilm formation and on mature biofilm of *Staphylococcus epidermidis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) which cause infections. These pathogens lead to infections by simply targeting and weakening the immune system. Choosing two bacteria forms contributes simply to enlarge the scope of application. Some species specific genes of *S. epidermidis* and *P. aeruginosa* are known to effect biofilm development. Consequently, *AtlE* gene sequencing of *Staphylococcus epidermidis* and *rpoS* gene sequencing of *Pseudomonas aeruginosa* is analysed in order to determine any genotypic changes caused by ELF-EMF exposure.

P. aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium with unipolar-motility. Cell-surface polysaccharides play a critical role on the life cycle of bacteria as they stand as barriers between the cell wall and surroundings. By doing so, they mediate interaction between the layers and coating which eventually form the structural components of biofilms. On the other hand, *S. epidermidis* is a gram-positive and coagulase-negative staphylococcus. Most commonly, it lives on the human skin and mucosa. Catheters and implants can be infected by *Staphylococcus* species easily.

There are several approaches used to remove biofilm pathogens. Regarding the pathogens related to medical devices, one of the main strategies is to use systemic therapies with antimicrobial agents. However, it is rather difficult to prevent infections on implants as they are strongly resistant to antibiotics. Therefore, in order to succeed the therapy, the medical device should be removed from the implant. Another method for fighting the biofilm related infections is forming special functional surfaces. Clearly it is expected that these surfaces will prevent bacterial adhesion. In the past, mainly toxic materials have been used for the protective coating. However, today, because of environmental awareness, their use is very much limited, almost vanished. For the same aim, yet another approach is well known quorum-sensing which

technically corrupts the communication mechanism of bacteria, so that the bacteria fail to form biofilms. Although there are some successful outcomes where synthetic analogs are used in natural structures, clinical trials could not be done because of the toxic products. Besides, a small range of pathogens could be targeted by quorum-sensing mechanisms. In several in vitro studies, it is observed that phages also infect biofilm forming cells. Phages inducing the production of depolymerases have an advantage, since they can penetrate the inner layers of the biofilm by degrading components of the biofilm exopolymeric matrix. However, phages should be integrated to implants in order to be used clinically. Also phages require prior identification of the phage or polysaccharide depolymerase capable of infecting the bacterial cells and degrading the polysaccharide within the biofilm.

There has been an extended research on whether electromagnetic fields have any effect on the cells behavior. For this purpose, some studies have been carried out on animal models (*in vivo*) and cell cultures (*in vitro*). However, the findings are still inconclusive. On the other hand, some effects are observed with the application of electromagnetic field on biofilms. Biofilm formation is halted when electromagnetic fields with specific range of frequency are applied. This would be an alternative approach for the removal of biofilm considering the problems faced with the conventional approaches. Taking the originality point of the work into consideration, production and prevention of biofilms are achieved at two different surfaces and *P. aeruginosa* and *S. epidermidis* type bacteria are tested with the application of ELF-EMF.

This research is mainly composed of three steps: i) controlled biofilm formation with two different model microorganisms *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*; ii) removal and/or prevention of biofilms by exposure to extremely low frequency electromagnetic fields (ELF-EMFs); iii) *AtlE* gene sequencing of *S. epidermidis* and *rpoS* gene sequencing of *P. aeruginosa* exposed to ELF-EMF were performed so as to determine any genotypic changes.

A microtiter plate biofilm assay is used to monitor microbial attachment to an abiotic surface, which is a high-throughput method often referred to as the 96-well plate assay. In brief, cells are grown in microtiter dishes for a desired period of time, and then the wells are washed to remove planktonic bacteria. Cells remaining adhered to the wells are subsequently stained with a dye that allows visualization of the attachment pattern. Then, this surface-associated dye is solubilized for semi-quantitative assessment of the formed biofilm.

In order to emit ELF-EMFs, an experimental set-up was designed. The proposed experimental set-up consists of a solenoid that has 160 mm internal and 170 mm external diameter, with a length of single stranded 440 mm, 180 turns of copper wire. The device is supposed to deliver variable, homogeneous, sine-wave alternating magnetic fields regulated and defined along the center line with 45–300 Hz frequency and intensities ranging between 0.1 and 1.0 mT ±2%. The solenoid is powered by a power supply which is capable of forming a sine-wave output with a closely regulated frequency and voltage. Proposed experimental set-up consists of polystyrene and polypropylene microtiter plates for biofilm growth placed at the center of the cylindrical Solenoid, where assuming that the maximum homogeneity of electromagnetic field is obtained.

To investigate *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilm formation, each strain is inoculated in two different round-bottomed 96-well microtiter plates that are polystyrene and polypropylene in structure. Two separate biofilm formations of *P. aeruginosa* and *S. epidermidis*, one of each exposed to ELF-EMF within the Solenoid and the others used as controls, are incubated at room temperature and analyzed for 24 h and 48 h. *P. aeruginosa* and *S. epidermidis*, exposed to 45 Hz, 50 Hz, 100 Hz, 200 Hz and 300 Hz frequency with 1 mT intensity are analysed for their biofilm formation on polystyrene and polypropylene surfaces.

In order to explore the effect of EMF-ELF on *rpoS* and *atlE* genes, genomic DNA of ELF-EMF exposed bacteria were isolated. Then *rpoS* and *atlE* genes, which are responsible for biofilm development were sequenced and analysed for any DNA mutations. For DNA sequencing, such as those involving genomic DNA isolation, forward and reverse primer design, PCR, agarose gel electrophoresis and DNA sequencing, standard procedures were followed. ELF-EMF exposed cultures and the respective sham exposed controls were studied for DNA sequence analysis.

Exposure of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to 45 Hz and 50 Hz at 1.0 mT decrease the cell viability and inhibit the biofilm formation by 40% after 48 hours, both on polystyrene and polypropylene surfaces. Conversely, there is no significant difference to biofilm formation both on polystyrene and polypropylene surfaces exposed to 100 Hz, 200 Hz and 300 Hz frequency and 1 mT intensity of ELF-EMF. Quantification of ELF-EMF (1mT 50 Hz) effect at 3 hr time intervals over 48 hrs, showed that the formation of *S. epidermidis* biofilm is halted by ELF-EMF exposure after 24 hrs, as similar growth is obtained between 24 hrs and 48 hrs time intervals. Nevertheless, ELF-EMF has significantly little effect on planktonic *P. aeruginosa* cultures, as the cells continued growing both at 24 hrs and 48 hrs compared to non-exposed control samples. With regard to the analysis of *rpoS* and *atlE* genes, similar DNA sequence profiles were observed in each experimental condition examined both in exposed and non-exposed cultures during biofilm formation.

ÇOK DÜŞÜK FREKANSLI ELEKTROMANYETİK ALAN (ÇDF-EMA) UYGULANARAK BAKTERİYEL BİYOFİLMLERİN KONTROLÜ

ÖZET

Bakteriler genellikle birbirinden bağımsız olarak düşünülüp planktonik formda incelenirler. Fakat birçok araştırma göstermiştir ki bakteriler biyofilm olarak adlandırılan topluluklar halinde birbirleriyle etkileşerek çoğalmaktadırlar. Biyofilm oluşumu hem canlı hem cansız sistemler üzerinde kendiliğinden ortaya çıkabilen ve bakterilerin hayatta kalmalarını sağlayan önemli bir mekanizmadır. Bu durum bakterilerin simbiyotik olarak besinlere ulaşma ve hayatta kalma şansını büyük oranda arttırmaktadır. Biyofilmler, ürettikleri ekzopolimer matriks içerisinde yaşamaları ile karakterize edilen, mikrokoloniler oluşturarak yüzeye yapışan bakteri gruplarıdır. Matriksi oluşturan hücre dışı polimerik yapılar ağırlıklı olarak polimerik şekerlerden meydana gelmekle birlikte, proteinler, nükleik asitler ve lipidler de bu polimerik yapıların içeriğini oluşturmaktadır.

Matriks yapısını oluşturan biyopolimerler nedeniyle, biyofilmi oluşturan bakteriler, daha değişken metabolik yol izlerine sahip ve geleneksel antimikrobiyal ajanlara karşı dirençlidirler. Biyofilm enfeksiyonları dünyada birçok ölüme yol açmakta ve yüksek sağlık harcamalarına sebep olmaktadır. İnsan vücudunda biyofilm oluşumuna yol açan başlıca etkenler arasında medikal cihazların kullanımı ve implantlar gelmektedir. Biyofilm yapılar normal hücre yapılarıyla karşılaştırıldıklarında makrofaj ve antibiyotiklere karşı daha dirençli oldukları için oluşumlarını engellemek oldukça güçtür.

Bu çalışmada, tıbbi cihazlar ve implantlar üzerinde biyofilm oluşturarak enfeksiyona yol açtığı bilinen *Staphylococcus epidermidis* (Gram +) ve *Pseudomonas aeruginosa* (Gram -) bakterilerinin, çok düşük frekanslı elektromanyetik alan (ÇDF-EMA) uygulanması sonucunda biyofilm oluşturmalarının yavaşlatılması veya engellenmesi amaçlanmıştır. Bu patojenler özellikle bağışıklık sistemini hedef alarak ve zayıflatarak enfeksiyona yol açmaktadır. *S. epidermidis* ve *P. aeruginosa*' nın türe özgü bazı genlerinin biyofilm gelişimini etkilediği bilinmektedir. Bu genlerden *S. epidermidis*'e ait *atlE* geni ve *P. aeruginosa*'ya ait *rpoS* geninin bakteriler arası iletişim (quorum-sensing) mekanizmasında görev aldığı ve biyofilm oluşumundan sorumlu oldukları rapor edilmiştir. Bu kapsamda, ÇDF-EMA'ya maruz bırakılan bakterilerin özellikle *atlE* ve *rpoS* genlerinde mutasyonların meydana gelip gelmediği incelenerek biyofilm oluşumunda sorumlu genlerin nasıl etkilendiği araştırılmıştır.

P. aeruginosa gram (-), katalaz-pozitif ve aerobik çubuk şekilli bir patojen bakteridir. Hücre-yüzey polisakkaritleri bakterinin hayat döngüsünde önemli rol oynar. Bu polisakkaritler hücre duvarı ve çevre arasında bariyer görevi görerek, konak-patojen etkileşimlerine aracılık eder ve biyofilm oluşumuna yol açan yapısal bileşenleri oluştururlar. Diğer yandan, *S. epidermidis* gram (+), koagülaz-negatif ve katalaz-pozitif özellikte olan streptokoklardan bir bakteri türüdür. Birden fazla tabaka oluşturma özelliğine sahip olması nedeniyle, yapay yüzeylerde kolaylıkla biyofilmler oluşturabilmektedir.

Biyofilm oluşturan patojenleri uzaklaştırmada literatürde çeşitli yöntemler uygulanmaktadır. Medikal cihazlara bağlı enfeksiyonları engellemede kullanılan başlıca stratejilerden birisi standart antimikrobiyal ajanların kullanıldığı sistemik terapilerdir. Ancak implantlar üzerindeki enfeksiyonları engellemek oldukça güçtür. Çünkü biyofilmler yapıları gereği antibiyotiklere karşı direnç göstermektedir ve başarılı bir terapi için medikal cihazın çıkarılması gerekmektedir. Biyofilm enfeksiyonları ile mücadelede kullanılan bir diğer yöntem, bakterilerin yapışmasını engelleyen fonksiyonel yüzeylerin oluşturulmasına yöneliktir. Geçmiş dönemlerde koruyucu kaplamaların yapısında toksik maddeler yaygın olarak kullanılmaktaydı. Ancak günümüzde çevresel etkileri göz önüne alındığında kullanımlarının azaldığı söylenebilir. Bu azalmada bazı maddelerin yasaklanması da ayrıca önemli etken olmuştur. Yoğun olarak devam eden bir başka yaklaşımda ise bakterilerin iletişim mekanizmasını (quorum-sensing) bozarak biyofilm oluşumunun engellenmesi amaçlanmıştır. Quorum-sensing mekanizması kullanılarak doğal yapılarda sentetik analoglar vasıtasıyla başarılı sonuçlar elde edilse de, oluşan toksinlerden dolayı klinik aşamaya geçilememiştir. Ayrıca quorum-sensing yöntemlerinde sadece belirli patojenler hedef alınabilmektedir. Birçok *in vitro* çalışmada, fajların da biyofilm oluşturan hücreleri enfekte ettiği görülmüştür. Fajlar, biyofilmlerin ekzopolimerik matriksine ait yapıları parçalayarak iç tabakalara nüfuz edebilir ve biyofilm oluşumunu engelleyebilir. Fajların klinik olarak kullanılabilmesi için bir şekilde implantlara entegre edilmesi gerekmektedir. Ancak bu yöntemde fajların bakteriyel hücreleri enfekte ettiğinin ve biyofilm üzerindeki polisakkaritleri parçalayabildiğinin belirlenmesi gerekmektedir.

Elektromanyetik alanlar'ın (EMA) hücre davranışları üzerinde herhangi bir etkisinin olup olmadığı uzun süredir araştırılmaktadır. EMA'nın etkileri üzerine çeşitli hayvan modelleri (*in vivo*) ve hücre kültürleri (*in vitro*) üzerinde çalışmalar yürütülmüş olup etkileri üzerine kesin bir yargıya varılamamıştır. Ancak çeşitli biyokimyasal çalışmalarda birçok enzimin yapı ve fonksiyonlarının, ayrıca bazı hücre organellerinin yapı ve fonksiyonlarının radyofrekans dalgalarına bağlı olarak bozulduğu yaygın olarak kabul edilmektedir. Günümüzde uygulanan yöntemler göz önüne alındığında biyofilme bağlı enfeksiyonları engellemek ve kontrolünü sağlamak için alternatif yöntemler geliştirmeye acil ihtiyaç vardır. Elektromanyetik alan uygulayarak yapılan birçok deneysel çalışmada belirli frekanslardaki elektromanyetik alanların biyofilm oluşumlarını etkilediği görülmüştür. Biyofilm oluşumunu engellemeye yönelik geleneksel yöntemlerin problemleri göz önüne alındığında elekromanyetik alan yöntemi ile biyofilm oluşumunun engellenmesi alternatif bir yol olabilir. Çalışmanın özgünlük noktasını biyofilmlerin üretimi ve uzaklaştırılmasının aynı yerde ve aynı ortamda gerçekleştirildiği göz önüne alınarak, *P. aeruginosa* ve *S. epidermidis* bakterileri ÇDF-EMA'ya maruz bırakılarak test edilmiştir.

Üç aşamada gerçekleşen bu çalışmada: i) iki farklı model mikroorganizmadan (*P. aeruginosa* ve *S. epidermidis*) kontrollü olarak biyofilm eldesi; ii) biyofilmlerin ÇDF-EMA'ya maruz bırakılarak uzaklaştırılması ve/veya tamamen ortadan kaldırılması ve iii) ÇDF-EMA'ya maruz bırakılan *S. epidermidis* ve *P. aeruginosa*'ya özgü *atlE* ve *rpoS* genlerine dizileme yapılarak gen düzeyinde meydana gelen değişiklikler incelenmiştir.

Abiyotik bir yüzeye mikrobik bağlanmayı incelemek için, çoğunlukla 96-kuyucuklu plaka deneyi olarak da adlandırılan ve yüksek verimli bir yöntem olan mikrotitre plakası biyofilm testi kullanılır. Bu yöntemde, hücreler mikrotitre plakalarda istenilen süre kadar büyütülür ve daha sonra planktonik bakterileri uzaklaştırmak için kuyular

yıkanır. Daha sonra kuyulara yapışan hücreler, bağlanma oranının görselleştirilmesini sağlayan bir boya ile boyanır. Bu yüzey-bağımlı boya, oluşan biyofilmin yarı-kantitatif oranını belirlemek için çözünür hale getirilir.

ÇDF-EMA'ları oluşturmak için bir deney düzeneği tasarlanmıştır. Tasarlanan deney düzeneğinin iç ve dış çapı 160-170 mm, uzunluğu 440 mm ve 180 sarımlı bakır telden oluşan bir Solenoiddir. Cihazın, 45-300 Hz frekans aralığında ve 0.1 ile 1.0 mT ±%2 yoğunlukları arasında değişen, merkez hat boyunca düzenlenen ve tanımlanan homojen, sinüs dalgası değişimli manyetik alanlar vermesi beklenmektedir. Solenoid, ayarlanmış bir frekans ve gerilimle sinüs dalgası çıkışı oluşturabilen bir güç kaynağına bağlıdır. Deney düzeneği, elektromanyetik alanın maksimum homojenliğinin elde edildiği varsayılan silindir yapıdaki Solenoidin merkezine biyofilmlerin büyümesi için yerleştirilen polistiren ve polipropilen mikrotitre plakalarından oluşmaktadır.

Pseudomonas aeruginosa ve *Staphylococcus epidermidis* biyofilm oluşumunu araştırmak için, her suş polistiren ve polipropilen yapıdaki iki farklı yuvarlak tabanlı 96-kuyucuklu mikrotitre plakalara inoküle edilmiştir. *P. aeruginosa* ve *S. epidermidis* oluşumlarının herbiri için Solenoid içinde ÇDF-EMA'ya maruz bırakılan ve maruz bırakılmayan kontroller olmak üzere, birer örneği oda sıcaklığında inkübe edilmiş ve 24 saat ile 48 saat zaman aralıklarında analiz edilmiştir. 45 Hz, 50 Hz, 100 Hz, 200 Hz ve 300 Hz frekanslarında 1 mT yoğunluğa maruz bırakılan *P. aeruginosa* ve *S. epidermidis*'in, polistiren ve polipropilen yüzeylerinde biyofilm oluşumları incelenmiştir.

ÇDF-EMA'nın *rpoS* ve *atlE* genleri üzerindeki etkisini araştırmak için, ÇDF-EMA'ya maruz bırakılan bakterilerin genomik DNA'sı izole edilmiş ve daha sonra biofilm gelişiminden sorumlu olan *rpoS* ve *atlE* genleri dizilenip herhangi bir DNA mutasyonu olup olmadığı incelenmiştir. DNA dizilemesi için genomik DNA izolasyonu, ileri ve geri primer tasarımı, Polimer Zincir Reaksiyonu (PZR), agaroz jel elektroforezi ve DNA dizilimi gibi standart prosedürler takip edilmiştir. DNA dizi analizi için ÇDF-EMA'ya maruz bırakılan kültürler ve maruz bırakılmayan kontrol grubu kullanılmıştır.

Pseudomonas aeruginosa ve *Staphylococcus epidermidis*'in 45 Hz ve 50 Hz'de 1.0 mT'ye maruz kalması sonucu hücre canlılığının azaldığı ve biyofilm oluşumunun 48 saat sonra hem polistiren hem de polipropilen yüzeyler üzerinde %40 oranında önlendiği gözlemlenmiştir. Bunun yanında 100 Hz, 200 Hz ve 300 Hz frekanslarında ve 1 mT yoğunluğunda polistiren ve polipropilen yüzeylerde ÇDF-EMA'ya maruz bırakılan bakterilerin biyofilmler oluşturması açısından anlamlı bir fark gözlemlenmemiştir. ÇDF-EMA (1mT 50 Hz) etkisinin, 48 saat boyunca 3 saatlik zaman aralıklarında nicel olarak incelenmesi sonucunda, *S. epidermidis* biyofilminin 24 saat sonra ÇDF-EMA etkisiyle azaldığı görülmüştür. ÇDF-EMA'nın planktonik *P. aeruginosa* üzerinde önemli bir etkisi olmadığı görülmüştür. *RpoS* ve *atlE* genlerinin analizi sonucunda, biyofilm oluşumu sırasında maruz kalan ve maruz kalmayan kontrol örneklerinden elde edilen *rpoS* ve *atlE* genlerinin DNA dizisi fragmanlarında farklılıklar gözlenmemiştir.

1. INTRODUCTION

Bacteria generally live as individual organisms known as planktonic form. However, many applications have shown that they grow in communities stated as biofilms. Biofilms, work symbiotically to enhance their ability to survive and obtain nutrients (Costerton & Stewart, 2001). Biofilm bacteria produce exopolymeric matrix which enhance them to attach and form microcolonies nearly on all kinds of surfaces and can be extremely difficult to remove (Scannapieco, 2004). Extracellular polymeric structures (EPS) are mainly composed of polymeric sugars, proteins, nucleic acids and lipids that form the matrix. (Blenkinsopp & Costerton, 1991; D. Davies, 2003; Ellwood, Keevil, Marsh, Brown, & Wardell, 1982). Biofilms, when compared to normal cell structures, are more resistant to macrophages and antibiotics, so they are more difficult to remove (Caubet *et al.*, 2004; Estrela, Heck, & Abraham, 2009). Biofilms are reported to be responsible for too many deaths around the world by causing infections, and these infections are spread through using medical devices and implants (Estrela et al., 2009).

There are several approaches for the removal of pathogenic bacterial biofilms. One of the most widely used strategies is systemic therapies which use standard antimicrobial agents. However, as bacterial biofilms are resistant to antibiotics, implants need to be removed for this stategy to be successful (Azeredo & Sutherland, 2008). Another approach to combat bacterial biofilm infections is the formation of functional surfaces that prevent bacteria to attach. In the past, toxic substances were widely used in protective coating matter, but nowadays considering environmental effects, their usage is reduced, even some of them are banned (Callow & Callow, 2002; Estrela et al., 2009; Flemming, 2002). In another different approach, the bacterial communication mechanism (quorum-sensing) is disrupted so that the bacteria fail to form biofilms. Although there are some successful outcomes where synthetic analogs are used in natural structures, clinical trials could not be done because of the toxic products. Besides, a small range of pathogens could be targeted by quorum-sensing mechanisms (Hentzer, Eberl, Nielsen, & Givskov, 2003). In several *in vitro* studies, it is observed

that phages also infect biofilm forming cells. Phages inducing the production of depolymerases have an advantage since they can penetrate the inner layers of the biofilm by degrading components of the biofilm exopolymeric matrix. However, phages should be integrated to implants in order to be used clinically. Also phages require prior identification of the phage or polysaccharide depolymerase capable of infecting the bacterial cells and degrading the polysaccharide within the biofilm (Azeredo & Sutherland, 2008).

Although these approaches prove to be affective there is an urgent need to develop alternative ways to prevent and control biofilm-associated infections (Di Campli, Di Bartolomeo, Grande, Di Giulio, & Cellini, 2010). One of these approaches could be by using extremely low frequency electromagnetic fields (ELF-EMFs). Defined EMFs have proven to prevent biofilm formation in several studies (del Pozo, Rouse, Mandrekar, Sampedro, et al., 2009; del Pozo, Rouse, Mandrekar, Steckelberg, & Patel, 2009; Andreas Obermeier, Florian Dominik Matl, Wolfgang Friess, & Axel Stemberger, 2009). If we consider the problems of conventional biofilm removal approaches, EMFs could be used as an alternative way for biofilm removal (McLeod & Sandvik, 2010).

The effects of Electromagnetic Fields (EMFs) on cells' behaviour, whether it is deadly or causing some other alterations, has been researched for decades in the scientific field. It is important to define these effects because the number of electrical home devices, cell phones, power distribution units and power lines which generate EMFs are increasing every day (Wertheimer & Leeper, 1979). In some biochemical studies it is reported that structure and function of many enzymes and some organelles is disrupted by radiofrequency waves (Stavroulakis, 2003).

1.1 Purpose of Thesis

In a variety of studies, it is indicated that EMFs may affect living cells in several ways. In this case if we consider prokaryotic cells, it is shown that EMFs change the binding properties of biofilm bacteria by causing stress effect leading to phenotypic and transcriptional changes (Di Campli et al., 2010). In this research our aim is to prevent biofilm formation or totally remove biofilms of *Staphylococcus epidermidis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) bacteria by using ELF-EMFs. These bacteria are known to be the main cause of infections by forming biofilms on medical devices

and implants. Simultaneously, phenotypic and genotypic alterations in gram (+) and gram (-) bacteria groups will be determined when exposed to EMFs. All these data are expected to give insight into the effects of ELF-EMFs on two different bacteria groups. Consequently, it is expected to remove bacterial biofilms on medical devices, implants even plaques on teeth by ELF-EMFs so that infections are partly or totally prevented. If successful results are obtained, our approach might be conducted with other biofilm removal tools so that more efficient biofilm removal tool can be reinforced when ELF-EMFs is applied.

1.2 Bacterial Biofilms

Many applications have shown that bacteria grow in communities known as biofilms, which work symbiotically to enhance their ability to survive and obtain nutrients. Bacteria produce extracellular polymeric structures (EPS) which form the matrix. That matrix is mainly composed of polymeric sugars, proteins, nucleic acids and lipids. Exopolymeric matrix enhance bacteria to attach and form micro-colonies on surfaces. (Blenkinsopp & Costerton, 1991; D. Davies, 2003; Ellwood *et al.*, 1982).

