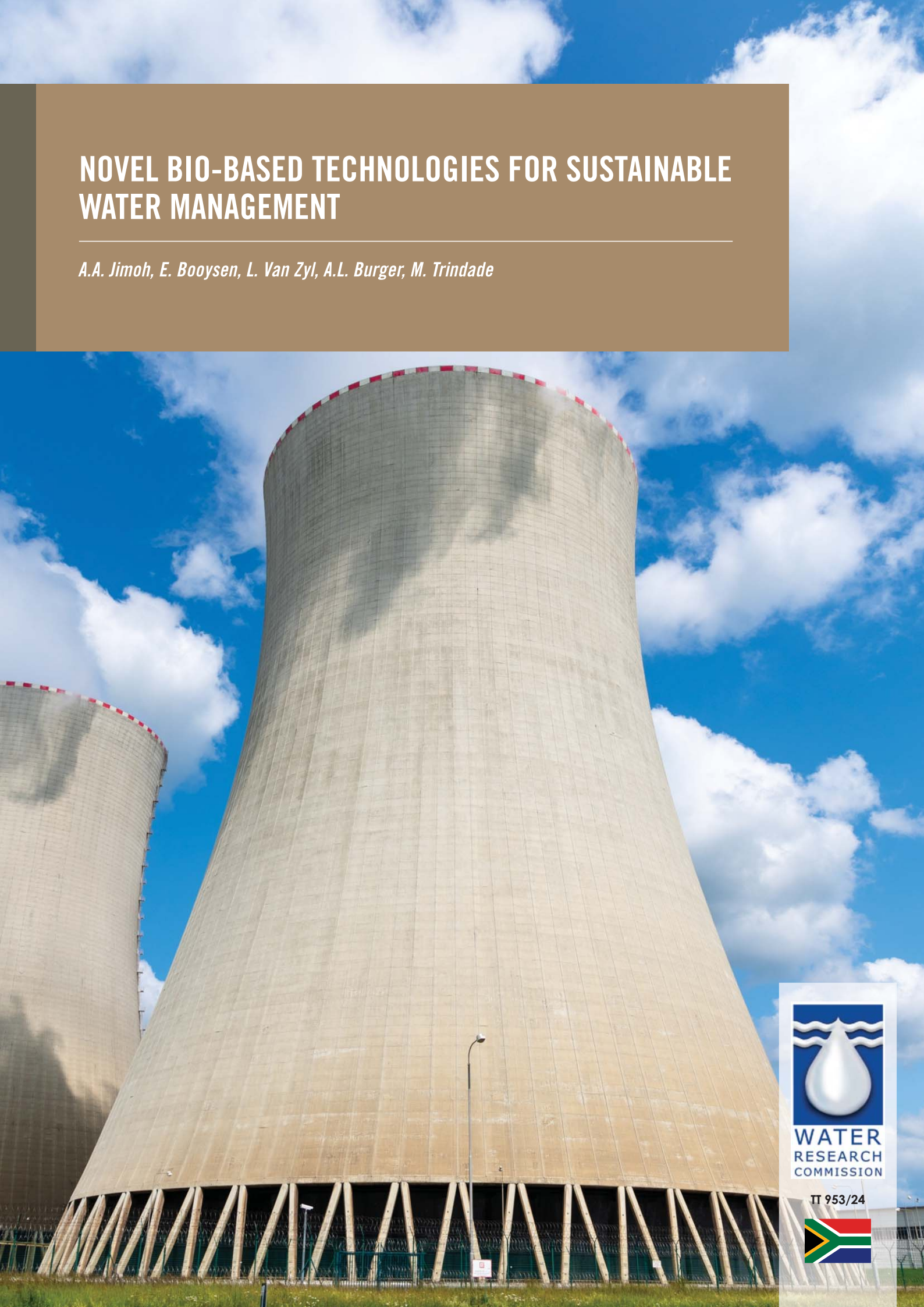
NOVEL BIO-BASED TECHNOLOGIES FOR SUSTAINABLE WATER MANAGEMENT

A.A. Jimoh, E. Booysen, L. Van Zyl, A.L. Burger, M. Trindade



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Novel Bio-Based Technologies for Sustainable Water Management