Microorganisms which form biofilms are also investigated according to their interactions with the microenvironments. The metabolism of bacteria is mostly affected by the environment. Therefore, biofilm research represents the best tool to examine growth in natural and ecosystems of interest. Planktonic bacteria are highly motile and have enormous access to nutrients and multiply rapidly, when compared to sessile bacteria.

Bacteria can form biofilms nearly on all surfaces from cellulose to silicone and glass to steel (which are significant materials used for the production of medical instruments) by communicating through quorum sensing (QS) pathways. For many years, medical devices have been sterilized by the medical industry with gaseous agents. However, the majority of the contamination occurs after the adhesion of the microorganisms (after biofilm is formed) and they can’t be effectively inhibited inside the human body (de Carvalho, 2007).

According to Doi et al. (2012), biofilms are defined as “aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface”. EPS, that

is integral in the formation of bacterial communities, consists of proteins, extracellular DNA and polysaccharides. The polysaccharide component of the matrix can provide many diverse benefits to the cells in the biofilm, including adhesion, protection, and structure. Aggregative polysaccharides, allow the bacterial cells to adhere to each other as well as surfaces by acting as molecular glue (Limoli, Jones, & Wozniak, 2015).

Once biofilms are formed, they are extremely difficult to eliminate. Many researchers and medical device developers have tried to stop them from forming in the first place. A biofilm starts when a few cells initiate specialized chemical hooks to adhere to a surface. These pioneering cells help to make a target surface more attractive to subsequent cells, which eventually mature into a complex, structured film (Figure 1.1) (Monroe, 2007).

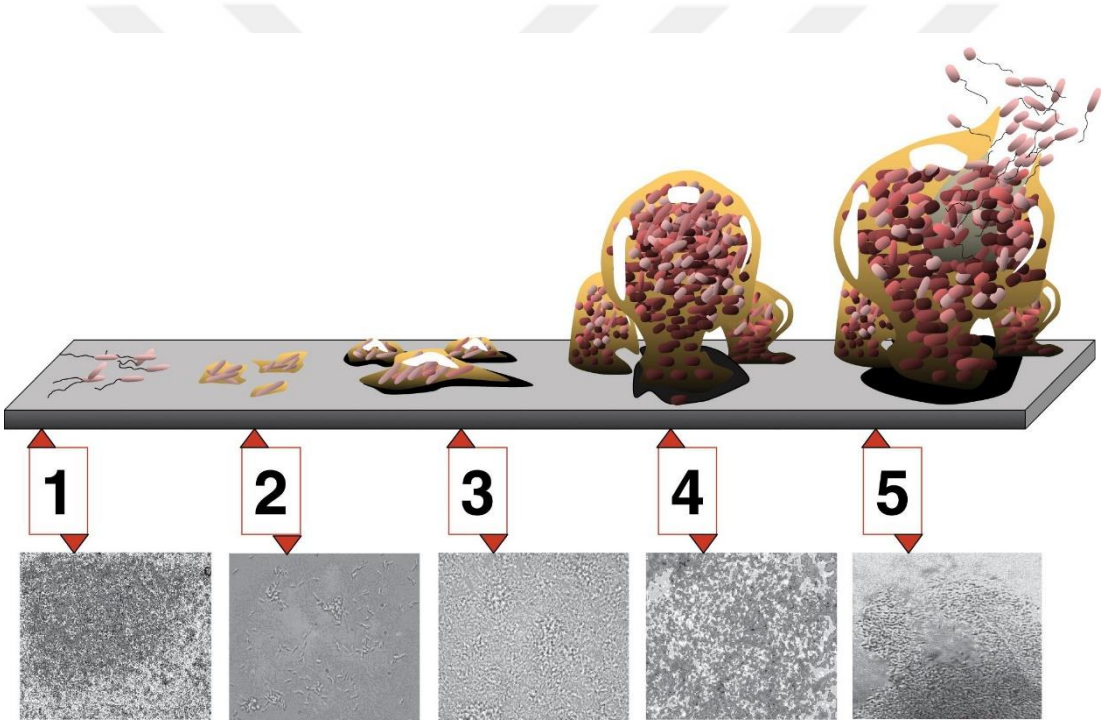


Figure 1.1 : Five stages of biofilm formation and development: 1) Initial attachment; 2) Irreversible attachment; 3) Maturation I; 4) Maturation II; 5) Dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to same scale (Monroe, 2007).

Either in surface-attached biofilms or in one layered bacterial formations, most cells in multilayered biofilms experience cell-to-cell contact that result in aggregates. Together with the properties of the matrix, the biofilm lifestyle is clearly distinct from that of free-living bacterial cells through intercellular interactions (Konopka, 2009).

Biofilms are associated with persistent infections in higher organisms, especially humans, and are also the main cause of contamination on medical devices and implants. Bacterial cells, together with the extracellular polymeric substances (EPS) that provide architecture and stability to the biofilm, can be considered to be habitat formers. Biofilms derive several properties that are not so similar to the free-living bacterial cells (Figure 1.2).

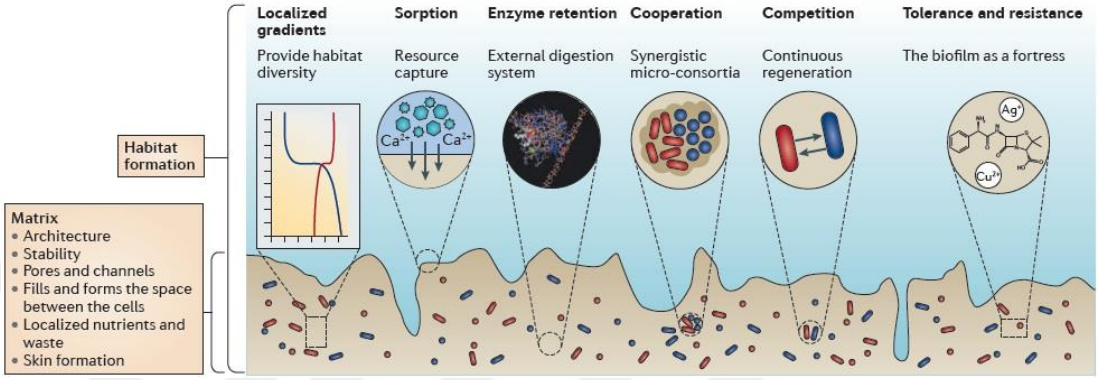


Figure 1.2 : Properties of biofilms and their habitat formation (Flemming et al., 2016).

The physical and social interactions of the bacterial cells provide them fundamental properties like enhanced rate of gene exchange and an increased tolerance to antimicrobials of the biofilm. The matrix formation is a dynamic process and depends on social competition, feeding by other organisms, synthesis and secretion of extracellular material, shear stress and nutrient availability (Flemming et al., 2016).

The ability of bacteria to form biofilms poses a major problem in various industrial and medical settings like contamination and infection, since biofilms are very difficult to remove. The impenetrable character of the biofilm, the slow growth rate of organisms, and the induction of resistance mechanisms have been proposed to explain the observed increased resistance of biofilms to antimicrobial agents (Donlan & Costerton, 2002).

The *rpoS* gene, which influences transcription of some *P. aeruginosa* genes, codes for an RNA polymerase σ subunit (Greenberg, 2000). Previous studies indicated that *rpoS* of *P. aeruginosa* may serve some role in biofilm development (Heydorn et al., 2000; Whiteley et al., 2001). Kjaergaard & Klemm (2003) found 46% of *RpoS*-dependent genes to be differently expressed in biofilms and deletion of *rpoS* rendered bacteria incapable of establishing sessile communities (Kjaergaard & Klemm, 2003).

Qin et al. (2007) have reported that *atlE*, encoding a major autolysin, is necessary for primary attachment and biofilm development of *S. epidermidis* in the wells of microtitre trays. Although the use of antibodies has failed to provide conclusive evidence that the AtlE protein is directly involved in mediating primary attachment, it is shown that the AtlE protein is located at the cell surface of *S. epidermidis*. It is therefore possible that, by promoting the release of extracellular DNA, AtlE plays an indirect role in *S. epidermidis* biofilm formation (Qin et al., 2007).

1.2.1 Biofilms of gram-positive bacteria and characteristic features of *Staphylococcus epidermidis*

The molecular mechanisms and factors involved in biofilm formation and subsequent dispersal of bacteria differ according to the nature of the microorganism and specific systems investigated. Focusing on a selection of Gram-positive bacteria including *Staphylococcus* spp, *Bacillus* spp, *Listeria monocytogenes*, and lactic acid bacteria (LAB), biofilms can display a wide range of phenotypes.

Biofilms of *Staphylococcus aureus* grown under static conditions consist of a dense layer of cells with an elaborate matrix harboring various types of polymers (Kovacs, Kuipers, & van der Veen, 2011). Likewise, biofilms of *Bacillus* spp form structured pellicles floating on culture media or architecturally complex colonies on solid agar media. Bacilli can also form submersed biofilms under static conditions at the air–liquid interface (de Leeuw, Moezelaar, Zwietering, & Abee, 2007). Another species of gram-positive bacteria, *L. monocytogenes* generally consist of a homogeneous layer of cells and microcolonies grown under static conditions. Biofilm cells display a morphology similar to that of planktonic cells. In addition, static biofilms of lactic acid bacteria (LAB) such as *Lactococcus lactis* and *Lactobacillus plantarum* consist of a dense layer of cells that are frequently embedded in polymeric substances (Kovacs et al., 2011).

Focusing on *Staphylococcus epidermidis*, which is a gram-positive and coagulase-negative bacterium, has become a serious nosocomial pathogen frequently causing infections associated with implanted materials (Figure 1.3). It is distinguished from coagulase-positive staphylococci such as *S. aureus* by lacking the enzyme coagulase (Otto, 2009a). In such device-associated infections, biofilm formation is a major factor determining *S. epidermidis* pathogenicity.

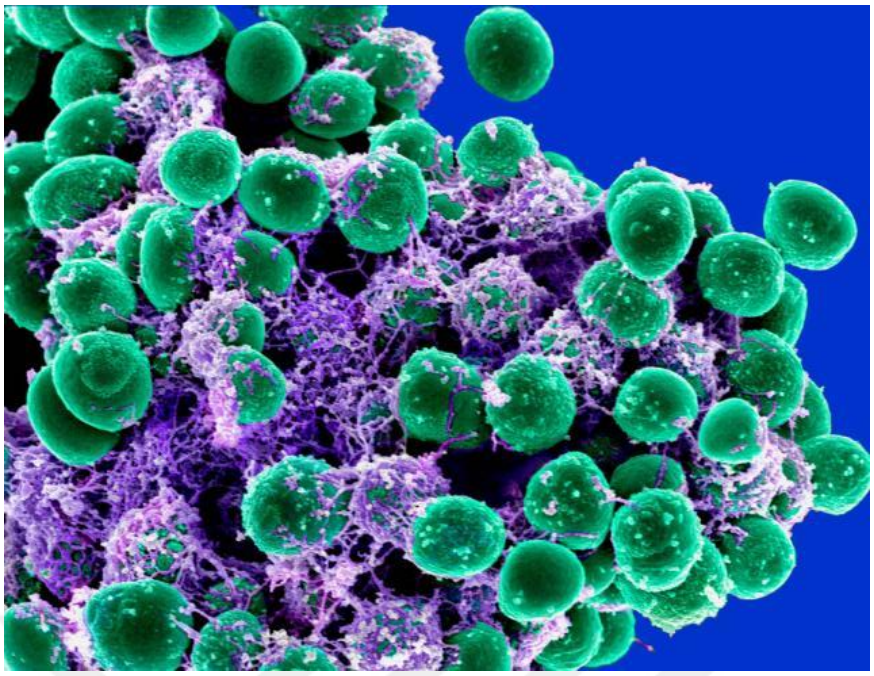


Figure 1.3 : Coloured scanning electron micrograph (SEM) of *Staphylococcus epidermidis* bacteria (green) embedded in an exopolysaccharide matrix (purple). These Gram-positive cocci (spherical bacteria) are able to form biofilms on plastics and so can cause infections around medical devices (Url-1).

S. epidermidis biofilm formation consists of a two-step process. The first stage includes attachment of cells to a surface (initial attachment phase). The second stage involves the formation of a multilayered architecture and cell–cell aggregation (accumulative phase). During biofilm formation, the polysaccharide intercellular adhesion (PIA) component of the EPS matrix of *S. epidermidis* is considered a major cell-to-cell interconnecting compound. However, other matrix components like extracellular DNA which has been shown to be important for biofilm formation of *Pseudomonas aeruginosa*, *Streptococcus intermedius* and *Streptococcus* mutans, may also be important for biofilm development of *S. epidermidis* (Qin et al., 2007). Initial attachment of *S. epidermidis* is mediated primarily by cell surface proteins (Limoli et al., 2015).

One autolysin protein, AtlE, facilitates bacterial attachment to the surface of medical devices and dictates pathogenesis for *S. epidermidis* biofilm-associated infections. During *S. epidermidis* growth through AtlE-mediated lysis of a subpopulation of the bacteria, extracellular DNA is generated. This process is required for initial bacterial attachment to surfaces and biofilm development (Liang, Parsons, Findlay, Molin, & Zhiqiang, 2012). AtlE, encoded by the *atlE* gene, is a bifunctional autolysin with an N-terminal alanine amidase domain, a central cell wall-anchoring (CWA) domain, and

a C-terminal glucosaminidase domain. The CWA domain of AtlE has adhesive properties and is probably involved in the interaction between staphylococcal cells and biomaterial like other CWA domains of gram-positive autolysins able to mediate bacterial adhesion (Figure 1.4) (Sivadon et al., 2009).

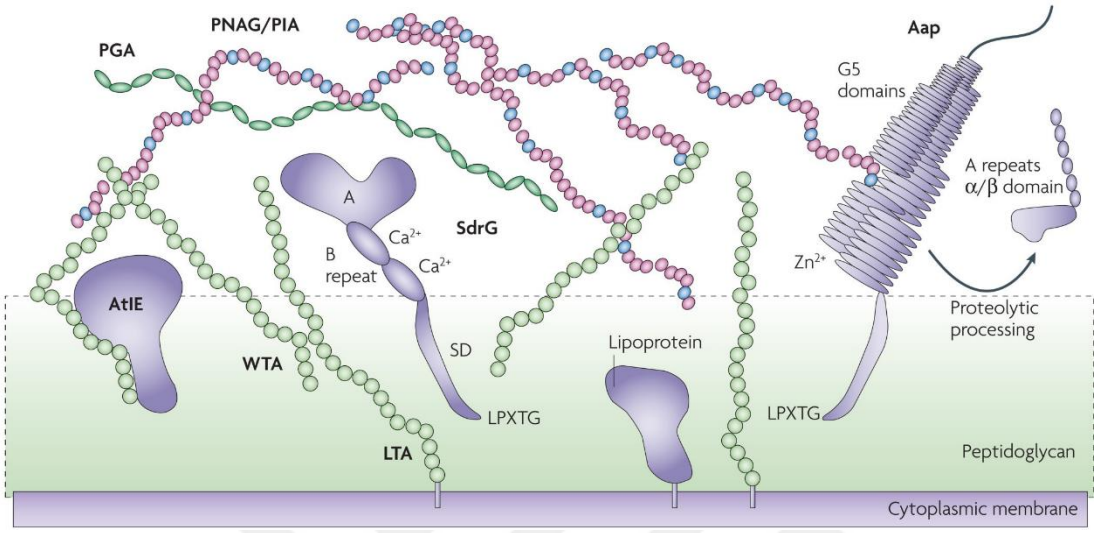


Figure 1.4 : The *S. epidermidis* cell surface. AtlE is a bifunctional adhesin/autolysin that contributres to biofilm formation by its surface hydrophobicity and to host matrix protein binding.

AtlE plays a role in binding of the cells to a naked polystyrene surface during early stages of adherence and also to plasma protein-coated polymer surfaces during later stages of adherence (Buttner, Mack, & Rohde, 2015; C. Heilmann, Hussain, Peters, & Gotz, 1997). Significantly, higher accumulation of AtlE was observed in the early exponential growth phase of the wild type and much higher accumulation of AtlE was detected in stationary growth phase of the mutant by proteome analysis of the extracellular secreted proteome of *S. epidermidis* (A. P. Davies, Harris, Rohde, Horstkotte, & Knobloch, 2007). Adhesion to abiotic surfaces such as catheters is mainly governed by bacterial cell surface hydrophobicity. The abundant surface protein AtlE is a specific protein that impacts surface adhesion in *S. epidermidis* (Otto, 2009b).

The *luxS* QS system is present in a variety of Gram-negative and Gram-positive bacteria. In several pathogens it is involved in virulence, which is shown that *luxS* is functional in *S. epidermidis* and impacts biofilm formation. Compared to the wild-type strain, the *luxS* mutant strain formed a thicker and more compact biofilm in an animal model of central venous catheter infection. *luxS* appears to influence biofilm formation via transcriptional regulation of the *ica* gene locus by altering production of an

exopolysaccharide called the polysaccharide intercellular adhesin (PIA). This points to a common scheme of QS dependent regulation of biofilm formation and biofilm associated infection in staphylococci (Kong, Vuong, & Otto, 2006).

Although, there are no noticeable differences in the growth patterns of luxS positive and negative strains of *S. epidermidis* biofilm growth, it is observed that luxS is involved in the activated methyl cycle and thus may alter the metabolism and biofilm formation of bacteria (Laverty, Gorman, & Gilmore, 2013).

1.2.2 Biofilms of gram-negative bacteria and characteristic features of *Pseudomonas aeruginosa*

Many persistent and chronic bacterial infections like periodontitis, biliary tract infection and endocarditis, are linked to the formation of biofilms in the medical field. These biofilms often serve as a source for recurrent infections and virtually all medical implants are prone to colonization by pathogenic bacteria. Sessile bacteria are up to 1000-fold more resistant to antibiotics, biocides, and hydrodynamic shear forces than their planktonic counterparts so bacterial biofilm infections are particularly problematic.

Within the Gram-negative bacteria, QS systems have been identified in bacterial species belonging to the α, β, and γ subclasses of Proteobacteria, including bacteria in the genera *Agrobacterium, Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia, Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahnella, Ralstonia, Rhodobacter, Rhizobium, Serratia, Vibrio, Xenorhabdus,* and *Yersinia*. All of these systems share several regulatory features. (Schembri, Givskov, & Klemm, 2002).

The Gram-negative bacterium *Pseudomonas aeruginosa* has become a model organism for independently studying QS and biofilm formation (Figure 1.5). It has been discovered that for the elaboration of mature, differentiated *P. aeruginosa* biofilms, QS was required (de Kievit, 2009). In addition, matrix components such as extracellular DNA may be important for biofilm development formation of *Pseudomonas aeruginosa* (Barken et al., 2006). Extracellular DNA functions as a structural component and cell–cell interconnecting compound and its production has been shown to be regulated via quorum sensing in *P. aeruginosa biofilms* (Qin et al., 2007).

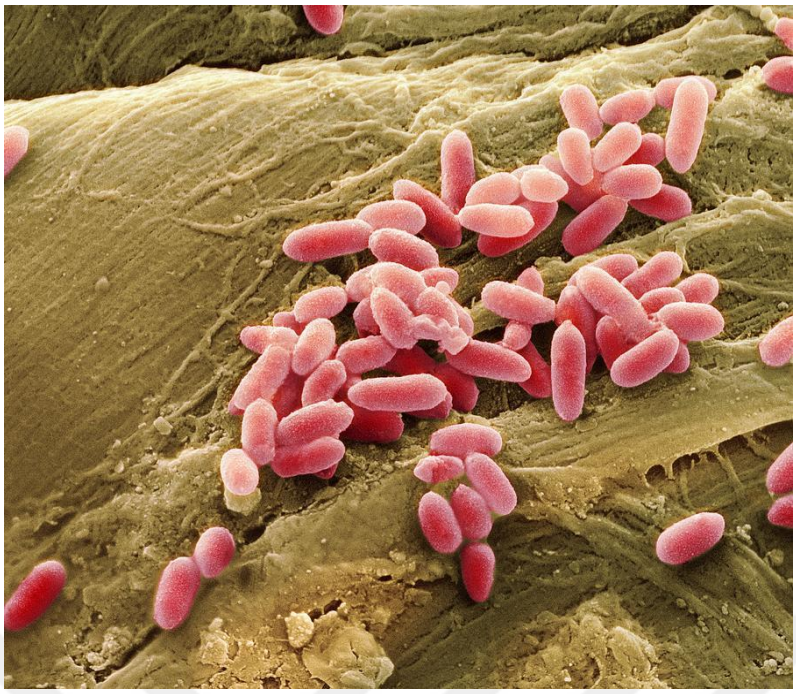


Figure 1.5 : *Pseudomonas aeruginosa* bacteria, coloured scanning electron micrograph (SEM). These Gram-negative rod-shaped bacteria can cause serious wound, lung, skin and urinary tract infections. These types of infections are most common in hospitals (Sandle, 2016).

It appears that exopolysaccharide, extracellular DNA and proteinaceous compounds can all function as matrix components in *P. aeruginosa biofilms*. Their relative importance as structural component may depend on the particular *P. aeruginosa* strain forming the biofilm, on the age of the biofilm and on the environmental conditions. Both *P. aeruginosa* PAO1 reference strain and clinical *P. aeruginosa* isolates show evidence for a role of extracellular DNA as cell-to-cell interconnecting compound in *P. aeruginosa* biofilms. It is reported that the cells in young PAO1 biofilms are held together by extracellular DNA whereas the cells in older PAO1 biofilms are held together primarily by the other compounds. Exopolysaccharides encoded by the *psl* genes appear to be the critical matrix component, while DNA makes up a substantial fraction of the matrix material in mature PAO1 biofilms. (Barken et al., 2006).

The *RpoS* sigma factor, has been described as a master stress-response regulator under various stress conditions and a central regulator of many stationary phase-inducible genes. Depending on various environmental conditions such as nutrient starvation, high osmolarity, heat shock, the presence of hydrogen peroxide and growth phase, significant physiological changes occur in bacteria. These environmental conditions can trigger the induction of stress-response genes. It was previously reported that the

genes which are under the control of *RpoS*, affect the antibiotic tolerance of stationary-phase cells. Genes involved in heat and osmotic induction are also known to be controlled by *RpoS*. The *RpoS* level in *P. aeruginosa* increases upon entry of the cells into the stationary phase. Furthermore, *RpoS* affects the expression of more than 40% of all quorum controlled genes and the production of extracellular alginate and biofilm formation (Bangera et al., 2001; Murakami et al., 2005).

1.2.3 Infections and biofilm

According to the Center for Disease Control and Prevention (Atlanta, GA), 65% of human diseases caused by human bacterial infections are associated with biofilms. The three-dimensional extracellular polymeric substance (EPS) matrix generated in bacterial biofilms forms physical and chemical barriers to antibiotics. It is thus very difficult to eradicate biofilms with antibiotic treatment (Kang, Jeong, Kim, Lee, & Jang, 2010).

A very large proportion of all implant-related infections, roughly four out of five, are caused by staphylococci mainly *Staphylococcus aureus* and *Staphylococcus epidermidis* respectively. Then follows the order in terms of prevalence of *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Infections caused by all existing pathogenic microbial species represent together only a minority of implant infections of just about 22%, except staphylococci. *Staphylococcus* genus therefore acquires a huge importance in implant-related infections (Campoccia, Montanaro, & Arciola, 2006).

In humans, the coagulase-negative *S. epidermidis* are among the most leading causes of nosocomial infections. Infections due to S. epidermidis typically are more subacute or even chronic. The most critical pathogenicity factor in these infections is the colonization of abiotic or biotic surfaces by the formation of a three-dimensional structure called a biofilm. Microorganisms within a biofilm are protected against the immune system of the host as well as against antimicrobial chemotherapy (Christine Heilmann & Götz, 2010).

It is now well-known that in many diseases like osteomyelitis, native valve, dental caries, endocarditis, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients, biofilm formation is an important factor. Bacterial infection in those implants, which generally

ends up with premature implant removal, have a potential of serious complications (Gupta & Kumar, 2008).

Since microorganisms of biofilm easily and rapidly develop resistance to any condition such as disinfectants, antibiotics and other stress factors, they require continuous, complex and combined treatment methods (Jefferson, 2004).

1.2.4 Approaches for the prevention of bacterial biofilms

Traditional strategies to control biofilm infections are based on the use of compounds that kill or inhibit the growth of planktonic bacteria. However, "biofilm-bound" bacteria are significantly less responsive to antibiotics and antimicrobial agents than freely suspended bacteria of the same species. Studies have shown that it is difficult for the antibodies to penetrate into bacteria in a biofilm. Therefore, it is crucial to develop new methods for prevention of bacterial colonization and biofilm formation (Ma & Katzenmeyer-Pleuss, 2017). Consequently, the first and preferred strategy against biofilm infection is to prevent invading bacteria from forming aggregates in the first place. Since the aggregates show increased tolerance to antibiotics, development of treatments that block surface attachment or other specific events in the early stages of aggregation and may interfere bacteria in a planktonic, susceptible state (Khan, Ahmad, Sajid, & Cameotra, 2014). The microorganism in an efficiently formed biofilm structure can tolerate antimicrobial agents at very high concentrations. That is 10 to 1000-times higher that needed to kill genetically equivalent planktonic bacteria. Moreover, biofilms are very difficult to eliminate from living hosts, which cause them to be extremely resistant to phagocytosis and degradation (Jefferson, 2004).

1.2.4.1 Formation of functional coatings

One of the most widely used antimicrobial technologies are coatings. There are a number of types of coating techniques (as described under) and the range of strategies maintains to increase as innovative new technologies are advanced. Table 1.1 summarizes examples, results and obstacles of these technologies (Z. Zhang, 2017).

Functional coatings are widely used when bulk properties of substrates needed to be conserved in order to protect surfaces against microbial attacks. In order to assemble the required functionality and permanence criteria, a fine thin film coating should have

resistence to corrosion, good adhesion to substrate and precise chemical control (Gupta & Kumar, 2008).

The micro-organisms attached to the surface may directly inactivate or kill and/or prevent their adhesion by addition of organic or inorganic compounds to superficial coatings. These coatings may be applied to pipes, fibres, glass, tiles, plastic, metals, food wrappers, etc. The idea of introducing biocidal agents, such as metals, within the surface material became an appealing solution, especially at long term. To prevent infections in tissue pockets, silver coatings may be used in implanted devices. Reactive groups may also be linked to the polymers such as polypropylene, silicone, polyester and polyurethane to produce surface functionalised polymers (de Carvalho, 2007).

1.2.4.2 Incorporation of detergents and biocides into surface materials

Biofilms are strongly hindered by biocides or antibiotics penetration. Factors that lead to inhibition of biofilm growth, disruption, or eradication of biofilms are being investigated to increase the efficiency of new treatment strategies against bacterial infections. Microbial products, enzymes, sodium salts, metal nanoparticles, antibiotics, acids, chitosan and its derivatives, or plant products all influence biofilm structure via various mechanisms and with different efficiencies (Khan et al., 2014).

Bacteria residing within a biofilm is protected against biocides or detergents to a certain degree by the surrounding extracellular matrix. For instance, bacteria spreaded from a contaminated catheter and growing as a biofilm in the human host, are more resistant to antibiotic treatment and less susceptible to the immune response (Brombacher, 2004). Either biopassive or bioactive approaches have been developed in modifying surfaces to be anti-fouling to bacteria. Biopassive surface modifications aim to prevent the initial adhesion of bacteria, which are also known as “nonadhesive” or “repellent,”. Bioactive surface modifications actively kill bacteria that contact with the surface. In this approach, biocides such as quaternary ammonium compounds, *N*-halamines, antimicrobial peptides and antibiotics or broad-spectrum antimicrobials like silver ions and nitric oxide are typically used. These biocides can be adsorbed onto the surface and released into the environment or covalently bound to the surface or physically entrapped. However, the use of biocides can pose a substantial environmental risk (Z. Zhang, 2017).

Table 1.1 : Types of anti-biofilm technologies and their characteristics (Z. Zhang, 2017).

Anti-biofilm technologies		Examples	Mechanisms	Limitations
Coatings	Anti-adhesive	PEO, zwitterionic polymer, topographical structure, superhydrophobic coating	Low surface energy chemistry and nano-/ micro-textured morphology reduce fouling, passive repelling	Stability, oxidation damage, in vivo efficacy may vary
	Antimicrobial loaded	Materials loaded with small molecule biocides, heavy metal, antibiotics, etc.	Active inhibition at the surface	Issues with optimizing release for effectiveness in preventing resistance, in vivo efficacy
	Controlled/active release	Temperature-responsive copolymer, hydrolytically degradable film, PH-sensitive releasing	Active inhibition, release in response to stimuli	Release profile, stability, in vivo efficacy
	Dual/ multifunctional	Differentially adhesive surfaces, low fouling, and antibacterial coating	Prevent colonization while promote tissue integration, combines modalities to reinforce efficacy	Complex to optimize
Antimicrobial/anti-biofilm agents	Antibiotics	Gentamicin, rifampicin, minocycline, doxycycline, etc	Bactericidal through multiple genetic and biochemical pathways	Resistance
	Metal ions, oxides, nanoparticles	Silver zeolite, copper oxide, zinc oxide, ferric ammonium citrate	Release metal ions that target bacterial cells, some actions not well understood	Allergy

Table 1.1 (Continued) : Types of anti-biofilm technologies and their characteristics (Z. Zhang, 2017).

Anti-biofilm technologies		Examples	Mechanisms	Limitations
	Cationic compounds	Cationic surfactant, polymers, peptides, lipids, etc.	Disrupting bacterial membranes, allowing the free exchange of intra- and extracellular ions	Complex process in extraction, isolation and purification, expensive
	Quorum sensing inhibitors (QSI)	Triazolyldihydrofuranone, cinnamaldehyde, hamamelitannin	Inhibit virulence factors and biofilm formation	New entities chemical, limited information
	Dispersing enzymes	DNase I, DspB, a-amylase, restriction endonucleases	Cause biofilm detaching	Expensive
	Bacteriophage	Caudovirales, ligamenvirales, some unassigned viruses	Viruses that infect bacterial cells	Host specified, purification
	Natural compounds	Phenol, phenolic compounds, etc.	Diverse, not well studied	Complex composition add difficulties to optimize efficacy
Biofilm removal techniques/ physical strategies		Manual debridement, pulsed electrical fields, ultrasound therapy, and other topical and combination therapies	Remove multispecies bioburden or devitalized host tissue	Promising yet the efficacy has yet to be proven in the clinic
Vaccines		Live, attenuated; toxoid; killed, whole cell; polysaccharide; polysaccharide—protein conjugate	Leveraging immune system	Critical phenotypes and factors are not adequately addressed
Combined Modalities		Polyphenolic compounds and antimicrobial agent, enzyme-based compounds combined with metal ions, antimicrobials with non-contact ultrasound therapy	Synergistic antibacterial mechanism	Complexity

Although biocides are non-antibiotic chemical compounds, they are able to inhibit microbial growth or even kill microorganisms with their disinfecting and antiseptic properties. These disinfecting properties depend on chemical properties (e.g. reactivity, temperature of activity and optimum pH), characteristics of microorganism (e.g. metabolic status, tolerance/resistance, number of organisms in the population) and environment (e.g. water activity, surface type, presence of other reactive compounds). The biocide should not be easily inactivated and have a wide range of activity against microorganisms and conditions of action. Some coatings act only on attached cells and may not be able to inhibit cell attachment. For instance, (-)- usnic acid-loaded polymers prevented the biofilm formation of *Staphylococcus aureus* by killing the adherent cells but did not prevent attachment of the cells. Polymers such as polypropylene, rubber, silicone, polyester and polyurethane could be coated with reactive groups to produce surface functionalised polymers (de Carvalho, 2007).

The microorganisms to be removed from the surfaces and the nature of the contaminating residue materials (carbohydrates, fat, proteins, mineral salts) are important to develop an effective control of undesirable biofilms. Moreover, the choice of detergents and disinfectants relies upon their efficacy, safety and simplicity of expulsion; specifically to the corrosive nature of the chemical treatments and the subsequent sensory value effects on the final products (Simões, Simões, & Vieira, 2010).

1.2.4.3 Quorum sensing and inhibition targets/strategies

Biofilm formation is regarded as a strategy for bacteria to survive. Some advantages like increased tolerance and adaptation to several responses are provided to bacteria by this unity. A special mechanism called quorum sensing, allows bacteria inside the biofilm structure to communicate with each other. By quorum sensing, bacteria produce small signal molecules, which can diffuse into their environment and provide a concentration-dependent interaction with special receptor proteins. Quorum sensing is evolved to sense and monitor the population density among the bacteria and is related with several vital processes like the control of expression of virulence factors, motility, protection, biofilm formation/maintenance etc. (Bjarnsholt & Givskov, 2008).

QS systems in gram-negative bacteria are controlled by autoinducers N-Acyl-L-Homoserine Lactones (AHL). These molecules take place in the quorum sensing mechanisms of many gram-negative bacteria and are widely conserved signal molecules. For the coordination of some cellular activities and regulation of some genes, the bacteria first release, then detect and respond to the accumulation of these signal molecules (Dong, Gusti, Zhang, Xu, & Zhang, 2002). Gram (+) bacteria also possess QS systems but are different in terms of signal molecules when compared to the systems of Gram (-) bacteria. The QS system in these bacteria work by using small signaling peptides, which are variable in length, Instead of the AHL molecule-based system in Gram (-) bacteria. These peptides are moved out by active transportation after they are cleaved and processed inside the cells. These transported peptides initiate a response inside the cell by interacting with transmembrane receptors of two-component regulatory systems. QS-regulated genes expression increases by the proliferation of bacterial density and the concentration of the signal peptides also increase (Bjarnsholt & Givskov, 2008).

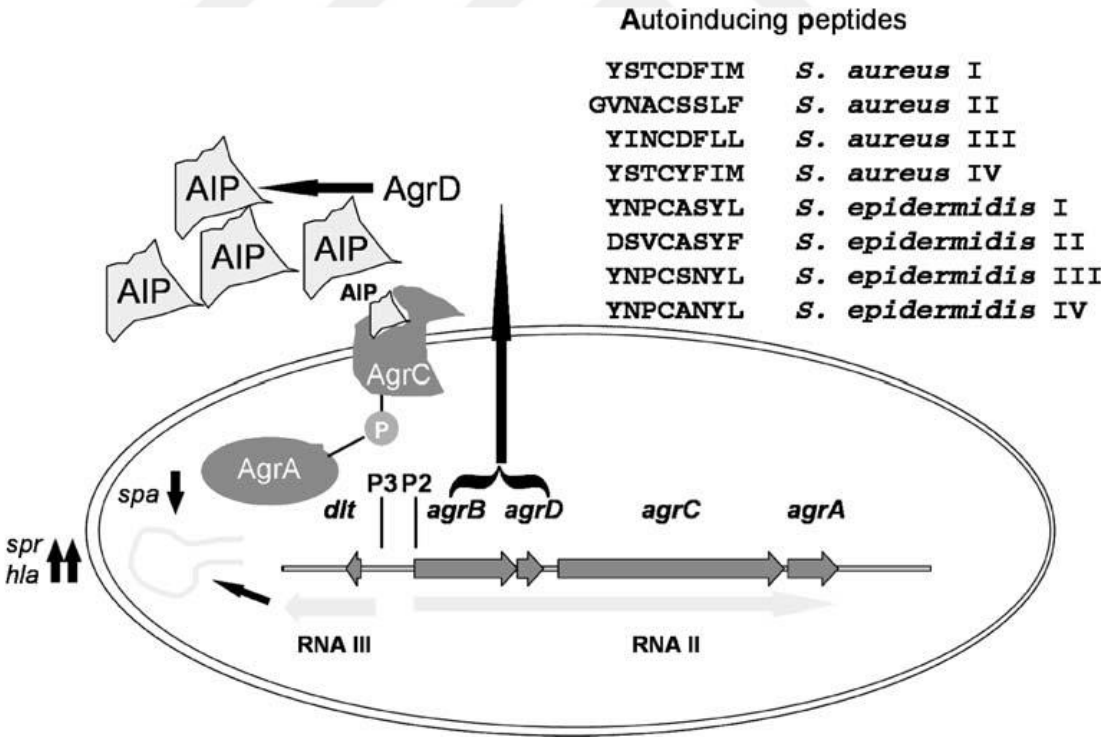


Figure 1.6 : Schematic representation of the function of the *agr* system of *Staphylococcus* (A. P. Davies et al., 2007).

In *Pseudomonas aeruginosa* and many other organisms, a notable correlation has been demonstrated between biofilm formation and HSL-mediated quorum-sensing mechanisms. In *Staphylococcus*, two quorum-sensing systems have been described to

date and both appear to have an impact on biofilm formation. The best known quorum sensing system is the accessory gene regulator (agr) system, which has been studied in great detail especially in *S. aureus*. The second known is the *Staphylococcus* luxS/AI-2 system (Figure 1.6). (A. P. Davies et al., 2007).

Davies et al. (1998) showed that in *P. aeruginosa* two different cell-to-cell signaling systems, lasR-lasI and rhlR-rhlI, were involved in biofilm formation. Acylated homoserine lactone signals were also detected in biofilms of gram-negative bacteria on urethral catheters (D. G. Davies et al., 1998; Donlan, 2002).

Some human life-threatening diseases are related to QS mechanisms which are controlled by bacterial biofilms coordinated behaviours such as virulence factor production and formation. Consequently, QS has been considered as an attractive target for the development of new anti-infective and therapeutic agents. Although different QS systems have been identified in bacteria, all of them function with the same principle that involves signal production, followed by their accumulation in the extracellular environment and finally signal detection by the specific receptor.

Main quorum-sensing inhibition strategies include, (i) inhibition of QS signal detection by receptor blockage, (ii) QS signal degradation in the extracellular environment, (iii) inhibition of QS signal biosynthesis and (iv) disruption of efflux pumps. For instance, compounds such as L/D-S-adenosylhomocysteine, sinefungin and butyryl-SAM have been reported to suppress the AHL synthesis and inhibit the first step of pathogenic *P. aeruginosa* in QS signalling (blockage of Acyl-Homoserine Lactone production in gram-negative bacteria). Similarly, in gram-positive bacteria, inhibition of ribosomes and peptidases responsible for the synthesis of Autoinducing Peptides (AIPs), creates bactericidal activity which results in in addition to quorum-quenching activity that theoretically increases the pressure on bacteria to develop resistance (Kalia, 2016). In addition, Alfaro et al. (2004) reported that the synthesis of two substrate analogues, S-anhydroribosyl-L-homocysteine and S-homoribosyl-L-cysteine, prevent the initial and final step of the QS mechanism. (Alfaro, Zhang, Wynn, Karschner, & Zhou, 2004).

1.2.4.4 Enzymes

Replacement of biocides with non-toxic alternatives such as enzymes could be one of the solutions to overcome the problem with biofilm formation (Kristensen et al., 2008).

Ranging from food industry to large scale biocatalysis, enzymes have been used in several industries and are also used for the removal and degradation of bacterial biofilms. Several enzymes like hydrolases and lyases, individually and/or their combinations, could be used to target the complex Extracellular Polymeric Substances (EPS) described above. These enzymes could disintegrate the polymeric networks composing the biofilm matrix and detach the biofilm from the surface it is attached (Kristensen et al., 2008; Leroy, Delbarre, Ghillebaert, Compere, & Combes, 2008). Besides these enzymes, bacterial cell-to-cell communications can also be directed by some enzymes that specifically block the bacterial population-density-dependent attack. This strategy is called quorum-quenching mechanism that can be used in controlling bacterial pathogens and to build up a proactive defense barrier (L. H. Zhang, 2003).

Overwhelming majority of gram-negative bacteria possess signal molecules for QS systems which include *N*-acylhomererine lactones (AHL or AI-1), that interact with a LuxR-type receptor protein. AHL-dependent system plays an important role in microbial pathogenicity and in bacterial biofilm formation. So, it is logical to conclude that its inhibitors may probably be promising antibiofilm agents (Mart'yanov, Teteneva, & Zhurina, 2017).

V. Singh et al. (2006) reported that 5-methylthioadenosine/S-adenosylhomocysteine nucleosidase, is suppressed by immucillin A (V. Singh et al., 2006). Activity of another enzyme, triclosan suppresses AHL synthesis by interaction with the enzyme enoyl-acyl carrier protein reductase, which catalyzes the synthesis of AHL precursors (Phan & Marquis, 2006).

Yet another approach is destruction or modification of existing signal molecules by specific enzymes (lactonases, acylases, amidases, and oxidoreductases). Most commonly used enzymes are hydrolyzing the major EPM components (polysaccharides, proteins, and DNA). For instance, polysaccharide hydrolase (dispersin B) was a useful agent against staphylococci biofilms, which hydrolyzes Poly-N-acetylglucosamine. Similarly, gentamycin sensitivity of *P. aeruginosa* biofilms is significantly increased by alginate lyase, which hydrolyzes alginate of the EPM. Another enzyme hydrolyse, was used for hydrolysis of *P. aeruginosa* and *Burkholderia cenocepacia* biofilms. In some cases, hydrolysis of extracellular DNA (eDNA), which is present in the EPM of many biofilms, results in biofilm dispersion or makes the biofilm cells susceptible to biocides. Hence, it is a convenient target for

biofilm treatment. staphylococcal biofilms formed on glass, plastic, or titanium treated with DNase, resulted in biofilm disruption. Especially the mixture of DNase and alginate lyase, significantly increased the sensitivity of *P. aeruginosa* biofilms to aminoglycosides (Mart'yanov et al., 2017).

1.2.5 Approaches for the removal and killing of established biofilms

1.2.5.1 Using oxidizing biocides

There is a wide range of chemical disinfectants, which may also differ in their ability to kill target microorganisms. These disinfectants can be divided according to their mode of action: surface active compounds including quaternary ammonium compounds (QACs) and acid anionic compounds; oxidizing agents including chlorine-based compounds, hydrogen peroxide, ozone and peracetic acid; and iodophores. Disinfectants containing hydrogen peroxide or peracetic acid decompose into oxygen and water (or acetic acid) so they are regarded as environmentally friendly. Hydrogen peroxide is widely used in disinfectants, has been found to be effective against biofilm cells and affects the biofilm matrix (Khan et al., 2014).

Medical instruments have been sterilised with gaseous agents such as ethylene oxide and chlorine dioxide for fifty years. They are commercialised in nonflammable blends with inert carrier gases to overcome their explosive character. Though, many of the corrosion or fouling processes occur inside the human body after adhesion and growth of microorganisms. Therefore, rough treatments of the surfaces of the devices to prevent and/or destroy cell adhesion are hindered. Antimicrobial affluences required to destroy biofilms are not only toxic to microorganisms but may also be toxic to the patient. Some microorganisms may produce specific compounds able to destroy the biocide molecule at the same time causing allergic reactions to human body (de Carvalho, 2007).

1.2.5.2 Standart antimicrobial agents

Each bacterial community in a biofilm develops multiple mechanisms and defences for survival against biocides, antibiotics, and host immunity, as well as defenses against phagocytosis, UV radiation, viral attack, shear stress and dehydration. Biofilm organisms typically exhibit a high resistance to antimicrobial agents , like 100 to 1000 times the concentrations of antibiotics and biocides compared to their planktonic

counterparts (Wolcott & Ehrlich, 2008). Doyle (1999) reported that reactive biocides such as hyperchlorite and hydrogen peroxide had limited penetration into a biofilm. In contrast, other types of antimicrobials such as antibiotics penetrate quickly into the biofilm but still have limited efficacy compared to planktonic cells. It is also reported that as planktonic cells adhere to a surface and form biofilms, a large number of genes are upregulated. Such genetic transformations may also play a role in the antimicrobial resistance of biofilms while changing from the planktonic to the sessile state (Doyle, 1999).

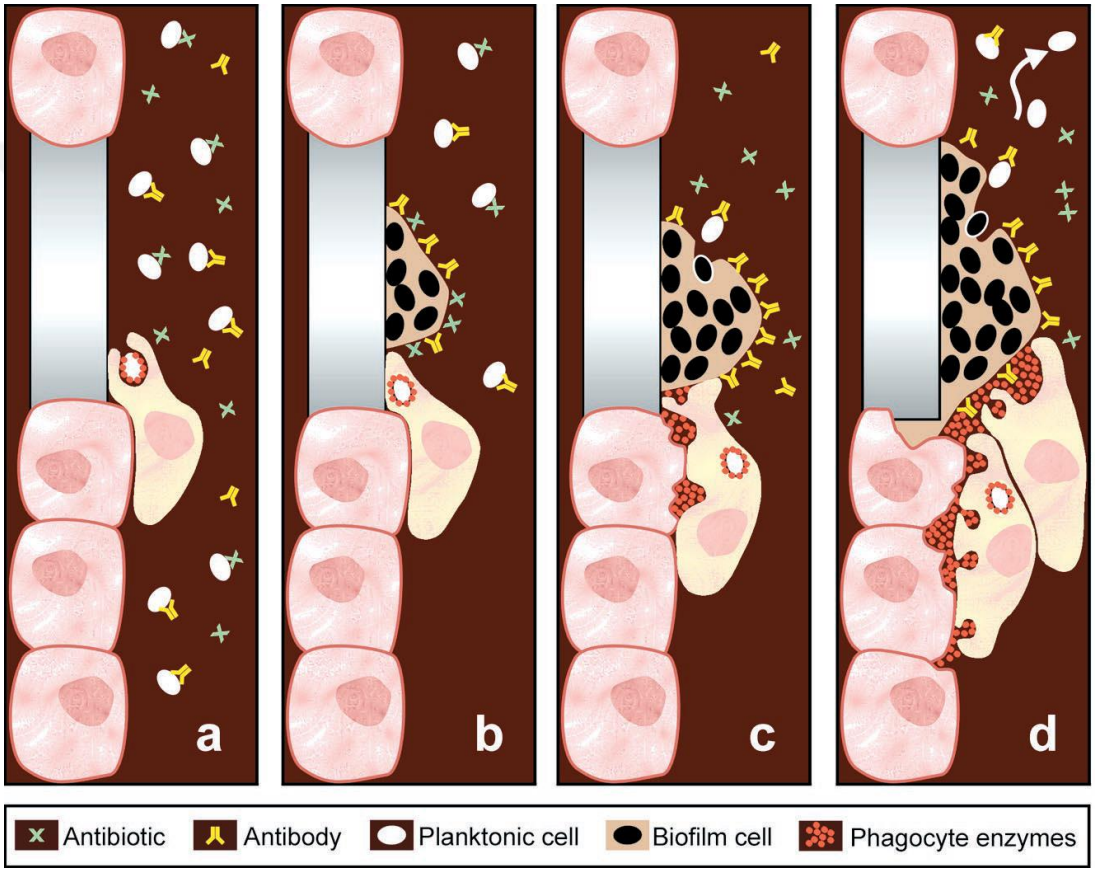


Figure 1.7 : Diagram of a medical biofilm. a) Planktonic bacteria can be easily cleared by antibodies and are susceptible to antibiotics b) Biofilms on inert surfaces are resistant to antibodies, phagocytes, and antibiotics. c) Phagocytes are attracted to the biofilms; phagocytic enzymes are released. d) Planktonic bacteria are released from the biofilm and cause infection (Costerton, Stewart, & Greenberg, 1999).

There are three hypothesis proposed to understand biofilm resistance to antimicrobial agents (Figure 1.7). One hypothesis is the failure of an agent to penetrate the full depth of the biofilm. A second mechanism suggests that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state, the state where cells are not very susceptible to many antimicrobial agents. A third

mechanism suggests that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype causing reduced biofilm susceptibility (Costerton et al., 1999). The finding that a strain of bacteria is susceptible to a specific antibiotic is a reliable indicator of the effectiveness of that drug in most clinical situations. For instance, the activity of β-lactam antibiotics is poor and the aminoglycosides have limited efficacy (Prince, 2002).

1.2.5.3 Removal of biofilms using carbon dioxide aerosols

In the CO_2 aerosol cleaning technique, tiny CO_2 particles are generated by supersonic expansion and these particles are projected towards contaminated surfaces at high velocity (Sherman, 2007). By passing through a small nozzle, the high-pressure CO_2 gas expands, condensation nuclei form and the gas then becomes supersaturated. Due to an adiabatic decrease in temperature, the nuclei grows and solidifies on further expansion. The solid CO_2 particles hitting the surface, drive out surface contaminants. Moreover, the solid CO_2 particles form a solvent at the solid CO_2–surface interface by melting on an impact. The contaminants that adhere to the surface are suspended by the solvent. The removal of surface contaminants is both initiated by momentum transfer and solvent action; the CO_2 gas then carries them away from the surface (Figure 1.8) (Kang et al., 2010).