Report
to the Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Many industries, hospitals, institutions, and office buildings utilise some type of cooling tower. Microbial fouling through the development of biofilms represents one of the major problems in these cooling systems, leading to several problems, including a loss of cooling efficacy, blockages, and system corrosion, in addition to raising risks to safety and health. Therefore, the treatment of cooling tower water is necessary to prevent fouling, and since the treated water is often discharged to the sanitary sewer system, this needs to be managed efficiently. Most recirculating cooling water system maintenance plans include controlled additions of conditioning chemicals. Although there are many options for the treatment of this water to improve the cleanliness, longevity, and efficiency of these systems, it can be challenging because individual systems are unique. One of the most efficient treatment strategies involves the simultaneous use of a biocide and a dispersant (a surfactant). The biocide kills the bacteria while the dispersant limits their attachment to the system surfaces due to lowering of the surface tension of the aqueous environment. Optimisation of different combinations of surfactants and biocides could lead to improved system efficiency, optimise operating costs, reduce maintenance and system shut-down periods, and aid safety management.

This project investigated whether the use of a biosurfactant could provide a more superior, optimised, and environmentally-friendly treatment than what is currently being used for three different industrial water sources, offering multiple solutions for more effective management of the most difficult to treat component.

AIMS

The project aimed to deliver new anti-biofilm products specifically designed to treat different industrial wastewaters. This was addressed by investigating the dispersant capability of the patented lyso-ornithine (LOL) biosurfactants through co-formulation with two proprietary biocides (DECONT-A and DECONT-M) and assessing whether there is improved and/or more efficient biofilm control.
Therefore, the objectives of this report were as follows:

1. To investigate optimal production conditions and maximize the quantity of LOL produced to facilitate a more cost-effective bioprocess
2. High-throughput evaluation of IMBM-BS1 (LOL) against biofilms, co-formulated with DECONT-A and DECONT-M in industrial water sources collected from the cooling tower ponds from 3 undisclosed power generating facilities
3. Assess the antibiofilm activity of a Decont-A and LOL co-formulation in a continuous flow system by measuring metabolic activity and biofilm dispersal in real-time

METHODS

Fermentation of the recombinant strain expressing the LOL (hereon referred to as the “B” strain) was optimized by testing various conditions, including temperature, IPTG concentration, L-ornithine concentration, and media pH to achieve an optimal bioprocess. The LOL synthesis was visualized and quantitatively compared by high-performance liquid chromatography (HPLC) analysis. Scaled-up production of LOL was performed based on the optimized parameters (25^{o}C, 1 mM IPTG, 1 mM, 20 mM L-ornithine, pH 7). The LOL congeners were purified using the Bio-Rad NGC medium-pressure liquid chromatography system. Fractions were pooled and analyzed by HPLC to determine purity.

The ability of industrial wastewater sources to form biofilms was evaluated in a 96-well high-throughput assay, followed by determining the effect of the lyso-ornithine lipids and Decont-A and M biocides on the biofilms developed in these wastewaters. The minimum biofilm inhibitory concentration for the lyso-ornithine lipid,

Decont-A and M was established, followed by assays using co-formulations of lyso-ornithine lipid and Decont-A or M. All the co-formulation assays were performed on the industrial water source that performed the best under the single compound assays.

The effect of the Decont-A and LOL formulations that showed the highest activity in the 96-well high throughput assays was assessed in a continuous flow system. The metabolic activity was measured using a carbon dioxide evolution measurement system (CEMS). A concentrated inoculum of the microbial community present in the selected wastewater was used to inoculate the system, and sterilized wastewater was used as a growth medium. The industrial wastewater was used as both inoculum and growth media to better mimic the conditions in industrial setups. The metagenomic profile of the biofilms, pre- and post-treatment, was analysed to assess which community members were most affected by the treatments.

RESULTS AND DISCUSSION

The optimised cultivation parameters for the *E. coli* "B" strain increased the quantity of LOL synthesised, facilitating a more cost-effective bioprocess. The four major LOL species (C14:0, C16:0, C16:1, C18:0) were optimally produced at 25°C, 1 mM IPTG, 20 mM L-ornithine, and medium pH 7. The optimised expression conditions also revealed that we can manipulate the ratio of the fatty acid chain length profiles. This outcome will change the biosurfactant's hydrophilic-lipophilic balance and thereby change its application as LOLs with longer fatty acids have application in one field (as wetting agents or water-in-oil emulsifiers), whereas LOLs with shorter fatty acids may have applications in a completely different field (as detergents and oil-in-water emulsifiers).

Cooling tower wastewater collected from 3 different power generating facilities (hereon referred to as cooling tower pond 1, 2, or 3) established biofilms. Although the microbial community in cooling tower pond three water formed a biofilm after 7 days, it was significantly less developed compared to the biofilms formed by the water from ponds 1 and 2. The cooling tower pond 1 water contained the highest population of biofilm-forming bacteria and was used as the water source for the biofilm studies. The minimum biofilm inhibitory concentration of LOL was 60 µg/mL and 0.5 ppm for Decont-A and M when tested on this biofilm-forming community using a high-throughput 96-well biofilm assay. Co-formulating Decont-A and LOL significantly improved the inhibition of biofilm formation, while the biofilm inhibitory effects of Decont-M were not improved when co-formulated with LOL.

The most effective co-formulation (0.5 ppm Decont-A + 10 µg/mL LOL) was tested in a continuous flow system. In the preliminary studies we found that 0.5 ppm Decont-A + 10 µg/mL LOL was not effective at dispersing or disrupting the biofilm. A higher concentration of Decont-A (60 ppm) was co-formulated with LOL and resulted in significantly (19%) improved efficacy. A significant shift in the microbial community pre- and post-treatment was also observed for all three treatment groups (10 µg/mL LOL, 60 ppm Decont-A and 60 ppm Decont-A + 10 µg/mL LOL), investigated through metagenomic analyses. Most importantly, the targeted effect of the LOL on the antimicrobial resistant members of the biofilm, which are typically the most difficult to eradicate, and why biofilms are recalcitrant to antibiotic treatments, represents a major finding.

GENERAL

The aim of this project was to develop a novel biocide-biosurfactant co-formulation to improve biofilm removal in industrial water systems such as cooling towers used for generating power or in mining industries. The co-formulation developed in this study, containing the natural LOL as a biodispersant, improved the bioactivity of the biocide. Therefore, the aim of this project was achieved as a novel biocide-biosurfactant formulation with improved antibiofilm activity was developed.

CONCLUSIONS AND RECOMMENDATIONS

Biofilms are ubiquitous in all environments and in industrial water systems, such as cooling towers. They are generally detrimental to power plant operations and can lead to financial losses. In this report we developed and evaluated a novel biocide-biosurfactant co-formulation for antibiofilm activity. The use of high-throughput assessment represented a useful approach for narrowing down the number of formulations to be tested in a continuous flow system, providing savings on reagents and time. However, it can sometimes be misleading as the doses established in the high-throughput screening did not correlate to the results found in the continuous flow systems. Therefore, system optimisation must be conducted for each setup type and cannot be assumed to be directly transferable. Although the antibiofilm activity of Decont-A was improved by 19% when combined with LOL in the continuous flow system, this needs to be assessed in a scaled-up process (2000 L capacity). To achieve the LOL yields required to assess at this scale, the fermentation-based production of LOL also needs to be scaled. Further optimisation of the LOL yield through genetic engineering of the recombinant “B” strain, together with the development of a fed-batch fermentation process at <250 L, is required to achieve the required LOL yields.

In nature, biofilms are found as heterogeneous communities compared to homogenous communities traditionally used in lab experiments to evaluate biofilms. Studies using homogenous biofilms do not emulate nature and can therefore be misleading. The use of the wastewater as both an inoculum and sterilised growth media is a major advantage in our study compared to many others because it provides better biofilm comparisons to what is faced in industrial settings. However, one major disadvantage of using natural inoculums is that growing and cultivating the biofilm takes significantly longer in the continuous flow systems when compared to lab strains. Given the novelty of our approach and a lack of comparable studies, we significantly underestimated the length of time a biofilm would take to establish and were unable to perform more extensive evaluations. Therefore, extended biofilm evaluations and optimization are still required before this technology can be transferred to a scaled-up process.

The metagenomic evaluation of the effect of the developed co-formulation on the biofilm composition served as a qualitative approach to assess how the biofilm was impacted by the treatments. This forms the foundation for future studies to determine the mode of action of the developed formulation and to perform more detailed assessment of the community evolution, especially with respect to developing resistance to the applied treatment.