That periodic jet of carbon dioxide (CO_2) aerosols, used to remove biofilms from various substrate surfaces, is a very quick and effective mechanical technique. However, the impact of the aerosols has never been evaluated on the viability of bacteria during treatment. When CO_2 aerosols were used to disperse biofilms of *Escherichia coli*, it was found that they led to a significant loss of viability, with approximately 50% of the dispersed bacteria killed in the process. This CO_2 aerosol technique is similar to high-pressure water sprays. Therein biofilms are removed primarily by mechanical impact or momentum transfer. However, the momentum that is delivered to the surface is much smaller in the CO_2 aerosol technique, as a result a negligible amount of damage occurs at the surface. (R. Singh, Monnappa, Hong, Mitchell, & Jang, 2015).

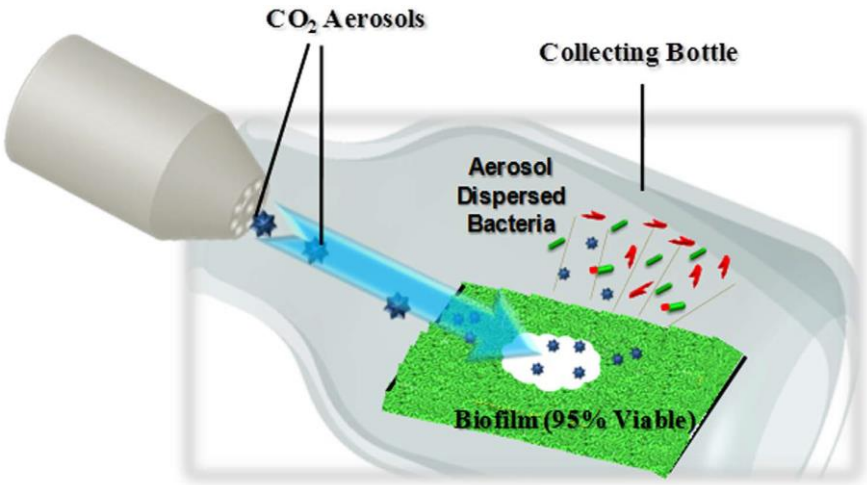


Figure 1.8 : Schematic illustration of the CO_2 aerosol technique. Used to expose bacterial biofilms to CO_2 aerosols and capture dispersed cells (Dwidar, Hong, Cha, Jang, & Mitchell, 2012).

1.2.5.4 Ozone and ultrasound

One of the proposed biofilm control strategy is the application of ozone and ultrasound. However, many such treatments may not be suitable for medical devices, because of concerns with device compatibility or effects on the patient. It was demonstrated that ultrasound used in combination with gentamicin, improved the efficacy of the antibiotic against *P. aeruginosa* biofilms on steel (Donlan & Costerton, 2002).

Ozone, also has been used successfully to kill and remove some biofilms, which is an even stronger oxidiser (Meyer, 2003). Ozone treatment in combination with ultrasound, significantly reduced biofilms from stainless steel surfaces, higher than by either treatment alone. These results indicated that ultrasound and ozonation used in combination may also be an effective treatment for biofilm removal (Baumann, Martin, & Feng, 2009).

1.3 Extremely Low Frequency (ELF) Electromagnetic Fields (EMFs)

Stationary charges create an electric field, whereas a magnetic field requires moving charges. Voltage and current, stationary and moving charges come together to produce electromagnetic fields (Consales, Merla, Marino, & Benassi, 2012). Natural and man-made sources both produce static electric and magnetic fields, as well as low-frequency fields. The natural fields are static or very slowly varying. On the other hand, most man-made sources are at extremely low frequencies. Humans are widely exposed to ELF fields of the order of 10-100 V/m and 0.1-1 μT, and occasionally to

much stronger fields, by the generation, transmission, distribution and usage of electricity at 50 or 60 Hz. Interest and concern about potential hazards of ELF fields are comprehensible, because ELF fields can interact with biological systems. The effects of electric and magnetic fields on cells behaviour, whether it is deadly or causing some other alterations, has been researched for decades in the scientific field (Humans, 2002).

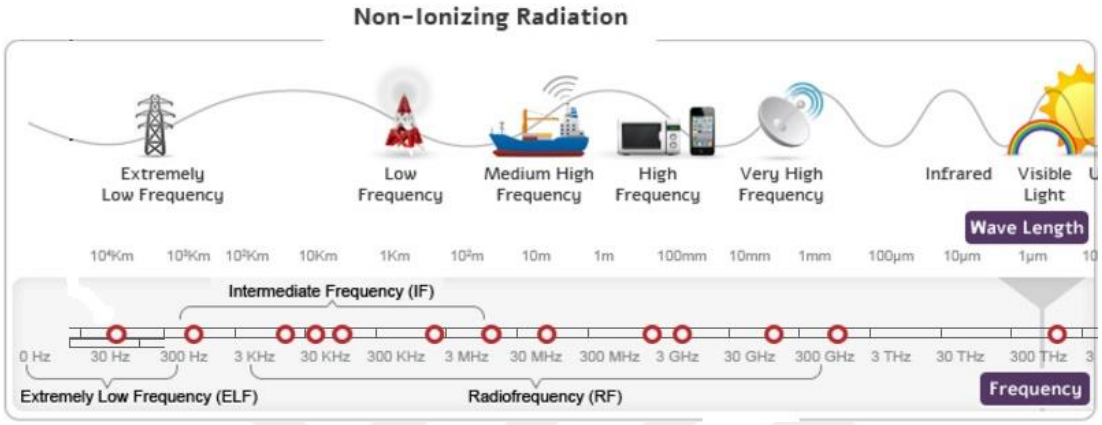


Figure 1.9 : The Electromanetic Non-Ionizing Radiation Spectrum and Extremely Low Frequency ("The Electromanetic Radiation Spectrum," 2015).

Electromagnetic fields are classified as ionizing and non-ionizing electromagnetic fields in two groups (Figure 1.9) (Souques, 2004). Non-ionizing electromagnetic fields include microwaves, radiofrequency fields, high frequency fields, as well as extremely low frequency fields and have frequencies below 300 GHz (Consales et al., 2012). The ratio of the wavelength of the EM fields to the object size defines the characteristics of EM fields and their interactions with objects. For objects about the size of people, the wavelength will be large in comparison to the object size at frequencies below 1 MHz. The extremely low frequency (ELF) band is designated as the band of frequencies from 30 to 300 Hz (particularly high wavelengths, from 1000 to 10000 km, respectively). are at this range. Many naturally occurring electric and magnetic effects, as well as commonly used power line frequencies of 50 and 60 Hz are seen in this low-frequency range. Much research has been performed to determine these fields safety or hazardous effects in small doses which are now so pervasive in our environment (Furse, Durney, & Christensen, 2009).

1.4 Biological Responses to Extremely Low Frequency (ELF) Electromagnetic Fields (EMFs)

In living organisms, various biological effects of extremely low-frequency electromagnetic fields (ELF-EMF) (< 0.05 mT) have been reported. Though, the different experimental conditions used and the biological uniqueness of each analyzed organism have made it difficult to establish the effects of ELF-EMF in biological systems. Only a few investigations into the effects of ELF-EMF on bacterial cells have been assumed. Bacteria are a good model organism for this type of study because of their short cell cycle and easy handling (Barauna et al., 2015).

Electromagnetic microwaves influence living organisms with different effects. The exposure to electromagnetic fields for prokaryotic systems, in particular causes phenotypic and transcriptional changes on free cells by stress effects and affects the surface adhesion on cells organized in biofilm (Di Campli et al., 2010). 50 Hz EMF with different intensities ranging between 0.1-1.0 mT changed cell morphology of *E. coli* by increasing the number of coccoid cells, through transcriptional modification which is a typical response to a stress factor (Cellini et al., 2008). Another study showed that cell viability and morphology changed in *H. pylori* cells exposed to 50 Hz EMF. Adhesion to polystrene surface was reduced and biofilm formation was inhibited (Di Campli et al., 2010).

Besides, Behari & Rajamani (2012) reported that a variety of clinical conditions such as leukaemia, brain tumours, infertility through inducing apoptosis, altering gene transcription and translation are associated with ELF-EMFs (Behari & Rajamani, 2012).

Defined EMFs have proven to prevent biofilm formation in several other studies (del Pozo, Rouse, Mandrekar, Sampedro, et al., 2009; del Pozo, Rouse, Mandrekar, Steckelberg, et al., 2009; A. Obermeier, F. D. Matl, W. Friess, & A. Stemberger, 2009). In some biochemical studies it is reported that structure and function of many enzymes and some organelles is disrupted by radiofrequency waves (Stavroulakis, 2003). If we consider the problems of conventional biofilm removal approaches, EMFs could be used as an alternative way for biofilm removal (McLeod & Sandvik, 2010).

1.5 Hypothesis

In a variety of studies, it is indicated that EMFs may affect living cells in several ways. In this case if we consider prokaryotic cells, it is shown that EMFs change the binding properties of biofilm bacteria by causing stress effect leading to phenotypic and transcriptional changes. Exposure to 50 Hz, 1.0 mT electromagnetic field was shown to have no significant effect on mature biofilm whereas it affected both cell morphology and cell viability significantly during biofilm formation. It showed that ELF-EMF interfere with the sessile morphology of *H. pylori* cells and decrease the adhesive properties resulting in lower cell viability (Di Campli et al., 2010). However, there were not any significant studies that investigated the effects of electromagnetism with higher frequencies such as 100-300 Hz. In another study, it was reported that a mutant of *S. epidermidis* which lost the expression of four cell surface proteins had lost the ability to adhere to a polystyrene surface. (Cheng, Zhang, Chen, Bryers, & Jiang, 2007).

In this research our aim is to prevent biofilm formation or totally remove biofilms of *Staphylococcus epidermidis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) bacteria by using ELF-EMFs. These bacteria are known to be the main cause of infections by forming biofilms on medical devices and implants. Simultaneously, genotypic alterations in gram (+) and gram (-) bacteria groups will be determined when exposed to ELF-EMFs. All these data are expected to give insight into the effects of ELF-EMFs on two different bacteria groups. Consequently, it is expected to remove bacterial biofilms on medical devices, implants even plaques on teeth by ELF-EMFs so that infections are partly or totally prevented. If successful results are obtained, our approach might be conducted with other biofilm removal tools so that more efficient biofilm removal tool reinforced with ELF-EMFs is achieved.

This research is mainly composed of three steps: 1) controlled biofilm formation with two different model microorganisms; 2) removal and/or prevention of biofilms by exposure to extremely low frequency electromagnetic fields (ELF-EMFs); and 3) *AtlE* gene sequencing of *Staphylococcus epidermidis* and *rpoS* gene sequencing of *Pseudomonas aeruginosa* exposed to ELF-EMF to determine any genotypic changes. Complete *atlE* sequencing of *Staphylococcus epidermidis* and partial sequencing of

the *rpoS* gene of *Pseudomonas aeruginosa* is analysed to determine any changes of ELF-EMF effect on biofilm development.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments, mediums, chemicals and solutions

The compositions and preparation of mediums, chemicals and solutions are given below:

Equipments	**Suppliers**
Pipettes:	Eppendorf 10 µL, 20 µL, 100 µL 200 µL, 1000 µL, 5000 µL
96-well Microtiter Plates	
Flat-Bottom Microplates:	Costar
Spectrophotometer:	PerkinElmer Lambda25 UV/VIS Spectrometer
Microplate Spectrophotometer	BIO-RAD
Autoclave	TOMY SX-700E
AC Power Supply (GW Instek APS-9501):	İnfotek Test Ölçü Cihazları Ltd. Şti.
Gaussmeter:	İnfotek Test Ölçü Cihazları Ltd. Şti.
Home-made Solenoid:	İnfotek Test Ölçü Cihazları Ltd. Şti.
Freezer (-80°C):	New-Brunswick
Chemicals and Media	
Crystal Violet	Merck
Trypticase Soy Broth	Merck
Acetic Acid	Merck
Glucose	Merck

2.1.2 Laboratory equipment

The laboratory equipment used to generate Exremely Low Frequency Electromagnetic Field is shown in Figure 2.1.

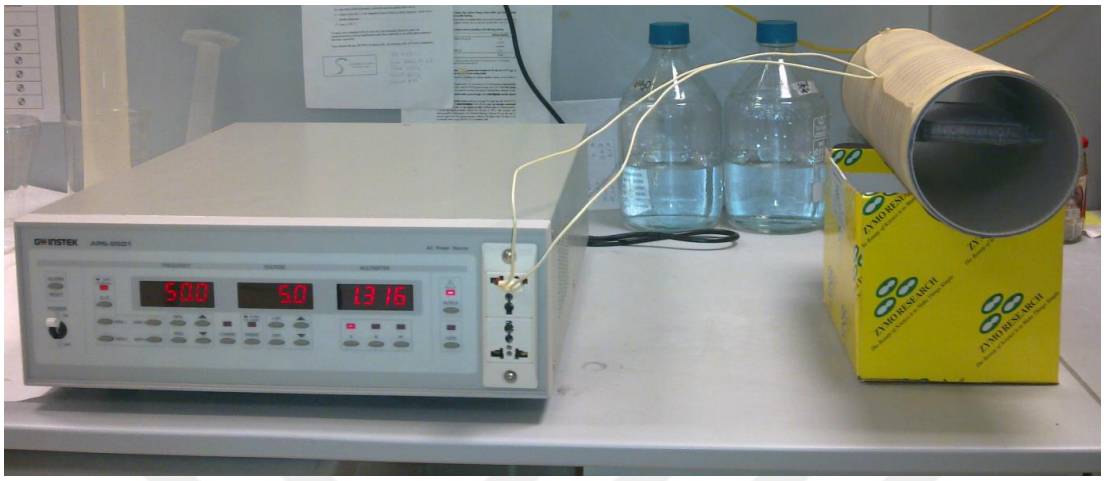


Figure 2.1 : Experimental set-up for ELF-EMF emission. Proposed set-up consists of a cylindrical solenoid and a microtiter plate for biofilm growth placed at the center where the maximum homogenity of electromagnetic field is obtained.

2.1.3 *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* strains

Pseudomonas aeruginosa PAO1 strain (ATCC 15692) and *Staphylococcus epidermidis* (ATCC 35984) were supplied from Hygiene Research Center ATCC, USA.

Pseudomonas aeruginosa PAO1 (ATCC 15692)	ATCC, USA
Staphylococcus epidermidis (ATCC 35984)	ATCC, USA

2.2 Methods

2.2.1 Cultivation of the cells

The bacterial species, *S. epidermidis* and *P. aeruginosa* PAO1, were used in this work. *S. epidermidis* and *P. aeruginosa* were first cultured in separate pure cultures overnight at 37°C on trypticase soy broth (TSB) for 16 hours. 10 µl of each culture was used to inoculate in fresh trypticase soy broth (TSB) and grown to 0.5 OD.

Pseudomonas aeruginosa and *Staphylococcus epidermidis*, which were inoculated in 5-ml Tryptic Soy Broth culture and grown to stationary phase, were diluted 1:100 in the same medium. 100 µl of each diluted culture and not cultured medium (control) was pipeted into each of four wells in a fresh microtiter plate that has not been tissue

culture treated. The plates were covered and incubated at 28°C for 24h and 48h at the center of the cylindrical solenoid in order to determine the effect of ELF-EMF on biofilm formation.

2.2.2 ELF-EMF exposure system design and Set-Up process

In order to emit ELF-EMFs, an experimental set-up was designed. The proposed experimental set-up consists of a solenoid that has 160 mm internal and 170 mm external diameter, with a length of single stranded 440 mm, 180 turns of copper wire (**Figure 2.2**). The device is supposed to deliver variable, homogeneous, sinewave alternating magnetic fields regulated and defined along the center line with 45-300 Hz frequency and intensities ranging between 0.1 and 1.0 mT ±2%. The solenoid is powered by a power supply capable of forming a sine-wave output with a closely regulated frequency and voltage.

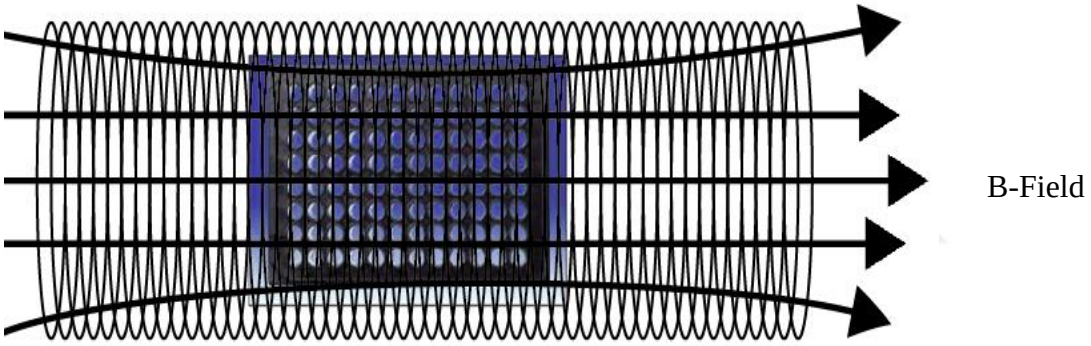


Figure 2.2 : Solenoid set-up for ELF-EMF emission. Proposed experimental set-up consists of microtiter plate for biofilm growth placed at the center of the cylindrical home-made solenoid where the maximum homogenity of magnetic field is obtained (Top view).

The biofilms of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to be analyzed, were formed in microtiter plates and located on a non-magnetic support in the maximum homogeneity part of the magnetic field at the center of the coil system. However, in order to determine the effect of ELF-EMF on biofilm formation, the proposed home-made set-up system should be finalized, and the procurement process is still ongoing.

Pseudomonas aeruginosa, which was inoculated in 5-ml Tryptic Soy Broth medium and grown to stationary phase, was diluted to 1:100 in the same medium. *Staphylococcus epidermidis* inoculated in 5-ml Tryptic Soy Broth + 0,25% Glucose medium and grown to stationary phase, was diluted to 1:100 in the same medium. 100

µl of each diluted culture and not cultured medium (control) was pipeted into each of five wells in fresh microtiter plates (polystyrene and polypropylene) that have not been tissue culture treated. The plates were covered and incubated at 28°C for 24h and 48h respectively at the center of the cylindrical solenoid in order to determine the effect of ELF-EMF on biofilm formation. The bacteria were exposed to 1 mT intensity at 45 Hz, 50 Hz, 100 Hz, 200 Hz and 300 Hz frequency.

2.2.3 Biofilm formation and ELF-EMF exposure analysis

2.2.3.1 Microtiter plate biofilm assay

This experimental system, which is often referred to as the 96-well plate assay, is a high-throughput method used to monitor microbial attachment to an abiotic surface. In brief, cells are grown in microtiter dishes for a desired period of time, and then the wells are washed to remove planktonic bacteria. Cells remaining adhered to the wells are subsequently stained with a dye that allows visualization of the attachment pattern. This surface-associated dye can also be solubilized for semiquantitative assessment of the biofilm formed (Merritt, Kadouri, & O'Toole, 2005).

In order to investigate *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilm formation, each strain was inoculated in two different round-bottomed 96-well microtiter plates which were polystyrene and polypropylene in structure. *P. aeruginosa* and *S. epidermidis* exposed to 45 Hz, 50 Hz, 100 Hz, 200 Hz and 300 Hz with 1 mT intensity were analysed for their biofilm formation on polystyrene and polypropylene surfaces for 24 h and 48 h.

2.2.3.2 Growing and analyzing static biofilms of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

Two separate biofilm formations of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, one exposed to ELF-EMF and the other used as a control, were inoculated at room temperature and analysed for 24 hrs and 48 hrs.

- 10 µl stock culture of each strain was inoculated in 5-ml Tryptic Soy Broth (Tryptic Soy Broth + 0,25% Glucose for *S. epidermidis*) and grown to stationary phase.
- Each culture was diluted 1:100 in the desired Tryptic Soy Broth medium. 150 µl of diluted culture was pipeted into each of five wells in a fresh microtiter

plate that has not been tissue culture treated. The plate was covered and incubated at 28°C for 24h and 48h.

- Two separate biofilm formations of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, one grown on polystyrene surface and the other grown on polypropylene, were exposed to defined ELF-EMF, were inoculated at room temperature and analysed for 24 h and 48 h.
- Four small trays were set up in a series and 1 to 2 inches of tap water was added to the last three. The first tray was used to collect waste, while the others were used to wash the assay plates.
- Planktonic bacteria were removed from each microtiter dish by briskly shaking the dish out over the waste tray. To wash wells, the plate in the first water tray was submerged and the liquid was shaken out vigorously over the waste tray. The water was replaced when it becomes cloudy.

2.2.3.3 Staining the biofilm

- 125 µl of 0.1% crystal violet solution was added to each well. Stained 10 min at room temperature.
- Each microtiter dish was shaken out over the waste tray to remove the crystal violet solution. The dishes were washed successively in each of the next two water trays. This step removes any crystal violet that is not specifically staining the adherent bacteria.
- Each microtiter dish was inverted and vigorously tapped on paper towels to remove any excess liquid. The plates were allowed to air-dry.

2.2.3.4 Quantifying the biofilm

- 200 µl of 30% acetic acid was added to each stained well. The dye was allowed to solubilize by covering the plates and incubating 10 to 15 min at room temperature.
- The contents of each well were briefly mixed by pipetting, and then 125 µl of the crystal violet/acetic acid solution was transferred from each well to a separate well in an optically clear flat-bottom 96-well plate. The optical density (OD) of each of these 125-µl samples were measured at a wavelength of 400 to 700 nm at 10 nm intervals and 585 nm was determined as the optimum wavelength. Consequently, all measuremnets of the samples were performed

at optical density (OD) of 585 nm wavelength. OD values from the wells that had not been inoculated with bacteria were used as negative controls.

2.2.4 Effect of ELF-EMF on planktonic (free-swimming) bacteria

In order to investigate the effect of ELF-EMF on planktonic *Pseudomonas aeruginosa* cultures, 10 µl stock culture of *P. aeruginosa* was inoculated in 5-ml Tryptic Soy Broth and grown to stationary phase for 16 hrs. After incubation, broth cultures were refreshed in TSB medium, with shaking at 37°C for 2 hrs and then adjusted in fresh TSB broth to obtain an optical density at 600 nm (OD_{600}) of ca. 0.05 corresponding to 105–106 CFU/ml. These cultures were used for experiments.

2.2.5 DNA sequence analysis of *rpoS* and *atlE* genes

Some species specific genes of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* are known to effect biofilm development. In order to investigate any genotypic effect of ELF-EMF on biofilm development, biofilm regulating *rpoS* gene of *S. epidermidis* and *atlE* gene of *P. aeruginosa* were DNA sequenced. Previous studies indicated that *rpoS* of *P. aeruginosa* serve some role in biofilm development (Whiteley et al., 2001). Furthermore, it is reported that *atlE* of *S. epidermidis* is necessary for primary attachment and biofilm development in the wells of microtitre trays. It is possible that *AtlE* plays an indirect role in *S. epidermidis* biofilm formation by promoting the release of extracellular DNA (Qin et al., 2007).

In order to determine ELF-EMF effect at the genomic level, the *rpoS* gene of *P. aeruginosa* and *atlE* of *S. epidermidis* has been sequenced. Complete *atlE* sequencing of *S. epidermidis* and partial sequencing of *rpoS P. aeruginosa* has been analysed to determine any changes of ELF-EMF effect on biofilm development. Firstly, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* cultures were exposed to ELF-EMF (50 Hz frequency–1 mT intensity) for 48 hrs in microtiter plates. Then, the planctonic forms have been removed and biofilms attached to the wells of microtiter plates were solubilized by pipetting with fresh medium. Solubilized biofilm cultures were then incubated overnight at 28°C respectively. Genomic DNA was isolated and genes responsible for biofilm development (*rpoS* and *atlE*) were sequenced and analysed for any DNA mutations.

2.2.5.1 Preparation of biofilm bacteria for DNA analysis

Pseudomonas aeruginosa, which was inoculated in 5-ml Tryptic Soy Broth medium and grown to stationary phase, was diluted to 1:100 in the same medium. *Staphylococcus epidermidis* inoculated in 5-ml Tryptic Soy Broth + 0,25% Glucose medium and grown to stationary phase, was diluted to 1:100 in the same medium. 100 µl of each diluted culture and not cultured medium (control) was pipeted into each of five wells in fresh microtiter plates (polystyrene) that have not been tissue culture treated. The plates were covered and incubated at 28°C for 48 hrs respectively at the center of the cylindrical solenoid in order to determine the effect of ELF-EMF on biofilm formation. The bacteria were exposed to 1 mT intensity at 50 Hz frequency.