An important outcome of this project is the development of a biological control formulation that provides superior biofilm inhibition activity than what is currently being employed for the treatment of water-cooling systems. Every species of microbe has different resistance to a particular biocide. Therefore, we can potentially address this growing problem of biocidal resistance by the development of this new formulation. Furthermore, many typically used biocides pose safety risks and are difficult to handle, whereas our formulation offers a renewable, potentially more cost-effective, and safe solution with reduced environmental impact when the water is discharged.

Although the primary application focus is for power station waters, there is scope to employ the developed formulation more broadly since so many different industries must manage biofilms in cooling towers and other processes. While a universal solution is possible, it is dependent on regional locality, product market needs, logistics, and other factors. This study has generated a baseline to inform economic decisions that any industry will face when looking at the chemicals they use to treat their cooling tower water. Moreover, the involvement of an industry partner, who has experience in developing economically feasible products for water treatment, will ensure that the detailed techno-economic studies coupled with a pilot study performed on a case study basis will provide important insight beneficial to many industries.

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Collaborators	**Affiliation**
Prof Gideon Wolfaardt	Department of Microbiology, Stellenbosch University
Dr Mark Kelly Mr Burt Rodrigues	Biodx
Dr Lucia Steenkamp	CSIR
Others	
Dr Elanna Bester	Department of Microbiology, Stellenbosch University

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ACRONYMS & ABBREVIATIONS

EPS	extracellular polymeric substances
HPLC	high performance liquid chromatography
LOL	lyso-ornithine lipids
MIC	microbially influenced corrosion
OL	ornithine lipids
QACs	quaternary ammonium compounds
SDS	Sodium dodecyl sulfate
CTP1	cooling tower pond 1
CTP2	cooling tower pond 2
CTP3	cooling tower pond 3
ESKAPE	nosocomial pathogens that exhibit multidrug resistance and virulence: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*
TSB	Tryptic soy broth
CEMS	Carbon dioxide evolution measurement system
BS	Biosurfactant
LP	Lipopeptides
ACP	Acyl-acyl carrier protein
LB	Luria broth
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MIC	Minimum inhibitory concentration
OD	Optical density

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Water cooling towers are major users of water worldwide and increased industrial development has led to a greater demand for water and natural resources, particularly in threshold countries such as China, India and South Africa (Cloete & Flemming, 2012). Micro-organisms in cooling towers attach to surfaces and develop into biofilms, highly structured micro-consortia that are immersed in a self-produced extracellular polymeric substances (EPS) (Flemming & Wingender, 2010) and which promote biofouling (Lutterbach & De Franca, 1996). Biofilms contribute to increased resistance in a number of ways, and in industrial water systems it shields the cells in the deeper layers against biocides. If the cooling water is not treated, the biofilms that develop can lead to possible production shutdowns due to serious damage to equipment as well as reduced heat transfer efficiency with consequent energy losses occurring. Conditions within these systems also promote the development of pathogenic organisms, such as *Legionella pneumophila* (Li *et al*. 2015). The problems inherent to cooling systems have important economic and even health impacts. Consequently, the prevention, monitoring and control of biofouling are key components of management strategies of cooling towers (Ludensky, 2003).

Current methods used to prevent and control biofilm formation are mainly based on treatment of the cooling systems with oxidizing and non-oxidizing biocides (Di Pippo *et al*., 2017). Considerably higher concentrations of toxic biocides are needed to effectively treat biofilms and encourage microorganisms to exist in the planktonic state. Increasing biocide concentration is costly, resulting in growing environmental burdens that threaten non-target organisms and may also promote the development of biocide resistance (Cloete *et al*., 1992; Donlan, 2000). The development of treatment programs that involve alteration between biocides to disrupt resistance development have provided improved control, but resistance is often not sustained. Anti-scaling, anti-foaming, and dispersant compounds have therefore become important constituents of biocides to enhance biocide effectivity (Cloete *et al*., 1992). In particular, the combination of dispersants (surfactants) with biocides offers significant improvement as these surface-active compounds disrupt the biofilm matrix by forming micelles. The dispersants break up the foulants into smaller particles keeping them suspended in cooling water and thereby facilitating improved penetration of biocides. This prevents deposit formation and enables foulant removal from the system. However, these added chemicals are toxic to the environment and therefore represent an ecological hazard.