The international reference strains *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were used for the experiments. Strain features and genes ***rpoS*** and ***atlE***, which are biofilm regulating genes, are as follows:

Organism: *Pseudomonas aeruginosa* strain PAO1
Gene: *rpoS*
NCBI Gene ID: 880421
Organism: *Staphylococcus epidermidis* strain RP62A
Gene: *atlE*
NCBI GeneBank Accession No: CP000029.1 (between 627656-631663)

Bacterial cultures of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were exposed to ELF-EMF (50 Hz frequency–1 mT intensity) for 48 hrs to allow bacterial cell adhesion on polystyrene surface. The planctonic forms of bacteria have been removed and biofilms attached to the wells of microtiter plates were solubilized by pipetting with fresh Tryptic Soy Broth medium. Solubilized biofilm cultures were then incubated overnight at 28°C. Genomic DNA of both bacterial straind were isolated, then *rpoS* and *atlE* genes, which are responsible for biofilm development were sequenced and analysed for any DNA mutations.

2.2.5.2 Growing biofilms

In order to investigate *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilm regulating genes *rpoS* and *atlE*, first the strains were inoculated in round-bottomed 96-well microtiter plates of polystyrene. Both bacterial strains were exposed to 50 Hz with 1 mT intensity on polystyrene surface at 28°C for 48 hrs.

- 10 µl stock culture of each strain was inoculated in 5-ml Tryptic Soy Broth and grown to stationary phase for 16 hrs.
- The overnight cultures were separately diluted into 1:100 fresh Tryptic Soy Broth medium for biofilm assays. As an alternative biofilm-promoting medium that stimulates less planktonic growth and a more robust biofilm.
- 150 µl of diluted culture was pipeted into each of four wells in a fresh microtiter plate that has not been tissue culture treated. The plate was covered and incubated at 28°C for 48 hrs.
- *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* grown on polystyrene surface were exposed to defined ELF-EMF during biofilm formation, and inoculated at room temperature for 48 hrs.
- The planctonic forms of bacteria have been removed and biofilms attached to the wells of microtiter plates were solubilized by pipetting with fresh 5-ml Tryptic Soy Broth medium.

Solubilized biofilm cultures of *P. aeruginosa* and *S. epidermidis* were then incubated overnight at 28°C.

2.2.5.3 Genomic DNA purification protocol

"Bioeksen Bacterial DNA Purification Kit" was used for genomic DNA isolation. 500 µl of overnight culture was used for each bacterial strain.

- 500 µL culture was pipetted to sample tube (2.0 mL microfuge tube).
- 20 µL of lysozyme was pipetted into the sample tube.
- Sample tube was vortexed and mixed for 10 sec.
- Sample tube was incubated for 15 minutes at 37°C.
- 500 µL of GIT buffer and 10 µL PK was added to the sample tube.
- Sample tube was vortexed and mixed for 10 sec.
- Sample tube is incubated at 95°C for 15 min.
- 500 µl of IP is added to the sample tube, vortexed and mixed for 10 sec.
- The colon is placed on top of the centrifuge tube.
- 675 µL of sample from the tube was transferred onto the liquid column and waited for 15 sec.
- Centrifuged for 1 minute at 13.000 g.

- The liquid in the centrifuge tube is poured into the trash. The column is again placed over the centrifuge tube.
- 675 µL of sample from the tube was transferred onto the liquid column and waited for 15 sec.
- Centrifuged for 1 minute at 13.000 g.
- The liquid in the centrifuge tube is poured into the trash. The column is again placed over the centrifuge tube.
- All remaining liquid in the sample tube was transferred to the column, waited for 15 sec.
- Centrifuged for 1 minute at 13.000 g.
- The liquid in the centrifuge tube is poured into the trash. The column is again placed over the centrifuge tube.
- 500 µl of Washing Buffer was added onto the column.
- Centrifuged for 1 minute at 13.000 g.
- The liquid in the centrifuged tube is poured into the trash. The column is again placed over the centrifuge tube.
- 500 µl of Washing Buffer was added onto the column.
- Centrifuged for 1 minute at 13.000 g.
- The centrifuge tube is discarded together with the liquid in the tube. The column is again placed over a new centrifuge tube this time.
- Centrifuged for 1 minute at 13.000 g.
- The centrifuge tube is discarded together with the liquid in the tube.
- The column is placed on the ET tube (1.5 mL microfuge tube).
- Elution Buffer is heated to 60°C and 100 µl Elution Buffer is transferred onto the column. Waited for 1 min.
- Centrifuged for 1 minute at 13.000 g.

The isolated DNA is stored at -20°C.

2.2.5.4 PCR protocol for *rpoS* and *atlE* gene amplification

In order to obtain *rpoS* gene, we amplified the *rpoS* gene by PCR using primers PA_rpoS_F and PA_rpoS_R from biofilm of ELF-EMF exposed *P. aeruginosa* PAO1 DNA used as a template. Similarly, in order to obtain *atlE* gene, we amplified the *atlE* gene by PCR using primers SE_atlE_F and SE_atlE_R from *S. epidermidis* biofilm

DNA used as a template, which was exposed to ELF-EMF. Indicated primer properties and sequences, used for DNA amplification are as follows:

P. aeruginosa forward primer: PA_rpoS_F 5'- AGGTCAAGGTAGGGCAATC - 3'
P. aeruginosa reverse primer: PA_rpoS_R 5'- TTAAATTCACCGAGCGTTTT - 3'
S. epidermidis forward primer: SE_atlE_F 5' - TGACAATGTTCCCAGCATAA - 3'
S. epidermidis reverse primer: SE_atlE_R 5'- TCGAAGCAGTGACAGGATAA - 3'

PCR prosedure used to amplify *rpoS* and *atlE* genes is as follows:

PCR Protocol for Amplification

- 5 µl 2x Bioeksen PCR Mix
- 2 µl 1/100 Diluted gDNA
- 0.5 µl 10 µM F Primer
- 0.5 µl 10 µM R Primer
- 2 µl DNase RNase-free Water

PCR Thermal Cycle Protocol

- 1 cycle
- 98°C 3 min.
- 45 Loops
- 98°C 10 sec.
- 52°C 30 sec.
- 72°C 120 sec.
- 1 cycle
- 72°C 3 min.

2.2.5.5 PCR purification protocol

"Bioeksen PCR Clean-Up Kit" was used for the purification of obtained PCR products. 100 µl PCR product was used for each PCR product.

- The PCR products were taken into a microcentrifuge tube and if less than 20 µl, completed to 20 µl with DB.

- Then BB1 is added up to 3 times the total volume of the first step sample volume. For example, if the PCR product is 10 µl and 10 µl of DB is added, we obtain 20 µl sample. So we add 60 µl BB1 to that sample.
- Vortexed (at maximum speed) for 10 seconds.
- BB2 is added equal to the total volume of the first step sample. For example, if the PCR product is 10 µl and we add 10 µl of DB, we obtain total volume of 20 µl. Than we add 60 µl BB1 on top of it. We add 20 µl BB2 to that sample.
- Then vortexed (at maximum speed) for 10 seconds.
- DNA column is placed in the Collection Tube.
- The mixture is added to the DNA column. Centrifuge at 14.000 g for 1 minute. Take the liquor from the Collection Tube and put the Collection Tube back into the DNA Column.
- 500 µl WB is added to the DNA column, centrifuge at 14.000 g for 1 minute. The liquid is taken from the Collection Tube and the Collection Tube is put back into the DNA Column.
- 8th step is repeated, so that 500 µl WB is used twice in total.
- The DNA Column is centrifuged blank at 14.000 g for 1 min. Then the Collection Tube was discard and the DNA Column was inserted into into a new microcentrifuge tube.
- 50 µL of EB was pipetted to the DNA column. Incubated for 1 minute at room temperature (between + 15 ° C and + 25 ° C). Centrifuge at 14.000 g for 1 minute. The isolated PCR products were eluted to the bottom microcentrifuge tube dissolved in EB.
- PCR products are stored at -20 ° C if not used immediately. If used immediately, store at + 4 ° C until use.

2.2.5.6 DNA sequence analysis of *rpoS* and *atlE* genes

DNA sequencing of the PCR products of *rpoS* and *atlE* gene fragments were obtained by using the following primers. Two primers were used for *rpoS* sequencing and seven primers were used for *atlE* sequencing:

P. aeruginosa forward primer : ***PA_rpoS_SqF1*** *5'-GATGATCGAGAGCAACCTG - 3'*

P. aeruginosa forward primer : ***PA_rpoS_SqF2*** *5'-GACGGAACTCACCGACAA - 3'*

S. epidermidis forward primer : ***SE_atlE_SqF1*** *5'-GATGCGAATCAAAATCAAACG-3'*

S. epidermidis forward primer : ***SE_atlE_SqF2*** *5'- CAATTCACCCCATTTAGTGC - 3'*

S. epidermidis forward primer : ***SE_atlE_SqF3*** *5'-GCTGAAAACGATGGAAGAGG- 3'*

S. epidermidis forward primer : ***SE_atlE_SqF4*** *5'- GGTACACCAAAACAAGTTGC - 3'*

S. epidermidis forward primer : ***SE_atlE_SqF5*** *5'- GAACAGTTTCAGGTAAAGGC - 3'*

S. epidermidis forward primer : ***SE_atlE_SqF6*** *5'-CAATTACTAGCACCTAATACGC-3'*

S. epidermidis forward primer : ***SE_atlE_SqF7*** *5'- TATGGATACAAAGCGTTTAGC - 3'*

DNA sequencing of the PCR products were performed with the Sanger Dideoxy Chain Termination Method and 3730XL Sequencer (Applied Biosystems, Inc.).

Tools used for sequence alinment and blast:

DNA Baser Sequence Assembler v4

DNA Sequence Assembler is easy to use software for DNA sequence assembly/alignment, DNA sequence analysis, DNA Sequence manipulation and conversion, contig editing and mutation detection (Url-2; Url-3).

BLAST

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families (Url-4).

Clustal Omega

Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences (Url-5).

Chromas AB1 Sequence Viewer

Chromas is a free trace viewer for simple DNA sequencing projects which do not require assembly of multiple sequences (Url-6).

3. RESULTS AND DISCUSSION

3.1 Cell Staining and Quantification of Biofilms

Biofilms stained by crystal violet were mesured by using microplate spectrophotometer and microplate manager program at 585 nm. Stained biofilms form a ring at the air-liquid interface and at the bottom of microplate wells. Results are shown in Figure 3.1. The qualitative microtiter dish assay adherence test depends on the visual assessment of the degree of adherence of bacteria to the bottom and/or to the air-liquid interface of each well. Microtiter plate biofilm assay is certainly the most commonly used method for investigating bacterial attachment.

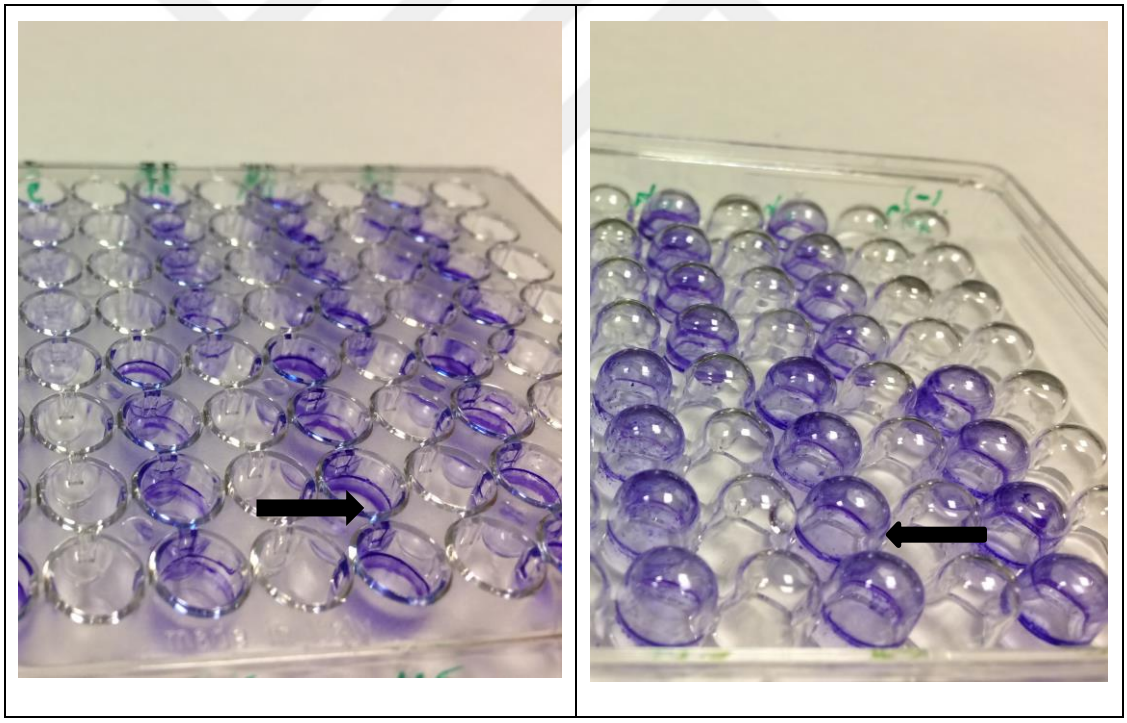


Figure 3.1 : Biofilm formation assay. Rings of crystal violet-stained biofilm are visible in the wells of a polystyrene microtiter plate (indicated by *arrow*). **(a)** Looking into the wells from the *top*. A side view of the wells with biofilms (**b)** Plate is inverted. A bottom view of the wells with biofilms.

Bacterial cells were grown in the wells of a specific microtiter plate. We used polystyrene and polypropylene microtiter plates. The wells were emptied and washed to remove planktonic cells before staining the biomass attached to the surface of the

wells at different time points. Attached cells remain adhered to the wells were subsequently stained with crystal violet that allows visualization. The dye was solubilized with 30% acetic acid. Consequently, all samples were measured at optical density (OD) of 585 nm wavelength.

3.2 Effect of ELF-EMF on Biofilm Formation

In order to verify the effect of 45 Hz, 50 Hz, 100 Hz, 200 Hz and 300 Hz and 1 mT ELF-EMF intensity on the biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, bacterial cultures exposed to ELF-EMF for 24 hrs and 48 hrs on two different surfaces which are polystyrene and polypropylene. Biofilms were compared to the respective non-exposed controls.

The measurement of biofilm cell mass of *P. aeruginosa* and *Staphylococcus epidermidis*, quantified by crystal violet staining, displayed a different effect of ELF-EMF in the inhibition of biofilm formation and a reduction in adhesion to polystyrene and polypropylene surfaces in respect to the non-exposed control samples.

In line with the data obtained from experiments, *P. aeruginosa* and *S. epidermidis* biofilm formation is observed to decrease nearly 40% after 48 hours in the frequency range of 45 Hz and 50 Hz and 1mT intensity of ELF- EMA compared to normal conditions both on polystyrene and polypropylene surfaces.

Although we observed some minor changes of biofilm formation of the bacteria exposed to 100 Hz, 200 Hz and 300 Hz frequency and 1 mT intensity of ELF-EMF, there is no significant difference to biofilm formation both on polystyrene and polypropylene surfaces.

A significant effect of ELF-EMF in the inhibition of biofilm formation and a reduction in adhesion to a polystyrene surface with in respect to non-exposed samples is observed at 48 hrs with the *S. epidermidis* samples (Figure 3.12). Results show that the formation of *S. epidermidis* biofilm is halted by ELF-EMF exposure after 24 hrs, as no increase is obtained between 24 hrs and 48 hrs time intervals. Nevertheless, ELF-EMF has no significant effect on *P. aeruginosa* biofilm formation, as the cells continued growing both at 24 hrs and 48 hrs compared to non-exposed control samples.

3.2.1 Biofilm formation on polypropylene surface exposed to 45 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polypropylene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 45 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 45 Hz with 1.0 mT intensity are given in Appendix A, Table A.1.

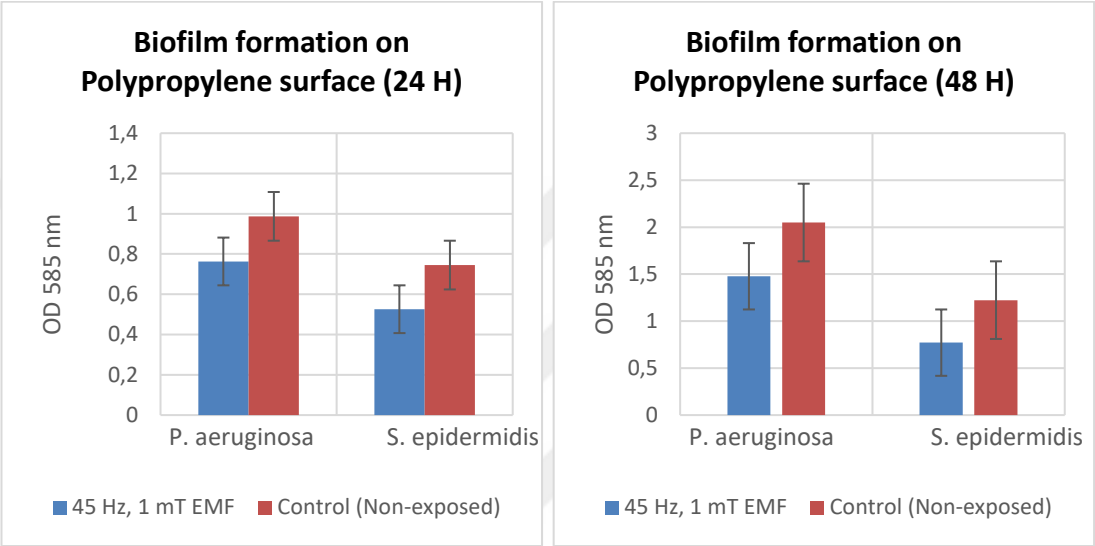


Figure 3.2 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polypropylene surface exposed to 45 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.2, adhesion of *P. aeruginosa* was slightly different from *S. epidermidis*. The surface was covered by *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h by ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 23% after 24 hours and 28% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Similarly, *S. epidermidis* biofilm formation is observed to decrease approximately 30% after 24 hours and 37% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. These results show that *Staphylococcus* species are sligthtly more effected than *Pseudomoans* species by ELF-EMF exposure at 45 Hz frequency (1 mT intensity) polypropylene surface.

3.2.2 Biofilm formation on polystyrene surface exposed to 45 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polystyrene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 45 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 45 Hz with 1.0 mT intensity are given in Appendix A, Table A.1.

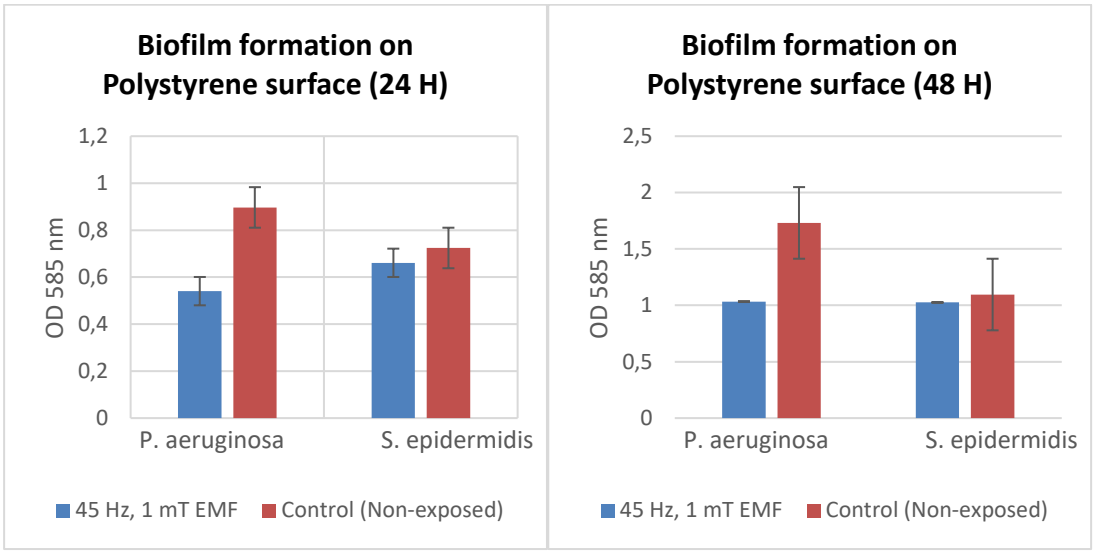


Figure 3.3 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polystyrene surface exposed to 45 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.3, inhibition of *P. aeruginosa* was different from *S. epidermidis*. The biofilm formation of *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h is observed despite ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 39% after 24 hours and 40% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. On the contrary, *S. epidermidis* biofilm formation is observed to decrease approximately 9% after 24 hours and 7% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. These results show that *Pseudomonas* species are more effected than *Staphylococcus* species by ELF-EMF exposure at 45 Hz frequency (1 mT intensity) on polystyrene surface.

3.2.3 Biofilm formation on polypropylene surface exposed to 50 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polypropylene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 50 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 50 Hz with 1.0 mT intensity are given in Appendix A, Table A.2.

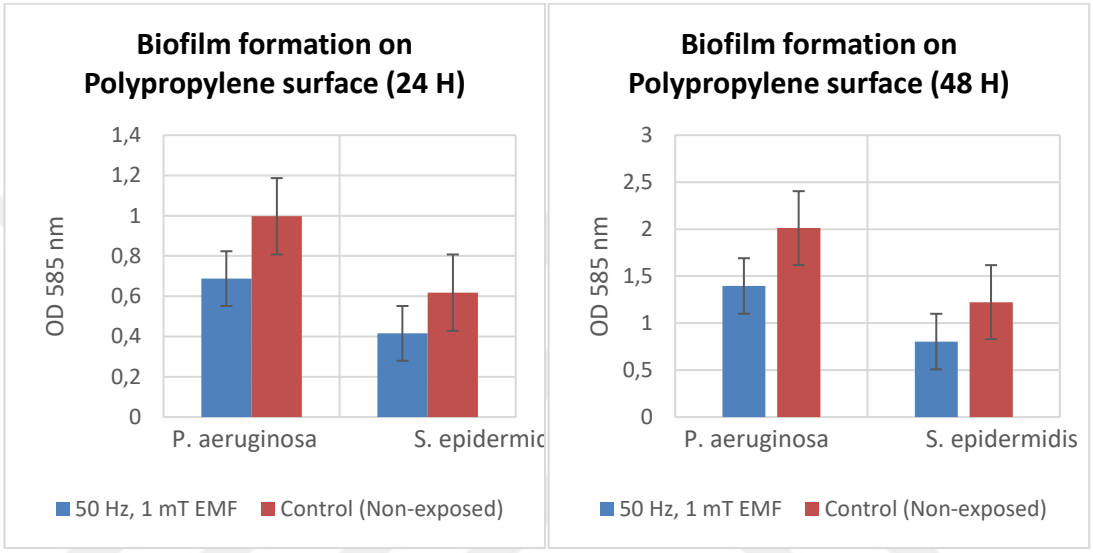


Figure 3.4 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polypropylene surface exposed to 50 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.4, adhesion of *P. aeruginosa* was slightly different from *S. epidermidis*. The surface was covered by *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h by ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 30% after 24 hours and 31% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Similarly, *S. epidermidis* biofilm formation is observed to decrease approximately 33% after 24 hours and 35% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. These results show that *Staphylococcus* species are sligthtly more effected than *Pseudomoans* species by ELF-EMF exposure at 50 Hz frequency (1 mT intensity) polypropylene surface.

3.2.4 Biofilm formation on polystyrene surface exposed to 50 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polystyrene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 50 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 50 Hz with 1.0 mT intensity are given in Appendix A, Table A.2.

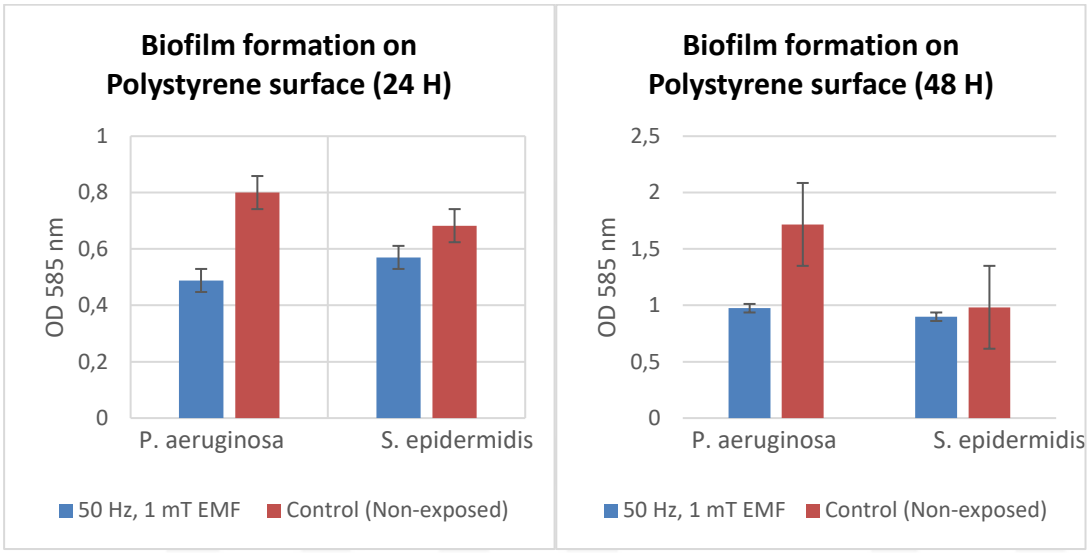


Figure 3.5 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polystyrene surface exposed to 50 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.5, inhibition of *P. aeruginosa* was different from *S. epidermidis*. The biofilm formation of *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h is observed despite ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 39% after 24 hours and 44% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. Contrarily, *S. epidermidis* biofilm formation is observed to decrease approximately 17% after 24 hours and 10% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. These results show that *Pseudomoans* species are more effected than *Staphylococcus* species by ELF-EMF exposure at 50 Hz frequency (1 mT intensity) on polystyrene surface.