The application of biocides in the cooling water systems, and other effluent water streams, carries with it the risk of developing microbial resistance. The traditional approach to restricting this undesirable effect is to utilize two or more biocides on an alternating sequence. The biocide is applied as a single “slug” dose and during the period between doses, a low concentration of dispersant is continuously dosed. As the available dispersants are manufactured from synthetic materials, the development of a natural biodispersant offers the opportunity to further reduce the negative environmental impacts of the microbial control systems offered to large industrial cooling water systems such as those utilized in power generation. The motivation for formulating a product containing both the biosurfactant and the biocide is to enhance the efficacy of the biocide, and thus reduce the amount needed to achieve a satisfactory level of control. Biosurfactants possess surfactant properties that enable them to penetrate the complex EPS network and pre-condition the growth surfaces. Biosurfactants have recently become an important bioeconomy product for diverse applications. They are characterized by high biodegradability, lack of potential bioaccumulation, non-toxicity, and non-carcinogenicity, thereby representing reduced environmental impact caused by other toxic chemicals (Di Pippo *et al*., 2018). Their use in biofouling and biocorrosion treatment has not been widely investigated (Płaza & Achal, 2020). Therefore, additional research must be performed to assess the capability of biosurfactants to function as biodispersants

for use in industrial systems and other water management applications in relation to biofilm control and disruption.

1.2 PROJECT AIMS

The project aimed to develop new anti-biofilm products specifically designed for the treatment of three relevant industrial water sources, i.e., large cooling water systems such as power generation systems, industrial process water, and food production industry effluent. This aim was addressed by investigating the dispersant capability of the LOL biosurfactant through co-formulation with the biocides DECONT-A and DECONT-M and assessing whether there is improved and/or more efficient biofilm control in these waters.

The objectives are therefore as follows:

1. To optimise the production of LOL through the genetically modified *E. coli* “B” strain expressing lyso-ornithine lipids
2. High throughput evaluation of LOL biosurfactant against biofilms formed with three water sources, co-formulated with Decont-A and Decont-M biocides
3. To evaluate biofilm dynamics for biosurfactant/biocide combinations

1.3 SCOPE AND LIMITATIONS

This study focuses on improving the bioactivity of a propriety biocide, Decont-A, by co-formulating with a recombinantly produced biosurfactant, lysine-ornithine lipid (LOL), identified in the metagenome of lake sediment (Williams *et al*., 2019). Decont-A, LOL, and the co-formulation were tested against biofilm forming communities present in industrial wastewater collected from three industrial cooling towers. The formulations were assessed for antibiofilm activity in both high-throughput assays as well as continuous flow systems. In the continuous flow system, the biofilm was monitored using a carbon dioxide evolution monitoring system (CEMS) and through metagenomic analyses to evaluate the impact of the treatments on the biofilm communities.