3.2.5 Biofilm formation on polypropylene surface exposed to 100 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polypropylene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 100 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 100 Hz with 1.0 mT intensity are given in Appendix A, Table A.3.

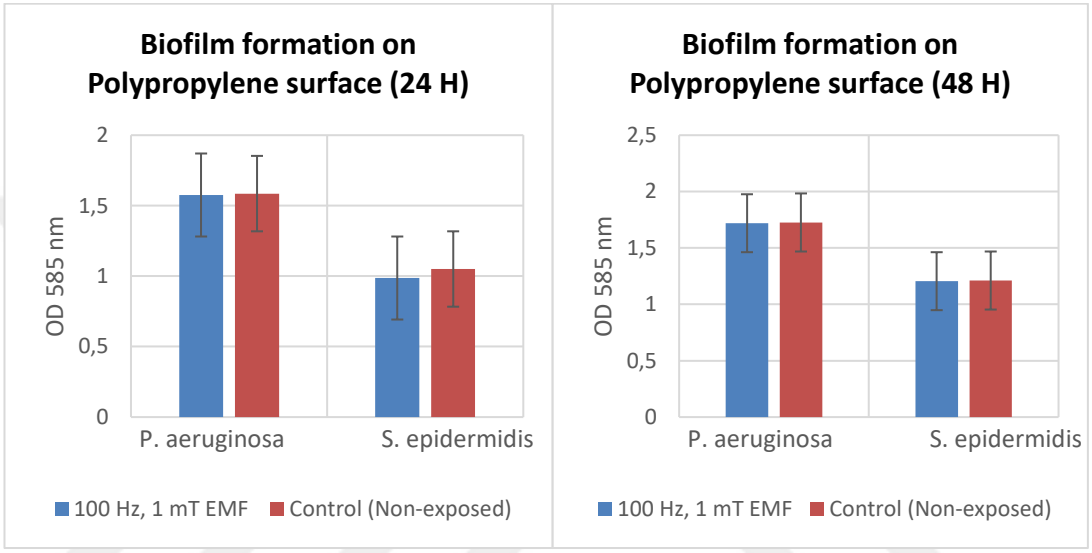


Figure 3.6 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polypropylene surface exposed to 100 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.6, adhesion of *P. aeruginosa* was slightly different from *S. epidermidis*. The surface was covered by *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h by ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 1% after 24 hours and 1% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Similarly, *S. epidermidis* biofilm formation is observed to decrease approximately 7% after 24 hours and 1% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. These results show that both *Staphylococcus* and *Pseudomoans* species are not affected by ELF-EMF exposure at 100 Hz frequency (1 mT intensity) on polypropylene surface.

3.2.6 Biofilm formation on polystyrene surface exposed to 100 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polystyrene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 100 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 100 Hz with 1.0 mT intensity are given in Appendix A, Table A.3.

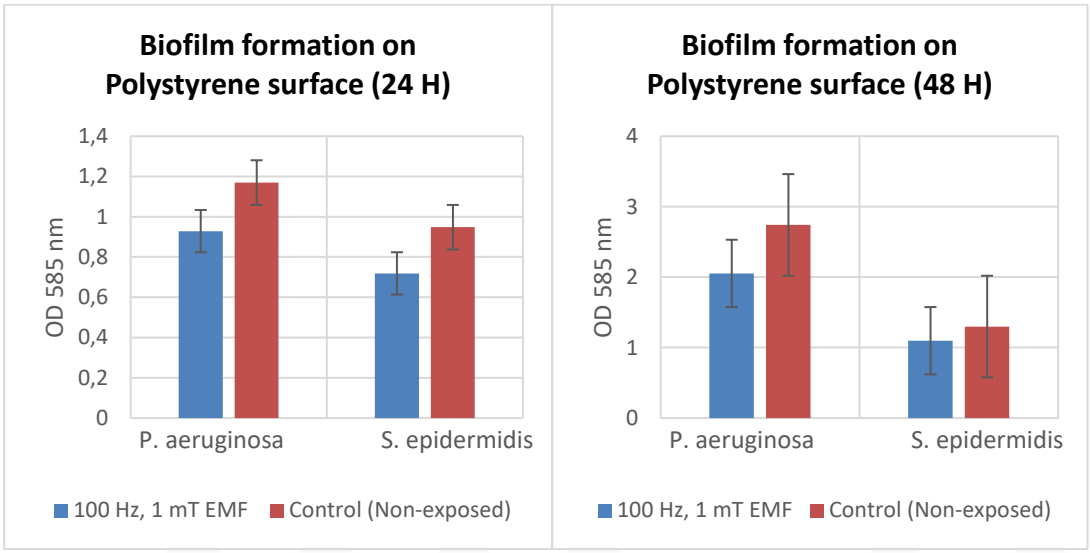


Figure 3.7 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polystyrene surface exposed to 100 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.7, inhibition of *P. aeruginosa* was different from *S. epidermidis*. The biofilm formation of *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h is observed despite ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 20% after 24 hours and 25% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. Contrarily, *S. epidermidis* biofilm formation is observed to decrease approximately 25% after 24 hours and 16% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. These results show that *Pseudomoans* species are more effected than *Staphylococcus* species by ELF-EMF exposure at 100 Hz frequency (1 mT intensity) on polystyrene surface.

3.2.7 Biofilm formation on polypropylene surface exposed to 200 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polypropylene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 200 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 200 Hz with 1.0 mT intensity are given in Appendix A, Table A.4.

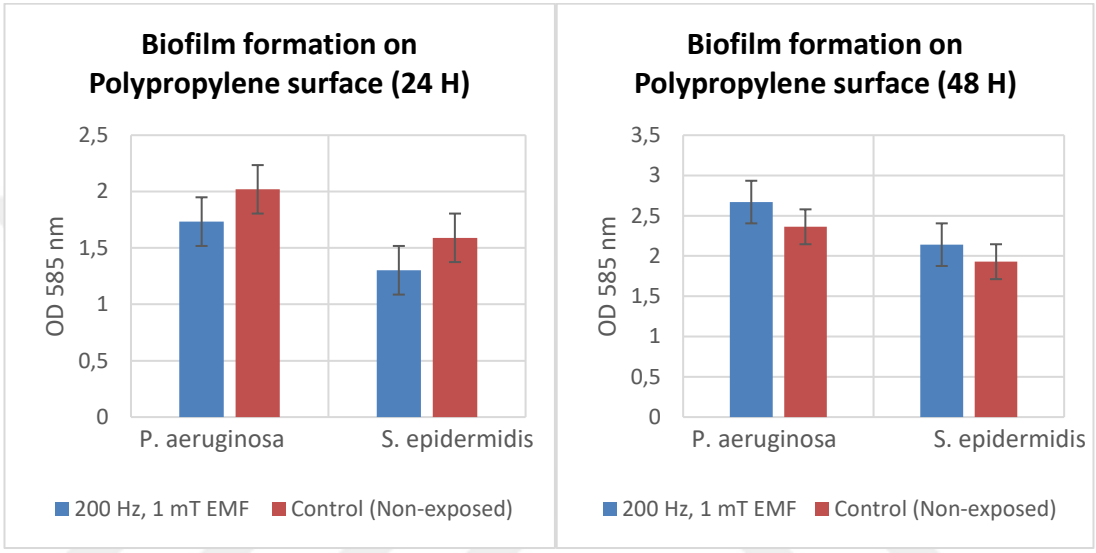


Figure 3.8 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polypropylene surface exposed to 200 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.8, adhesion of *P. aeruginosa* was slightly different from *S. epidermidis*. The surface was covered by *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h by ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 15% after 24 hours and increase 12% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Similarly, *S. epidermidis* biofilm formation is observed to decrease approximately 19% after 24 hours and increase 10% after 48 hours of ELF-EMA exposure, compared to non-exposed controls on polypropylene surface. These results show that both *Staphylococcus* and *Pseudomoans* species are slightly effected by ELF-EMF exposure at 200 Hz frequency (1 mT intensity) polypropylene surface.

3.2.8 Biofilm formation on polystyrene surface exposed to 200 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polystyrene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 200 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 200 Hz with 1.0 mT intensity are given in Appendix A, Table A.4.

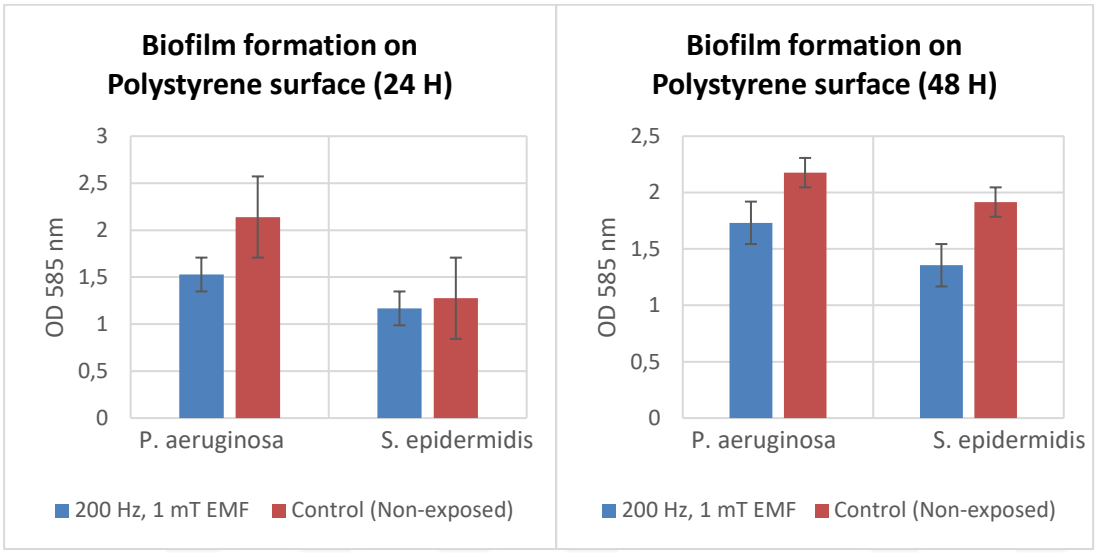


Figure 3.9 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polystyrene surface exposed to 200 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.9, inhibition of *P. aeruginosa* was different from *S. epidermidis*. The biofilm formation of *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h is observed despite ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 29% after 24 hours and 21% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. Contrarily, *S. epidermidis* biofilm formation is observed to decrease approximately 9% after 24 hours and 30% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. These results show that both *Pseudomoans* and *Staphylococcus* species are similarly effected by ELF-EMF exposure at 200 Hz frequency (1 mT intensity) on polystyrene surface.

3.2.9 Biofilm formation on polypropylene surface exposed to 300 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polypropylene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 300 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 300 Hz with 1.0 mT intensity are given in Appendix A, Table A.5.

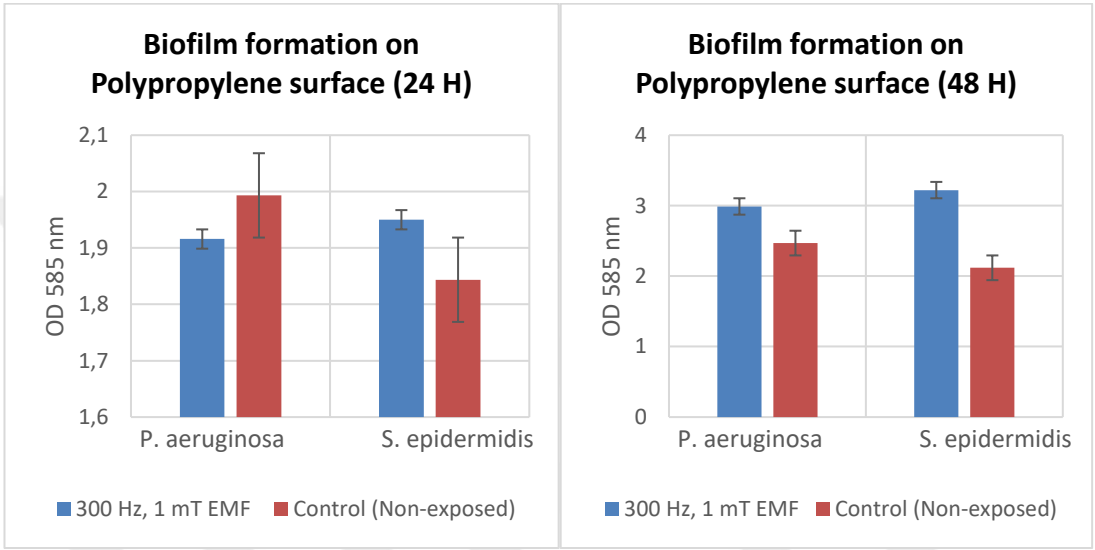


Figure 3.10 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polypropylene surface exposed to 300 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.10, adhesion of *P. aeruginosa* was slightly different from *S. epidermidis*. The surface was covered by *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h by ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease 4% after 24 hours and increase 18% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Similarly, *S. epidermidis* biofilm formation is observed to increase approximately 6% after 24 hours and 35% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polypropylene surface. Results show that ELF-EMF exposure at 300 Hz frequency (1 mT intensity) initiate biofilm development for *Staphylococcus* and *Pseudomoans* species on polypropylene surface. This may be because of the expression of some surface proteins functioning in surface attachment of cells.

3.2.10 Biofilm formation on polystyrene surface exposed to 300 Hz (1 mT) ELF-EMF

For the 24-h and 48-h adhesion and biofilm development study on polystyrene surface, the bacterial species *S. epidermidis* and *P. aeruginosa* PAO1 that were exposed to 300 Hz (1 mT intensity) ELF-EMF were analyzed. Absorbance values at 585 nm for ELF-EMF exposed microorganisms and controls that were incubated for 24 and 48 hours at 300 Hz with 1.0 mT intensity are given in Appendix A, Table A.5.

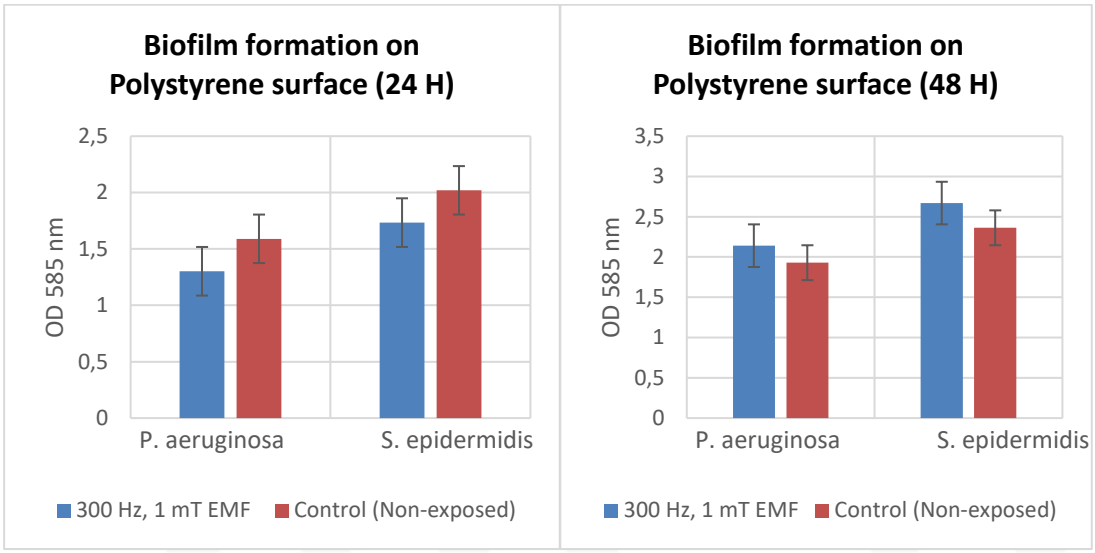


Figure 3.11 : *P. aeruginosa* and *S. epidermidis* biofilm formation on polystyrene surface exposed to 300 Hz ELF-EMF (1 mT). Biofilm development is analyzed in the wells of microtitre plates for 24 h and 48 h in Tryptic Soy Broth (+ 0,25% Glucose for *S. epidermidis*) medium. Average values were calculated from 5 different experiments.

As shown in Figure 3.11, inhibition of *P. aeruginosa* was different from *S. epidermidis*. The biofilm formation of *P. aeruginosa* as well as *S. epidermidis* after 24 h and 48 h is observed despite ELF-EMF exposure. The bacterial accumulation of *S. epidermidis* biofilm is quantitatively less than *P. aeruginosa* biofilm. *P. aeruginosa* biofilm formation is observed to decrease approximately 19% after 24 hours and increase 10% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. Contrarily, *S. epidermidis* biofilm formation is observed to decrease approximately 15% after 24 hours and increase 12% after 48 hours of ELF- EMA exposure, compared to non-exposed controls on polystyrene surface. These results show that both *Pseudomoans* and *Staphylococcus* species are

slightly effected by ELF-EMF exposure at 300 Hz frequency (1 mT intensity) on polystyrene surface.

3.3 Time-dependent Change of the Effect of ELF-EMF on Planktonic (Free-swimming) Bacteria and Biofilms

In order to investigate the effect of ELF-EMF on planktonic *Pseudomonas aeruginosa* cultures, 10 µl stock culture of *P. aeruginosa* was inoculated in 5-ml Tryptic Soy Broth and grown to stationary phase for 16 hrs. After incubation, broth cultures were refreshed in TSB medium, with shaking at 37°C for 2 hrs and then adjusted in fresh TSB broth to obtain an optical density at 600 nm (OD600) of ca. 0.05 corresponding to 100-105 CFU/ml. These cultures were used for experiments.

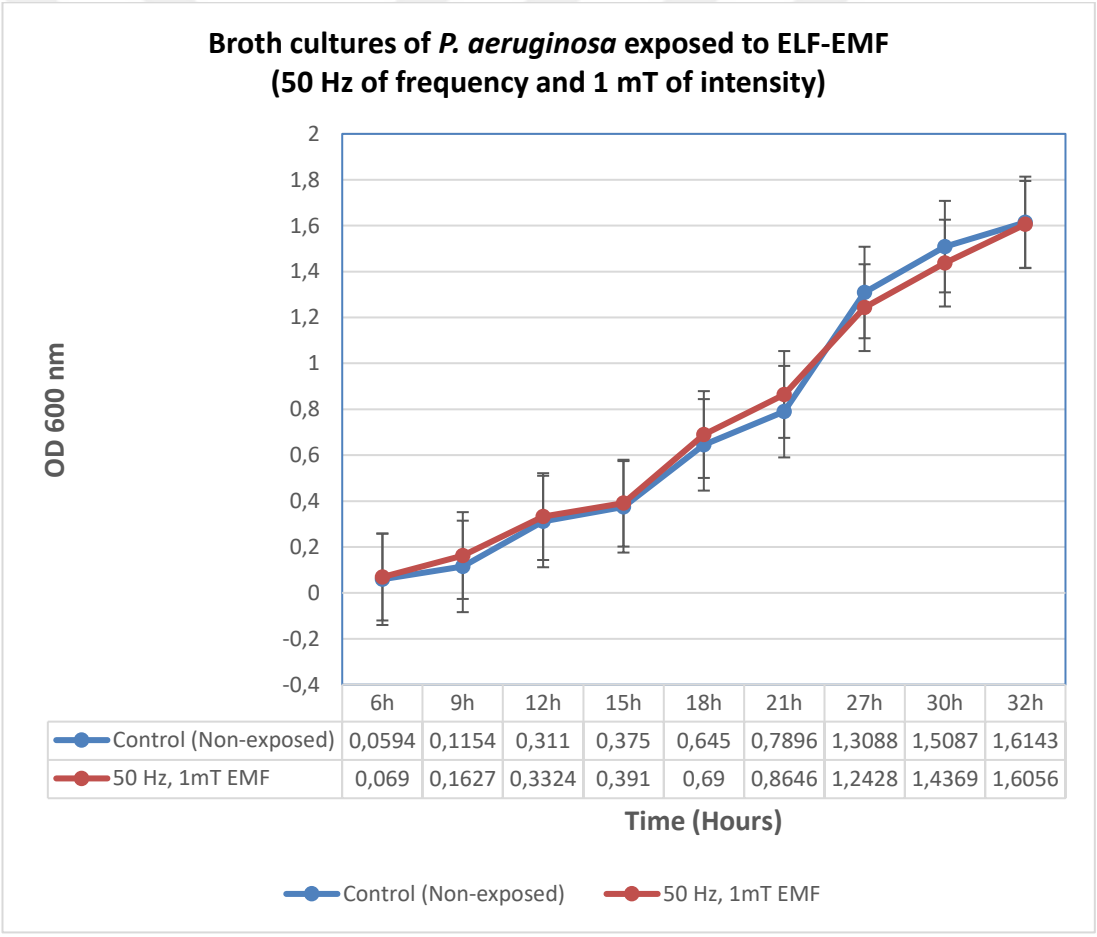
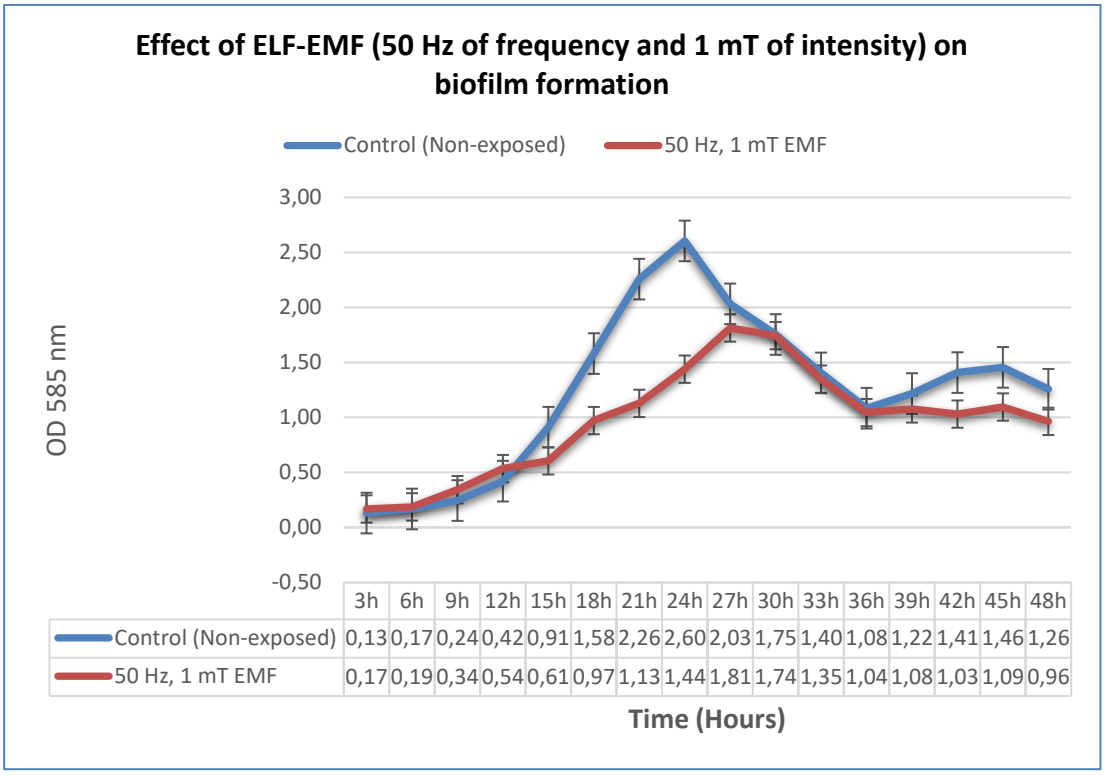


Figure 3.12 : Effect of ELF-EMF (50 Hz of frequency and 1 mT of intensity) on planktonic state (free-swimming) of *P. aeruginosa*. The ELF-EMF effect was monitored for 32 hours in 3 hour intervals. Planktonik state of *P. aeruginosa* grown in Tryptic Soy Broth medium (48 hrs, 28°C) exposed to ELF-EMF were analysed and monitored by spectrophotometry at OD 600 nm.

P. aeruginosa exposed to 50 Hz with 1 mT intensity were analysed for 32 hrs in 3 hrs intervals in order to determine the effect of ELF-EMF on planktonic bacteria. Every 3 hrs, 1 ml of sample was taken out (1 ml ELF-EMF exposed and 1 ml non-exposed) and analysed at 600 nm (OD600) for total cell counts. Results are shown in Figure 3.12. ELF-EMF exposured planktonic cells and non-exposed cells show similar growth pattern. This shows that ELF-EMF could possibly be acting on exopolymeric matrix or disrupting quorum sensing, or even effecting on cells' surface proteins so that bacterial cells could not attach easily on surfaces.



	3h	6h	9h	12h	15h	18h	21h	24h	27h	30h	33h	36h	39h	42h	45h	48h
Control (Non-exposed)	0,13	0,17	0,24	0,42	0,91	1,58	2,26	2,60	2,03	1,75	1,40	1,08	1,22	1,41	1,46	1,26
50 Hz, 1 mT EMF	0,17	0,19	0,34	0,54	0,61	0,97	1,13	1,44	1,81	1,74	1,35	1,04	1,08	1,03	1,09	0,96

Figure 3.13 : Quantification of ELF-EMF (1mT 50 Hz) effect at various time points over 48 hr, based on absorbance readings made at 585 nm. The ELF-EMF effect was monitored for 48 hours in 3 hour intervals. Biofilm attachment of *P. aeruginosa* grown in Tryptic Soy Broth medium (48 hrs, 28°C) to polystyrene surface on the wells of microtitre plates, were analysed. Average values were calculated from 4 different experiments.

The measurement of biofilm cell mass of P. aeruginosa, quantified by crystal violet staining, displayed a different growth pattern within 48 hrs (Figure 3.13). Effect of ELF-EMF on adhesion of bacteria to a polystyrene surface in respect to the non-exposed samples decreased by 55% in first 24 hrs. After 24 hrs, the prevention effect of ELF-EMF started to decline. This shows that ELF-EMF effect may vary according

to time. Biofilm development after 24 hrs start to increase and could not be easily prevented in and extended time range.

3.4 Effect of ELF-EMFs on *rpoS* and *atlE* Genes Responsible for Biofilm Formation in *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*

The aim of this work was to investigate the effects of exposure to extremely low-frequency electromagnetic fields (ELF-EMF) on biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* and explore the effect of EMF-ELF on *rpoS* and *atlE* genes. Bacterial cultures of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were exposed to ELF-EMF (50 Hz frequency–1 mT intensity) for 48 hrs to assess their effect on the cell adhesion, respectively. The planctonic forms of bacteria have been removed and biofilms attached to the wells of microtiter plates were solubilized by pipetting with fresh Tryptic Soy Broth medium. Solubilized biofilm cultures were then incubated overnight at 28°C. Genomic DNA was isolated, then *rpoS* and *atlE* genes, which are responsible for biofilm development were sequenced and analysed for any DNA mutations. For DNA sequencing, such as those involving genomic DNA isolation, forward and reverse primer design, PCR, agarose gel electrophoresis and DNA sequencing, standard procedures were followed. ELF-EMF exposed cultures and the respective sham exposed controls were studied for DNA sequence analysis.

In order to verify the effect of 50 Hz, 1 mT ELF-EMF intensity on biofilm regulating genes *rpoS* and *atlE* of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, bacterial cultures were exposed to ELF-EMF for 48h on polystyrene surface. The planctonic forms of bacteria have been removed and biofilms attached to the wells of microtiter plates were solubilized by pipetting with fresh 5-ml Tryptic Soy Broth medium. Solubilized biofilm cultures of *P. aeruginosa* and *S. epidermidis* were then incubated overnight at 28°C. Genomic DNA of each strain was isolated by Bioeksen Bacterial DNA Purification Kit. *rpoS* gene of *P. aeruginosa* PAO1 and *atlE* gene of *S. epidermidis* which were exposed to ELF-EMF were amplified by PCR with the indicated primers. PCR products of *rpoS* and *atlE* gene fragments were sequenced with the Sanger Dideoxy Chain Termination Method and 3730XL Sequencer (Applied Biosystems, Inc.).

The ELF-EMF exposed *P. aeruginosa* and *S. epidermidis* cultures and the corresponding non-exposed controls were compared for their DNA profiles to evaluate possible micro or macroevolutions induced by ELF-EMF. Obtained results are shown below in APPENDIX B. Previous studies indicated that *rpoS* of *P. aeruginosa* may serve some role in biofilm development (Heydorn et al., 2000; Whiteley et al., 2001). Kjaergaard & Klemm (2003) found 46% of *RpoS*-dependent genes to be differently expressed in biofilms and deletion of *rpoS* rendered bacteria incapable of establishing sessile communities (Kjaergaard & Klemm, 2003). Qin et al. (2007) have reported that *atlE*, encoding a major autolysin, is necessary for primary attachment and biofilm development of *S. epidermidis* in the wells of microtitre trays. (Qin et al., 2007). With regard to the analysis of *rpoS* and *atlE* genes, no notable differences were observed in *rpoS* and *atlE* genes DNA fragments obtained from exposed and non-exposed control samples during the biofilm formation.

4. CONCLUSIONS

In this thesis, the aim was to investigate the effects of Extremely Low Frequency Electromagnetic Fields (ELF-EMF) on biofilm formation of *Staphylococcus epidermidis* (Gram +) and *Pseudomonas aeruginosa* (Gram -) on two different surfaces, polystyrene and polypropylene. The effects of Electromagnetic Fields (EMFs) on cells behaviour, whether it is deadly or causing some other alterations, has been researched for decades in the scientific field. In a variety of studies, it is indicated that EMFs may affect living cells in several ways. In this case if we consider prokaryotic cells, it is shown that EMFs change the binding properties of biofilm bacteria by causing stress effect leading to phenotypic and transcriptional changes (Di Campli et al., 2010). In some biochemical studies it is reported that structure and function of many enzymes and some organelles is disrupted by radiofrequency waves (Stavroulakis, 2003). In this study, the effect of 45, 50, 100, 200, 300 Hz and 1 mT ELF-EMF exposure on biofilm formation was evaluated. In similar studies with other microorganisms such as *Helicobacter pylori*, exposure to 50 Hz, 1.0 mT electromagnetic field was shown to effect both cell morphology and cell viability significantly during biofilm formation. Di Campli (2010) showed that ELF-EMF interfere with the sessile morphology of *H. pylori* cells and decrease the adhesive properties resulting in lower cell viability (Di Campli et al., 2010). In this study, exposure of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* to 45 Hz and 50 Hz at 1.0 mT was shown to decrease the cell viability and inhibit the biofilm formation by 40% after 48 hours, in coherence with previous studies, compared to normal conditions both on polystyrene and polypropylene surfaces. Although we observed biofilm formation within 48 hrs, the time could be extended to 72 hrs so as to analyze biofilm behavior towards extended time intervals. Magnetic fields can affect specific types of chemical reactions, generally increasing concentrations of reactive free radicals in low fields. We chose 1.0 mT as the base intensity in order to obtain comparable data and normalize results. However, intensities ranging from 0.1 to 1.0 mT could also be studied in order to uncover the ELF-EMF effect on biofilms according to intensity range. This could also possibly decrease biofilm formation as

magnetic fields of less than ~1 mT could increase free radical concentration. As a result some enzymes using radicals could be effected leading to decreased biofilm formation.

In addition, effect of 100 Hz, 200 Hz and 300 Hz electromagnetic fields were also investigated. We observed some minor changes of biofilm formation of the bacteria exposed to 100 Hz, 200 Hz and 300 Hz frequency and 1 mT intensity of ELF-EMF both on polystyrene and polypropylene surfaces. Our observations show that both strains have diverse responses to different ELF-EMF frequencies and exposure times. ELF-EMF affecting the electrical load in the cell membranes of the cells to exhibit normal behavior by inhibiting biofilm matrix in the exopolimer matrix may lead to changes in the structure.

Quantification of ELF-EMF (1mT 50 Hz) effect at 3 hr time intervals over 48 hr, showed that the formation of *S. epidermidis* biofilm is halted by ELF-EMF exposure after 24 hrs, as little increase is obtained between 24 hrs and 48 hrs time intervals. Nevertheless, ELF-EMF has no significant effect on *P. aeruginosa* biofilm formation, as the cells continued growing both at 24 hrs and 48 hrs compared to non-exposed control samples. Effect of ELF-EMF (50 Hz of frequency and 1 mT of intensity) on planktonic state (free-swimming) of *P. aeruginosa* was also investigated. Our results show that ELF-EMF exposed and non-exposed planctonic bacteria have similar growt behavior. Obermeier et al. (2009) reported a significant decrease in the optical density of *S. aureus* in broth exposed to 0–30 Hz ELF-EMF at room temperature (A. Obermeier et al., 2009). These findings are comparable to our results showing that the ELF-EMF frequency determines the level of effectiveness in broth culture.

ELF-EMF exposed strain genes *rpoS* and *atlE* gene sequences were compared to the respective non-exposed controls for their DNA profiles to evaluate possible micro or macroevolutions induced by ELF-EMF. Previous studies indicated that the presence or absence of *rpoS* in *E. coli* did not significantly affect planktonic growth of the bacteria. In contrast, deletion of *rpoS* caused differences in biofilm cell arrangement. These studies suggest that *rpoS* is important for biofilm physiology (Adams & McLean, 1999). Similarly, Qin et al. (2007) have reported that *atlE* is necessary for primary attachment and biofilm development of *S. epidermidis* in the wells of microtitre trays (Qin et al., 2007). Results of the majority of studies suggest that ELF-EMF can act as a stressing factor on bacterial cells, inducing cell adhesion. It is known that bacterial stress response can trigger mutagenesis pathways, which

have the potential to increase genetic diversity. While some studies did not show ELF-induced genotoxicity, other ones have shown that ELF can both modify DNA and induce genotoxic effects (Giorgi, Marcantonio, Bersani, Gavoci, & Del Re, 2011). Due to their different biological and molecular structures, microorganisms are affected differently by the same ELF-EMF intensity. Most of the previous research has focused on the effects of a specific value of ELF-EMF. However the effect of ELF-EMF varies depending on the frequency, magnetic intensity, cell type and exposure time (Bayir, Bilgi, Sendemir-Urkmez, & Hames-Kocabas, 2015). Our observations show that both strains have diverse responses to different ELF-EMF frequencies and exposure times. With regard to the analysis of *rpoS* and *atlE* genes, similar DNA sequence profiles were observed in each experimental condition examined both in exposed and non-exposed cultures during biofilm formation. This leads to the fact that, biofilm inhibition at 45 Hz and 50 Hz (1 mT) is not the result of gene mutations. Stavroulakis (2003) stated that in some biochemical studies, structure and function of some enzymes and organelles is disrupted by radiofrequency waves. Initial attachment of *S. epidermidis* is primarily mediated by cell surface proteins and adhesion to abiotic surfaces such as catheters is mainly governed by bacterial cell surface hydrophobicity. In our study, although ELF-EMF exposure shows similar DNA sequence with non-exposed cultures, expression of cell surface proteins could be affected by ELF-EMF leading to stress effect and decreasing biofilm formation at 50 Hz frequency range. Similarly, bacterial cell surface hydrophobicity could be disrupted by ELF-EMF that prevent bacteria to bind on surfaces.

The metabolism of bacteria is strictly affected by the environment. The *RpoS* sigma factor has been described as a master stress-response regulator under various stress conditions and a central regulator of many stationary phase-inducible genes in *P. aeruginosa*. Furthermore, Bangera *et al.* (2001) and Murakami *et al.* (2005) reported that *RpoS* affects the expression of more than 40% of all quorum-controlled genes and the production of extracellular alginate and biofilm formation. Heilmann *et al.* (1997) reported that autolysin AtlE is a surface-associated protein mediating attachment of bacteria to polystyrene surfaces. AtlE has also vitronectin-binding activity functioning not only in the early stages of adherence but also at later stages. This shows that, although we did not find sequence changes of biofilm facilitating genes at DNA level, transcription and translation of *rpoS* and *atlE* genes could be affected by ELF-EMF

that change the binding properties of bacteria. In addition, biofilm-initiating genes other than *rpoS* and *atlE*, could also be affected and lead to a decrease in biofilm formation. It is also possible that the expression of signal molecules or special receptor proteins of the quorum sensing mechanism could be disrupted so that bacteria fail to adhere to surfaces exposed to ELF-EMF.

It is indicated that 1) different bacteria may adhere differently to the same material; 2) the same bacteria may adhere differently to different device materials; 3) the same bacteria may adhere differently to the same device material placed under different circumstances (medium, temperature, etc.). Our results are conclusive with the literature that both studied gram-positive and gram-negative bacteria show different adherence properties to ELF-EMF exposed to frequencies ranging from 45 Hz to 300 Hz (1 mT intensity). Similarly, different binding properties of biofilms could be observed after changing the intensity of ELF-EMF exposure between 0.1 and 1.0.

It is reported that medical devices such as prostheses, bone replacement implants, drug delivery, tissue engineering and catheters are composed by polymers made mostly of polyethylene, polypropylene, polystyrene, silicone rubber, Teflon®, polyvinyl chloride and polyurethane (Treter & Macedo, 2011). Polystyrene and polypropylene are also used as components of medical devices, surgeons drapes (which have proven to be infection free) as well as for medical packaging (Url-7). Investigation of biofilm formation on these surfaces exposed to ELF-EMF in our study is important to elucidate and give insigt to new approaches and applications against biofilms in the medical field.

In conclusion, if these results can be reproduced and developed by further studies, ELF-EMF exposure might be used as an alternative strategy to inhibit the formation of *P. aeruginosa* and *S. epidermidis* biofilms at hospital environments. These encouraging results offer the potential to use ELF-EMF at defined ranges to combat pathogens in hospitals and become a promising candidate for biofilm treatment. Infections caused by formation and dispersal of *Pseudomonas aeruginosa* and *S. epidermidis* biofilms through types of medical equipments such as stents, catheters, indwelling medical devices might be prevented by using ELF-EMFs to inhibit biofilm formation on those hospital equipments.

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APPENDICES

APPENDIX A: OD_{585} Absorbance Values of ELF-EMF Exposed Biofilms
APPENDIX B: *atlE* and *rpoS* Gene Sequences

APPENDIX A: OD_{585} Absorbance Values of ELF-EMF Exposed Biofilms

Table A.1 : OD_{585} absorbance values of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* exposed to 45 Hz (1 mT intensity) ELF-EMF on polystyrene and polypropylene surfaces, obtained by spectrophotometric assay. $OD_{1,2,3,4,5}$ corresponds to five independent measurements of OD_{585}; OD_{avg} stands for arithmetic mean value of five independent OD_{585} measurements.

		Pseudomonas aeruginosa						*Staphylococcus epidermidis*					
Surface	Time	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}
Polypropylene (45 Hz exposed)	24 h	0,785	0,603	0,915	1,025	0,487	0,763	0,564	0,45	0,449	0,651	0,515	0,5258
Polypropylene (Non-exposed)		0,872	0,965	1,113	1,229	0,756	0,987	0,611	0,646	0,776	0,947	0,745	0,745
Polypropylene (45 Hz exposed)	48 h	1,572	1,341	1,605	1,545	1,323	1,4772	0,653	0,896	0,805	0,603	0,898	0,771
Polypropylene (Non-exposed)		2,147	1,846	1,841	2,315	2,098	2,0494	1,221	1,232	1,108	1,103	1,452	1,2232
Polystyrene (45 Hz exposed)	24 h	0,37	0,65	0,945	0,415	0,322	0,5404	0,354	0,495	0,815	0,986	0,655	0,661
Polystyrene (Non-exposed)		0,885	0,767	0,964	0,87	0,998	0,8968	0,615	0,478	0,895	0,754	0,879	0,7242
Polystyrene (45 Hz exposed)	48 h	0,795	0,768	1,121	1,145	1,339	1,0336	0,658	0,803	1,326	1,233	1,108	1,0256
Polystyrene (Non-exposed)		1,522	1,749	1,811	1,679	1,894	1,731	0,81	0,949	1,756	1,065	0,899	1,0958

Table A.2 : OD_{585} absorbance values of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* exposed to 50 Hz (1 mT intensity) ELF-EMF on polystyrene and polypropylene surfaces, obtained by spectrophotometric assay. $OD_{1,2,3,4,5}$ corresponds to five independent measurements of OD_{585}; OD_{avg} stands for arithmetic mean value of five independent OD_{585} measurements.

		Pseudomonas aeruginosa						*Staphylococcus epidermidis*					
Surface	Time	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}
Polypropylene (50 Hz exposed)	24 h	0,698	0,507	0,812	1,012	0,409	0,6876	0,364	0,399	0,449	0,451	0,415	0,4156
Polypropylene (Non-exposed)		0,815	0,985	1,127	1,356	0,703	0,9972	0,402	0,446	0,677	0,947	0,618	0,618
Polypropylene (50 Hz exposed)	48 h	1,412	1,212	1,561	1,505	1,285	1,395	0,653	0,869	0,905	0,603	0,988	0,8036
Polypropylene (Non-exposed)		2,052	1,896	1,788	2,209	2,108	2,0106	1,112	1,202	1,008	1,333	1,461	1,2232
Polystyrene (50 Hz exposed)	24 h	0,35	0,597	0,885	0,323	0,286	0,4882	0,453	0,359	0,698	0,784	0,556	0,57
Polystyrene (Non-exposed)		0,761	0,667	0,891	0,77	0,911	0,8	0,615	0,542	0,716	0,707	0,833	0,6826
Polystyrene (50 Hz exposed)	48 h	0,795	0,668	0,998	1,125	1,286	0,9744	0,685	0,759	0,93	1,112	1,008	0,8988
Polystyrene (Non-exposed)		1,423	1,698	1,91	1,663	1,894	1,7176	0,741	0,994	0,89	1,166	1,122	0,9826

Table A.3 : OD_{585} absorbance values of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* exposed to 100 Hz (1 mT intensity) ELF-EMF on polystyrene and polypropylene surfaces, obtained by spectrophotometric assay. $OD_{1,2,3,4,5}$ corresponds to five independent measurements of OD_{585}; OD_{avg} stands for arithmetic mean value of five independent OD_{585} measurements.

		Pseudomonas aeruginosa						*Staphylococcus epidermidis*					
Surface	Time	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}
Polypropylene (100 Hz exposed)	24 h	1,508	1,585	1,755	1,446	1,584	1,5756	0,867	0,975	0,866	1,237	0,988	0,9866
Polypropylene (Non-exposed)		1,482	1,56	1,689	1,528	1,669	1,5856	0,949	1,25	1,097	1,04	0,917	1,0506
Polypropylene (100 Hz exposed)	48 h	1,457	1,915	1,819	1,927	1,478	1,7192	1,263	1,304	1,014	1,242	1,203	1,2052
Polypropylene (Non-exposed)		1,83	1,887	1,952	1,543	1,418	1,726	1,466	1,422	1,243	1,151	0,773	1,211
Polystyrene (100 Hz exposed)	24 h	0,654	0,547	1,069	1,316	1,057	0,9286	0,658	0,527	0,434	0,966	1,008	0,7186
Polystyrene (Non-exposed)		1,101	1,262	1,022	1,286	1,177	1,1696	0,858	1,058	1,23	0,865	0,728	0,9478
Polystyrene (100 Hz exposed)	48 h	3,085	2,46	1,245	2,146	1,33	2,0532	1,278	1,027	1,281	1,0121	0,89	1,0976
Polystyrene (Non-exposed)		2,917	2,69	3,192	3,02	1,886	2,741	1,243	1,209	1,461	1,529	1,053	1,299

Table A.4 : OD_{585} absorbance values of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* exposed to 200 Hz (1 mT intensity) ELF-EMF on polystyrene and polypropylene surfaces, obtained by spectrophotometric assay. $OD_{1,2,3,4,5}$ corresponds to five independent measurements of OD_{585}; OD_{avg} stands for arithmetic mean value of five independent OD_{585} measurements.

		Pseudomonas aeruginosa						*Staphylococcus epidermidis*					
Surface	Time	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}
Polypropylene (200 Hz exposed)	24 h	1,872	1,752	1,778	1,614	1,651	1,7334	1,26	1,285	1,238	1,303	1,424	1,302
Polypropylene (Non-exposed)		2,233	1,819	2,099	2,078	1,87	2,0198	1,482	1,738	1,431	1,543	1,755	1,5898
Polypropylene (200 Hz exposed)	48 h	2,432	2,242	2,332	3,253	3,089	2,6696	2,109	1,986	2,166	2,189	2,253	2,1406
Polypropylene (Non-exposed)		2,698	2,341	2,022	2,389	2,,657	2,3625	2,143	1,704	1,799	1,959	2,041	1,9292
Polystyrene (200 Hz exposed)	24 h	1,377	1,844	1,455	1,405	1,558	1,5278	1,164	0,926	1,374	0,947	1,426	1,1674
Polystyrene (Non-exposed)		2,667	1,763	2,312	1,766	2,191	2,1398	1,421	1,056	1,388	1,417	1,095	1,2754
Polystyrene (200 Hz exposed)	48 h	1,721	1,694	2,368	1,578	1,297	1,7316	1,252	1,237	1,419	1,668	1,2	1,3552
Polystyrene (Non-exposed)		1,777	2,867	2,347	1,851	2,041	2,1766	1,349	1,806	2,374	2,283	1,766	1,9156

Table A.5 : OD_{585} absorbance values of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* exposed to 300 Hz (1 mT intensity) ELF-EMF on polystyrene and polypropylene surfaces, obtained by spectrophotometric assay. $OD_{1,2,3,4,5}$ corresponds to five independent measurements of OD_{585}; OD_{avg} stands for arithmetic mean value of five independent OD_{585} measurements.