A major limitation of this study pertains to the scale of investigation. Although continuous flow biofilm systems are more representative of biofilm structures that form in nature, these systems are not exact replicas of biofilms that form in cooling towers found in industry. To replicate this as far as possible, biofilms were established using the wild type bacteria cultured from the industrial water collected from the cooling towers. However, the structural composition of the biofilms formed could still differ significantly from the biofilms formed in cooling towers, due to a variety of external factors such as the attachment materials on which the biofilms settle as well as flow of the circulating water. Biofilms with different structural compositions will behave differently under different conditions and will respond differently to treatments. According to literature, wild type cultures develop biofilms more slowly compared to lab grown cultures, thus these cultures could negatively impact the timeline set out for the completion of this project. Furthermore, loadshedding and other unexpected power outages would negatively impact the study due to the requirement for these continuous flow systems to run uninterrupted for a period of 4 weeks, per test run.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Biofilm formation is of great concern to many industries, including marine, food processing, water, sugar-alcohol, mining, oil, thermoelectric, papermaking, medicines, desalination, and water treatment plants. The consequences of biofilms are enormous as it results in functional problems, added costs, and capital losses which are accrued on equipment and its components, as well as posing a public health risk due to the proliferation of thermotolerant pathogens in biofilms. This is a common occurrence of microbial fouling, which is a natural process resulting from biofilm activity that leads to the undesirable deposition of inorganic and/or organic matter through contact with exposed materials, thereby leading to the deterioration of the affected surfaces (Rao, 2012). Microbial fouling development is usually a dynamic, ubiquitous, and highly complicated process with the main agents involved being biofilm-forming microorganisms such as bacteria, diatoms, fungi, and microalgal spores (Maria da Gloria & Sarubbo, 2021). Industrially, microbial fouling poses severe problems in cooling circuits, reducing heat transfer efficiency (Shukla *et al*., 2021). Many industrial processes depend on quick heat removal from a production area, and there is always extreme difficulty in heat exchange between the hot-to-cool interphase if the water-cooling system pipe is coated with biofilms. A biofilm layer of simply 0.1 mm can decrease productivity so significantly that the related power expenses to drive plants can increase by a factor of four when contrasted with a framework containing a similar thickness of calcium carbonate (Vasilescu, 2017). Other complications in cooling water systems are breakdown/damage, reduced efficiency, blockages, system shutdown, and energy loss. Also, biofilm communities can promote the corrosion of fouled metal surfaces/alloy (referred to as microbially influenced corrosion) by altering the surface electrochemical properties (Płaza & Achal, 2020). Thus, 50% of total costs are lost to microbiological corrosion and, if left untreated, can have costly consequences on critical equipment in cooling systems, leading to downtime and expensive repair work (Vasilescu, 2017). The problems inherent to cooling systems have important economic and even health impacts and, consequently, the prevention, monitoring, and control of microbial fouling and corrosion are critical components of the management strategies of these cooling towers (Ludensky, 2003).

2.2 BIOFILM FORMATION

Biofilms were initially characterised as simple "slabs" of matrix-embedded materials in which immobile bacterial cells were randomly attached (Costerton *et al.*, 1987). The biofilm description was further clarified by Flemming & Wuertz (2019) as being aggregates of microorganisms with distinct sessile cells that form small clusters and microcolonies. The term 'aggregate' accounts for the cell-to-cell contact that most cells in multi-layered biofilms experience, either in flocs or surface-attached biofilms (Flemming *et al*., 2016b). Biofilm formation is a constant cause of pollution on most inanimate, biological, natural, or man-made surfaces (Satpute *et al*., 2016). As shown in **Figure 2.1** biofilm formation involves different stages including the development of the conditioned film, attachment to the surface, formation of microcolonies, and maturation and dispersal of the biofilms (Kirov, 2003; Sharma *et al*., 2021). Cells in biofilms subsist in harsh conditions as they are enclosed by high molecular weight EPS, stabilized by intra- and intermolecular linkages. Microbial EPS secreted by a significant variation of microbes are primarily composed of lipids, proteins, extracellular DNA, polysaccharides, amyloids, and polymeric substances such as peptidolipids and glycolipids (Di Pippo *et al*., 2018). The EPS matrix supports microbial adhesion to surfaces and functions as a three-dimensional framework that provides functions such as hydration, digestive capacity, stability, and protection (Flemming *et al*., 2016a).