		Pseudomonas aeruginosa						*Staphylococcus epidermidis*					
Surface	Time	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}	OD_1	OD_2	OD_3	OD_4	OD_5	OD_{Avg}
Polypropylene (300 Hz exposed)	24 h	1,731	1,859	2,068	1,888	2,033	1,9158	2,061	1,84	1,719	2,089	2,041	1,95
Polypropylene (Non-exposed)		1,973	1,849	2,115	2,013	2,016	1,9932	1,476	1,963	1,783	1,871	2,125	1,8436
Polypropylene (300 Hz exposed)	48 h	2,97	3,132	3,063	3,018	2,76	2,9886	3,184	3,657	3,118	3,25	2,896	3,221
Polypropylene (Non-exposed)		2,226	2,581	2,536	2,409	2,593	2,469	2,637	2,34	1,894	1,852	1,869	2,1184
Polystyrene (300 Hz exposed)	24 h	1,26	1,285	1,238	1,303	1,424	1,302	1,872	1,752	1,778	1,614	1,651	1,7334
Polystyrene (Non-exposed)		1,482	1,738	1,431	1,543	1,755	1,5898	2,233	1,819	2,099	2,078	1,87	2,0198
Polystyrene (300 Hz exposed)	48 h	2,109	1,986	2,166	2,189	2,253	2,1406	2,432	2,242	2,332	3,253	3,089	2,6696
Polystyrene (Non-exposed)		2,143	1,704	1,799	1,959	2,041	1,9292	2,698	2,341	2,022	2,389	2,,657	2,3625

APPENDIX B: *atlE* and *rpoS* Gene Sequences

a) ELF-EMF Exposed *Staphylococcus epidermidis atlE* gene Sequence Alinment (BLAST) with Non-Exposed Control

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ab1-Assembly    ATGGCGAAAAAATTCAATTACAAATTACCGTCTATGGTTGCTTTAACGTTATTTGGCACA
Gene            ATGGCGAAAAAATTCAATTACAAATTACCGTCTATGGTTGCTTTAACGTTATTTGGCACA
                ************************************************************

ab1-Assembly    GCTTTTACTGCACATCAAGCAAATGCTGCTGAACAACCACAGAATCAGTCTAATCATAAA
Gene            GCTTTTACTGCACATCAAGCAAATGCTGCTGAACAACCACAGAATCAGTCTAATCATAAA
                ************************************************************

ab1-Assembly    AATGTATTAGATGATCAAACTGCCCTCAAACAAGCAGAAAAAGCTAAAAGCGAAGTTACA
Gene            AATGTATTAGATGATCAAACTGCCCTCAAACAAGCAGAAAAAGCTAAAAGCGAAGTTACA
                ************************************************************

ab1-Assembly    CAATCAACTACAAATGTATCTGGTACACAAACATATCAAGACCCTACCCAAGTTCAACCT
Gene            CAATCAACTACAAATGTATCTGGTACACAAACATATCAAGACCCTACCCAAGTTCAACCT
                ************************************************************

ab1-Assembly    AAACAAGACACACAAAGTACTACATATGATGCATCATTAGATGAAATGAGTACTTATAAT
Gene            AAACAAGACACACAAAGTACTACATATGATGCATCATTAGATGAAATGAGTACTTATAAT
                ************************************************************

ab1-Assembly    GAAATTTCATCAAATCAAAAGCAACAATCTTTATCAACAGATGATGCGAATCAAAATCAA
Gene            GAAATTTCATCAAATCAAAAGCAACAATCTTTATCAACAGATGATGCGAATCAAAATCAA
                ************************************************************

ab1-Assembly    ACGAATTCTGTTACAAAAAATCAACAAGAAGAAACAAATGATTTGACACAAGAAGATAAA
Gene            ACGAATTCTGTTACAAAAAATCAACAAGAAGAAACAAATGATTTGACACAAGAAGATAAA
                ************************************************************

ab1-Assembly    ACATCCACTGATACAAATCAATTACAGGAGACACAATCTGTAGCAAAAGAAAATGAGAAA
Gene            ACATCCACTGATACAAATCAATTACAGGAGACACAATCTGTAGCAAAAGAAAATGAGAAA
                ************************************************************

ab1-Assembly    GATTTAGGAGCTAACGCAAATAATGAACAACAAGACAAGAAGATGACTGCAAGTCAACCT
Gene            GATTTAGGAGCTAACGCAAATAATGAACAACAAGACAAGAAGATGACTGCAAGTCAACCT
                ************************************************************

ab1-Assembly    TCCGAAAATCAAGCAATTGAAACTCAAACTGCTTCTAATGATAATGAAAGCCAACAAAAA
Gene            TCCGAAAATCAAGCAATTGAAACTCAAACTGCTTCTAATGATAATGAAAGCCAACAAAAA
                ************************************************************

ab1-Assembly    AGTCAGCAAGTAACTTCTGAACAAAATGAAACTGCTACACCTAAAGTATCAAATACAAAC
Gene            AGTCAGCAAGTAACTTCTGAACAAAATGAAACTGCTACACCTAAAGTATCAAATACAAAC
                ************************************************************

ab1-Assembly    GCATCTGGTTATAATTTTGATTACGATGATGAAGACGATGATAGCTCAACAGACCATTTA
Gene            GCATCTGGTTATAATTTTGATTACGATGATGAAGACGATGATAGCTCAACAGACCATTTA
                ************************************************************

ab1-Assembly    GAGCCTATCTCATTAAACAATGTGAATGCTACATCTAAACAAACTACTTCATATAAATAT
Gene            GAGCCTATCTCATTAAACAATGTGAATGCTACATCTAAACAAACTACTTCATATAAATAT
                ************************************************************

ab1-Assembly    AAAGAACCAGCTCAACGTGTAACAACTAATACTGTAAAAAAAGAAACGGCATCTAATCAA
Gene            AAAGAACCAGCTCAACGTGTAACAACTAATACTGTAAAAAAAGAAACGGCATCTAATCAA
                ************************************************************

ab1-Assembly    GCGACTATAGATACAAAGCAATTCACCCCATTTAGTGCAACTGCTCAACCGAGAACAGTT
Gene            GCGACTATAGATACAAAGCAATTCACCCCATTTAGTGCAACTGCTCAACCGAGAACAGTT
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ab1-Assembly    TATTCTGTATCTAGTCAAAAAACATCATCATTACCGAAATATACACCAAAGGTTAATTCT
Gene            TATTCTGTATCTAGTCAAAAAACATCATCATTACCGAAATATACACCAAAGGTTAATTCT
                ************************************************************

ab1-Assembly    TCAATAAATAACTATATTCGTAAAAAGAATATGAAAGCACCAAGAATTGAAGAAGATTAT
Gene            TCAATAAATAACTATATTCGTAAAAAGAATATGAAAGCACCAAGAATTGAAGAAGATTAT
                ************************************************************

ab1-Assembly    ACGTCATATTTCCCTAAATATGGCTATAGAAACGGTGTGGGACGTCCTGAAGGTATCGTT
Gene            ACGTCATATTTCCCTAAATATGGCTATAGAAACGGTGTGGGACGTCCTGAAGGTATCGTT
                ************************************************************

ab1-Assembly    GTTCATGATACTGCAAATGATAACTCAACAATCGATGGCGAGATTGCTTTCATGAAACGT
Gene            GTTCATGATACTGCAAATGATAACTCAACAATCGATGGCGAGATTGCTTTCATGAAACGT
                ************************************************************

ab1-Assembly    AATTACACAAATGCATTCGTACACGCATTTGTTGATGGCAATAGAATTATAGAAACAGCT
Gene            AATTACACAAATGCATTCGTACACGCATTTGTTGATGGCAATAGAATTATAGAAACAGCT
                ************************************************************

ab1-Assembly    CCGACAGATTACTTATCTTGGGGTGCAGGTCCATATGGAAATCAACGTTTTATCAATGTT
Gene            CCGACAGATTACTTATCTTGGGGTGCAGGTCCATATGGAAATCAACGTTTTATCAATGTT
                ************************************************************

ab1-Assembly    GAAATCGTCCATACACATGATTATGATTCATTTGCACGTTCAATGAACAACTACGCTGAT
Gene            GAAATCGTCCATACACATGATTATGATTCATTTGCACGTTCAATGAACAACTACGCTGAT
                ************************************************************

ab1-Assembly    TATGCTGCAACGCAATTGCAATATTATAATTTAAAACCTGATAGCGCTGAAAACGATGGA
Gene            TATGCTGCAACGCAATTGCAATATTATAATTTAAAACCTGATAGCGCTGAAAACGATGGA
                ************************************************************

ab1-Assembly    AGAGGAACAGTTTGGACACATGCTGCTATCTCTAACTTCTTAGGAGGTACTGATCACGCT
Gene            AGAGGAACAGTTTGGACACATGCTGCTATCTCTAACTTCTTAGGAGGTACTGATCACGCT
                ************************************************************

ab1-Assembly    GACCCTCACCAATATTTAAGAAGTCACAATTATAGCTATGCAGAATTATATGACTTAATT
Gene            GACCCTCACCAATATTTAAGAAGTCACAATTATAGCTATGCAGAATTATATGACTTAATT
                ************************************************************

ab1-Assembly    TATGAAAAATATTTAATTAAAACGAAGCAAGTAGCACCTTGGGGCACAACATCTACAAAA
Gene            TATGAAAAATATTTAATTAAAACGAAGCAAGTAGCACCTTGGGGCACAACATCTACAAAA
                ************************************************************

ab1-Assembly    CCGTCACAACCTTCTAAACCATCAGGAGGAACTAATAATAAGTTAACTGTGTCTGCTAAT
Gene            CCGTCACAACCTTCTAAACCATCAGGAGGAACTAATAATAAGTTAACTGTGTCTGCTAAT
                ************************************************************

ab1-Assembly    CGTGGTGTTGCTCAAATTAAACCAACAAATAATGGCTTATATACAACTGTTTATGACAGT
Gene            CGTGGTGTTGCTCAAATTAAACCAACAAATAATGGCTTATATACAACTGTTTATGACAGT
                ************************************************************

ab1-Assembly    AAAGGTCATAAGACTGATCAAGTACAAAAAACTCTATCCGTTACTAAAACTGCAACATTA
Gene            AAAGGTCATAAGACTGATCAAGTACAAAAAACTCTATCCGTTACTAAAACTGCAACATTA
                ************************************************************

ab1-Assembly    GGAAATAACAAATTCTATTTAGTTGAAGACTACAATAGCGGTAAAAAATACGGTTGGGTT
Gene            GGAAATAACAAATTCTATTTAGTTGAAGACTACAATAGCGGTAAAAAATACGGTTGGGTT
                ************************************************************

ab1-Assembly    AAACAAGGTGATGTTGTTTATAACACTGCTAAGGCACCAGTAAAAGTGAATCAAACATAT
Gene            AAACAAGGTGATGTTGTTTATAACACTGCTAAGGCACCAGTAAAAGTGAATCAAACATAT
                ************************************************************

ab1-Assembly    AATGTTAAAGCAGGGTCAACACTTTACACAGTTCCTTGGGGTACACCAAAACAAGTTGCT
Gene            AATGTTAAAGCAGGGTCAACACTTTACACAGTTCCTTGGGGTACACCAAAACAAGTTGCT
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ab1-Assembly    AGCAAAGTATCTGGTACTGGAAATCAAACATTTAAAGCAACTAAACAGCAACAAATTGAT
Gene            AGCAAAGTATCTGGTACTGGAAATCAAACATTTAAAGCAACTAAACAGCAACAAATTGAT
                ************************************************************

ab1-Assembly    AAAGCAACGTATCTTTATGGTACAGTGAATGGTAAATCTGGTTGGATTAGTAAATATTAC
Gene            AAAGCAACGTATCTTTATGGTACAGTGAATGGTAAATCTGGTTGGATTAGTAAATATTAC
                ************************************************************

ab1-Assembly    TTAACTACAGCATCTAAACCTAGCAATCCAACTAAACCTTCAACAAACAACCAATTAACA
Gene            TTAACTACAGCATCTAAACCTAGCAATCCAACTAAACCTTCAACAAACAACCAATTAACA
                ************************************************************

ab1-Assembly    GTGACTAACAATAGTGGTGTTGCTCAAATCAATGCAAAAAATAGTGGCTTATATACTACA
Gene            GTGACTAACAATAGTGGTGTTGCTCAAATCAATGCAAAAAATAGTGGCTTATATACTACA
                ************************************************************

ab1-Assembly    GTTTATGACACTAAAGGAAAGACAACAAATCAAATCCAACGTACATTGTCAGTGACGAAA
Gene            GTTTATGACACTAAAGGAAAGACAACAAATCAAATCCAACGTACATTGTCAGTGACGAAA
                ************************************************************

ab1-Assembly    GCTGCCACACTTGGTGATAAAAAATTCTATCTTGTTGGTGATTATAATACTGGTACAAAT
Gene            GCTGCCACACTTGGTGATAAAAAATTCTATCTTGTTGGTGATTATAATACTGGTACAAAT
                ************************************************************

ab1-Assembly    TATGGTTGGGTAAAACAAGATGAGGTCATTTACAACACAGCTAAATCACCTGTAAAAATC
Gene            TATGGTTGGGTAAAACAAGATGAGGTCATTTACAACACAGCTAAATCACCTGTAAAAATC
                  **********************************************************

ab1-Assembly    AATCAAACATACAACGTCAAACCTGGTGTTAAATTACACACAGTACCTTGGGGCACATAT
Gene            AATCAAACATACAACGTCAAACCTGGTGTTAAATTACACACAGTACCTTGGGGCACATAT
                ************************************************************

ab1-Assembly    AATCAAGTGGCTGGAACAGTTTCAGGTAAAGGCGATCAAACTTTTAAAGCAACTAAACAA
Gene            AATCAAGTGGCTGGAACAGTTTCAGGTAAAGGCGATCAAACTTTTAAAGCAACTAAACAA
                ************************************************************

ab1-Assembly    CAACAAATTGATAAAGCAACATATCTTTATGGTACAGTGAACGGTAAATCTGGTTGGATT
Gene            CAACAAATTGATAAAGCAACATATCTTTATGGTACAGTGAACGGTAAATCTGGTTGGATT
                ************************************************************

ab1-Assembly    AGTAAATACTATTTAACTGCACCATCAAAAGTTCAAGCTTTGTCTACTCAATCAACACCA
Gene            AGTAAATACTATTTAACTGCACCATCAAAAGTTCAAGCTTTGTCTACTCAATCAACACCA
                ************************************************************

ab1-Assembly    GCACCTAAACAAGTAAAACCATCTACACAAACTGTAAATCAAATTGCTCAAGTGAAAGCT
Gene            GCACCTAAACAAGTAAAACCATCTACACAAACTGTAAATCAAATTGCTCAAGTGAAAGCT
                ************************************************************

ab1-Assembly    AATAATTCTGGAATAAGAGCATCTGTATATGATAAAACAGCCAAAAGTGGTACGAAATAC
Gene            ATAATTCTGGAATAAGAGCATCTGTATATGATAAAACAGCCAAAAGTGGTACGAAATAC
                ************************************************************

ab1-Assembly    GCTAACCGTACATTCCTTATCAATAAACAACGTACTCAAGGTAATAACACGTATGTACTA
Gene            GCTAACCGTACATTCCTTATCAATAAACAACGTACTCAAGGTAATAACACGTATGTACTA
                ************************************************************

ab1-Assembly    CTTCAAGATGGAACAAGTAATACTCCATTAGGATGGGTAAACATTAATGATGTGACAACT
Gene            CTTCAAGATGGAACAAGTAATACTCCATTAGGATGGGTAAACATTAATGATGTGACAACT
                ************************************************************

ab1-Assembly    CAAAATATCGGAAAACAAACTCAGTCTATAGGTAAATATTCAGTAAAACCTACAAATAAT
Gene            CAAAATATCGGAAAACAAACTCAGTCTATAGGTAAATATTCAGTAAAACCTACAAATAAT
                ************************************************************

ab1-Assembly    GGTCTATATTCTATTGCTTGGGGTACTAAAAACCAACAATTACTAGCACCTAATACGCTA
Gene            GGTCTATATTCTATTGCTTGGGGTACTAAAAACCAACAATTACTAGCACCTAATACGCTA
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ab1-Assembly    GCTAATCAAGCATTTAATGCTTCCAAAGCTGTTTACGTTGGTAAAGATTTATATCTATAC
Gene            GCTAATCAAGCATTTAATGCTTCCAAAGCTGTTTACGTTGGTAAAGATTTATATCTATAC
                ************************************************************

ab1-Assembly    GGTACAGTCAATAACAGAACAGGATGGATTGCTGCTAAGGATTTAATCCAAAACAGTACT
Gene            GGTACAGTCAATAACAGAACAGGATGGATTGCTGCTAAGGATTTAATCCAAAACAGTACT
                ************************************************************

ab1-Assembly    GACGCTCAATCAACACCATATAACTATACTTTTGTTATCAATAATAGTAAAAGTTATTTC
Gene            GACGCTCAATCAACACCATATAACTATACTTTTGTTATCAATAATAGTAAAAGTTATTTC
                ************************************************************

ab1-Assembly    TATATGGATCCAACAAAAGCAAACCGATATTCTTTAAAACCATATTATGAACAAACTTTC
Gene            TATATGGATCCAACAAAAGCAAACCGATATTCTTTAAAACCATATTATGAACAAACTTTC
                ************************************************************

ab1-Assembly    ACAGTCATTAAGCAAAAAAATATTAATGGCGTTAAATGGTACTATGGTCAACTTTTAGAC
Gene            ACAGTCATTAAGCAAAAAAATATTAATGGCGTTAAATGGTACTATGGTCAACTTTTAGAC
                ************************************************************

ab1-Assembly    GGTAAATATGTTTGGATAAAATCAACTGACTTAGTTAAGGAAAAAATTAAATATGCATAT
Gene            GGTAAATATGTTTGGATAAAATCAACTGACTTAGTTAAGGAAAAAATTAAATATGCATAT
                ************************************************************

ab1-Assembly    ACTGGAATGACTTTAAATAACGCGATAAATATCCAATCTCGTCTTAAATATAAACCACAA
Gene            ACTGGAATGACTTTAAATAACGCGATAAATATCCAATCTCGTCTTAAATATAAACCACAA
                ************************************************************

ab1-Assembly    GTACAAAATGAGCCTTTGAAATGGTCAAATGCTAATTATAGTCAAATTAAAAATGCTATG
Gene            GTACAAAATGAGCCTTTGAAATGGTCAAATGCTAATTATAGTCAAATTAAAAATGCTATG
                ************************************************************

ab1-Assembly    GATACAAAGCGTTTAGCTAATGATTCATCCTTAAAATATCAATTCTTACGTTTAGATCAA
Gene            GATACAAAGCGTTTAGCTAATGATTCATCCTTAAAATATCAATTCTTACGTTTAGATCAA
                ************************************************************

ab1-Assembly    CCACAATACTTGTCAGCACAAGCTCTCAATAAATTATTAAAAGGCAAAGGTGTACTTGAA
Gene            CCACAATACTTGTCAGCACAAGCTCTCAATAAATTATTAAAAGGCAAAGGTGTACTTGAA
                ************************************************************

ab1-Assembly    AACCAAGGCGCTGCATTTAGCCAAGCTGCACGTAAGTATGGTCTAAATGAAATTTATCTT
Gene            AACCAAGGCGCTGCATTTAGCCAAGCTGCACGTAAGTATGGTCTAAATGAAATTTATCTT
                ************************************************************

ab1-Assembly    ATCTCACATGCTTTAGTAGAAACAGGTAATGGAACTTCACAACTTGCTAAAGGTGGAGAT
Gene            ATCTCACATGCTTTAGTAGAAACAGGTAATGGAACTTCACAACTTGCTAAAGGTGGAGAT
                ************************************************************

ab1-Assembly    GTTTCAAAAGGTAAATTCACAACTAAAACAGGTCACAAATACCATAATGTCTTTGGAATT
Gene            GTTTCAAAAGGTAAATTCACAACTAAAACAGGTCACAAATACCATAATGTCTTTGGAATT
                ************************************************************

ab1-Assembly    GGTGCATTTGACAATAATGCACTTGTAGATGGTATCAAATACGCTAAAAATGCTGGATGG
Gene            GGTGCATTTGACAATAATGCACTTGTAGATGGTATCAAATACGCTAAAAATGCTGGATGG
                ************************************************************

ab1-Assembly    ACTTCTGTCTCTAAAGCAATTATTGGTGGCGCTAAATTCATTGGAAATTCATACGTGAAA
Gene            ACTTCTGTCTCTAAAGCAATTATTGGTGGCGCTAAATTCATTGGAAATTCATACGTGAAA
                ************************************************************

ab1-Assembly    GCAGGACAAAATACGCTATATAAAATGCGTTGGAATCCTGCAAACCCTGGTACGCATCAA
Gene            GCAGGACAAAATACGCTATATAAAATGCGTTGGAATCCTGCAAACCCTGGTACGCATCAA
                ************************************************************

ab1-Assembly    TATGCAACTGATATTAATTGGGCAAATGTCAACGCACAAGTATTAAAACAATTTTATGAT
Gene            TATGCAACTGATATTAATTGGGCAAATGTCAACGCACAAGTATTAAAACAATTTTATGAT
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ab1-Assembly    AAAATTGGTGAAGTCGGTAAGTACTTCGAAATTCCAACATACAAATAA
Gene            AAAATTGGTGAAGTCGGTAAGTACTTCGAAATTCCAACATACAAATAA
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b) ELF-EMF exposed *Pseudomonas aeruginosa* partial *rpoS* gene Sequence Alinment (BLAST) with non-exposed control

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abi1-Assembly   G--CGCCCCCACCT----GGAACAGACA-----AAAC-------CGCTCGCGGGCCAAGA
Gene            GGGCGATCCCGCTGGTCGGAAGCGGATGATCGAGAGCAACCTGCGGTTGGTGGTGAAGAT
                *  **. ***.*      *.*.*.** .     .*.*        * * * **  .*..:

abi1-Assembly   CGATCGGCGCTATGTCCATCGCGGACTGTCCCTGCTCGACCTGATCAGAGGAAGGCAACC
Gene            CGCCCGGCGCTATGTCAATCGCGGACTGTCCCTGCTCGACCTGATC-GAGGAAGGCAACC
                **. ************ ***************************** *************

abi1-Assembly   TAGGCCTGATCCGCGCCGTGGAGAAGTTCGATCCGGAGCGCGGATTCCGGTTCTCGACCT
Gene            TAGGCCTGATCCGCGCCGTGGAGAAGTTCGATCCGGAGCGCGGATTCCGGTTCTCGACCT
                ************************************************************

abi1-Assembly   ACGCCACCTGGTGGATCCGCCAGACCATCGAGCGGGCCATCATGAACCAGACCCGGACCA
Gene            ACGCCACCTGGTGGATCCGCCAGACCATCGAGCGGGCCATCATGAACCAGACCCGGACCA
                ************************************************************

abi1-Assembly   TTCGCTTGCCGATCCATGTGGTCAAGGAGCTCAACGTCTACCTGCGTGCGGCGCGGGAAC
Gene            TTCGCTTGCCGATCCATGTGGTCAAGGAGCTCAACGTCTACCTGCGTGCGGCGCGGGAAC
                ************************************************************

abi1-Assembly   TGACCCACAAGCTCGACCACGAACCTTCACCCGAAGAAATCGCCAACCTGCTGGAGAAGC
Gene            TGACCCACAAGCTCGACCACGAACCTTCACCCGAAGAAATCGCCAACCTGCTGGAGAAGC
                ************************************************************

abi1-Assembly   CGGTCGCCGAGGTCAAGCGCATGCTCGGCCTGAACGAACGGGTGACTTCGGTAGACGTCT
Gene            CGGTCGCCGAGGTCAAGCGCATGCTCGGCCTGAACGAACGGGTGACTTCGGTAGACGTCT
                ************************************************************

abi1-Assembly   CTCTTGGTCCGGACTCGGACAAGACCCTGCTGGATACGCTCACCGACGATCGCCCCACCG
Gene            CTCTTGGTCCGGACTCGGACAAGACCCTGCTGGATACGCTCACCGACGATCGCCCCACCG
                ************************************************************

abi1-Assembly   ATCCGTGCGAGCTGCTGCAGGATGACGATCTCAGCGAAAGCATCGACCAGTGGCTGACGG
Gene            ATCCGTGCGAGCTGCTGCAGGATGACGATCTCAGCGAAAGCATCGACCAGTGGCTGACGG
                ************************************************************

abi1-Assembly   AACTCACCGACAAGCAGCGTGAGGTGGTGATTCGCCGCTTCGGCTTGCGCGGTCACGAAA
Gene            AACTCACCGACAAGCAGCGTGAGGTGGTGATTCGCCGCTTCGGCTTGCGCGGTCACGAAA
                ************************************************************

abi1-Assembly   GCAGCACGCTGGAAGAGGTCGGCCAGGAAATCGGCCTGACCCGCGAGCGGGTTCGTCAGA
Gene            GCAGCACGCTGGAAGAGGTCGGCCAGGAAATCGGCCTGACCCGCGAGCGGGTTCGTCAGA
                ************************************************************

abi1-Assembly   TCCAGGTCGAGGCGCTGAAGCGCCTGCGGGAGATTCTGGAGAAGAATGGCCTGTCGAGTG
Gene            TCCAGGTCGAGGCGCTGAAGCGCCTGCGGGAGATTCTGGAGAAGAATGGCCTGTCGAGTG
                ************************************************************

abi1-Assembly   ACGCGCTGTTCCAGTGACGGAAAACCTTACACCCAATGAAAAAACAGGGTTCGCGGGTTT
Gene            ACGCGCTGTTCCAGTGA----------------------------------------
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abi1-Assembly   TTTGCGTCCGCTCAGTAAGCTTAA
Gene            ------------------------
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PROFESSIONAL EXPERIENCE AND REWARDS:

- 2014-Present: İstanbul Arel University Technology Transfer Office, Project Development and Management Expert
- 2013-2014: Yildiz Technology Transfer Office, Project Specialist
- 2010-2012: Evre Danışmanlık ve Bilişim Hizmetleri, Consultancy in R & D Funds, Project Manager
- 2010-2011: Ender Turizm Seyahat ve Taşımacılık Ltd.Şti, EU 7th Framework Programme - CORNET Research Project, Researcher
- 2007-2009: İstanbul Technical University, Advanced Technologies Research Project, Project Support Specialist
- 2007-2008: İstanbul Technical University, EU 6th Frame Integrated Project (IP No: 505899), Project Assistant
- 2007-2007: İstanbul Metropolitan Municipality, Coastal sea sample collection and microbial analysis of the sea water in beaches of İstanbul, Microbial Analyst

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- Karaguler, T., **Kahraman, H.,** Tuter, M. 2017. Analyzing Effects of ELF-EMFs on Removing Bacterial Biofilm. Biocybernetics and Biomedical Engineering; 37:2, pp. 336–340.
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OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

- **Kahraman, H.,** Sahin, D., Sarikaya, M., Gul-Karaguler, N., Tamerler, C. Molecular vector construction for binding affinity comparison of genetically engineered inorganic binding peptides and his-tagged proteins. Joint Conference of the 33rd FEBS Congress/11th IUBMB Conference, Jun 28-Jul 03, 2008, Athens, Greece.
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