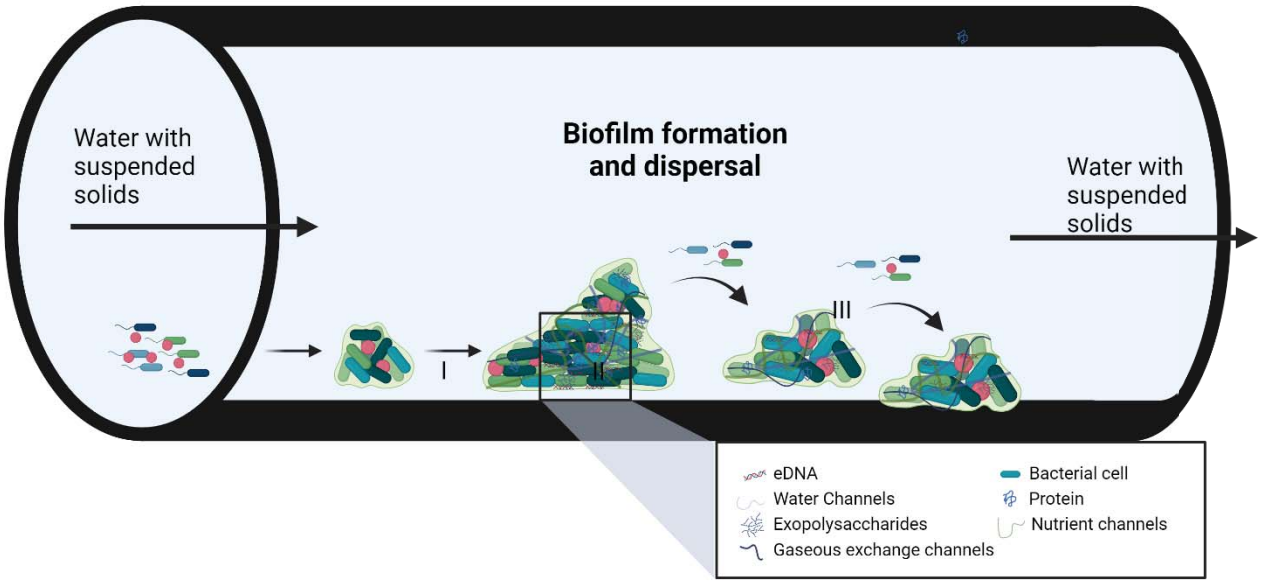


Figure 2.1. An illustration of the various processes associated with the formation of biofilms in cooling water systems (Adapted from Jimoh *et al.*, 2023)

2.3 MICROBIAL FOULING IN COOLING WATER SYSTEMS

The rapid increase in global population and industrialization exacerbates the challenges of water security, ineffective waste management, changing climatic conditions, biotic, and abiotic stress factors while adversely affecting the sustainability and protection of our environments. Manufacturing and other industrial processes require water use during their production process or for cooling equipment used in creating their products which has led to a demand on water that has exceeded the capacity in many regions of the world and has therefore become unsustainable (Cosgrove & Loucks, 2015). Different categories of water, such as deionized and feed water, will determine the treatment technologies to be used. Within the industrial water context, treatment systems are often designed to treat waste streams into effluent needed for reuse or to be safely discharged into the environment (AquaTech, 2019). Water treatment systems are used to protect cooling tower components from damage due to many contaminants present in circulation, feed, and blowdown water.

Biofilm communities colonize the surfaces of most cooling water systems of several industrial set-ups including steel mills, food, refineries, steel mills, chemicals, petrochemicals, petroleum, and power generation plants (Di Pippo *et al.*, 2018). Cooling systems release several thousand m^3 of steam per hour, ranging substantially in size from large industrial towers to small air conditioners (Pereira *et al.*, 2017). These cooling systems provide a conducive setting for excessive microbial growth, primarily supported by optimum temperature conditions (25 to 37^{o}C), neutral pH, exposure to sunlight, and constant aeration (Liu *et al.*, 2009). There is a compromise on the cooling systems due to requirements for optimum circulation and efficient heat transfer and removal (Di Pippo *et al.*, 2018). Additionally, different industrial equipment associated with cooling towers, such as fill materials, reservoirs, submerged sight glass and sensors, heat exchangers, and pipelines, possess structural surfaces that add to biofilm growth and development (Liu *et al.*, 2011; Rao, 2012). As the populations increase exponentially, there is a rapid increase in biofilm depth despite a prolonged start. Biofilm polymers enhance new cell attachment to the colonized surface and further accumulate inorganic debris from bulk water. The accumulative processes and the growth and replication of cells already on the surface ultimately generate microbial fouling, a significant problem associated with many applications that use water worldwide.

Biofilm development on these cooling systems also poses a public health risk due to the proliferation of thermotolerant pathogens, especially *Legionella pneumophila*. Many reports have proposed this strain as the main protagonist of about 90% of the worldwide cases and outbreaks of Legionnaires' disease, a potentially deadly form of pneumonia in developed countries (Walser *et al*., 2014). Another possible, though milder form of outbreak caused by this pathogen is Pontiac fever, presenting with flu-like symptoms (Diederen, 2008). *Legionella* species are facultative Gram-negative bacilli that proliferate in protozoan hosts and subsist in microbial biofilm communities (Wery *et al*., 2008). Studies have shown that the bulk water in cooling towers is a reservoir of highly diverse and species-rich microbial communities that include several *Legionella* spp., and a comprehensive understanding of their persistence is of high importance towards aspects of safety and public health (Pereira *et al*., 2017; Wery *et al*., 2008). Therefore, strategies to control fouling and corrosion are key to controlling the spread of epidemic disease.

2.4 MICROBIAL FOULING CONTROL METHODS

Microbial fouling is inevitable, challenging, and difficult to prevent. Thus, controlling microbial growth should be essential to any comprehensive industrial cooling water treatment programme. Various methods through physical, chemical, and biological processes have been developed to treat biofilm formation on surfaces and/or modify the effects of microbial fouling in general (Bereschenko *et al*., 2011; Li *et al*., 2018; Maria da Gloria & Sarubbo, 2021). These methods, detailed below, might be used individually or in combination to achieve optimal control efficacies. **Figure 2.2** represents different techniques reported in the literature to control microbial fouling.

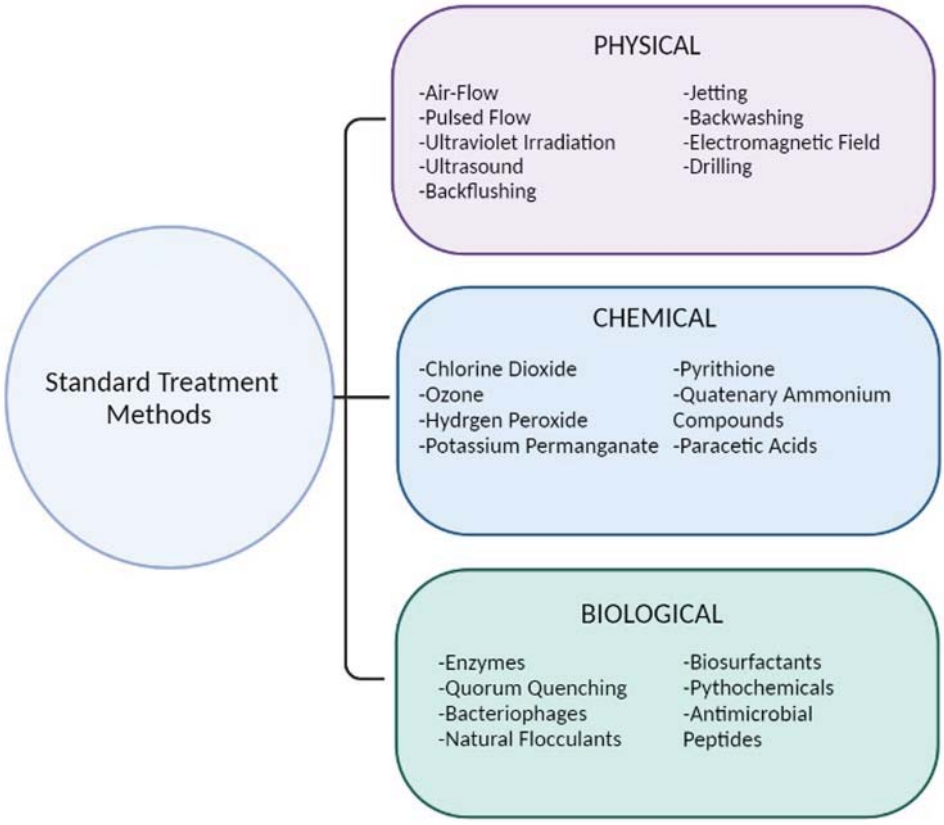


Figure 2.2. A diagrammatic summary of the standard treatment methods available in the literature to control biofouling.

2.4.1 Physical

There are several physical techniques that may be applied to cooling water systems to reduce the incidence of microbial fouling, but many are expensive and impractical. For instance, using heat exchangers to improve biofilm removal is high-energy dependent and requires much capital (Bott, 2009). Other physical techniques used to control microbial fouling in cooling towers include, but are not limited to, the circulation of sponge rubber balls and polymer fibres, use of ultrasound, and inserts (Bott, 2009). However, limitations to these techniques are well-reported and include loss in effectiveness, continuous cleaning, cost and difficulty in equipment transport, accumulation, associated blockage problems in cooling towers, increase in operational costs, and time delays. There have been attempts to mitigate the many effects of physical and chemical control methods. For example, research conducted using a consortium of ultrasound and ozone compounds showed more effectiveness than ozone alone against a *Pseudomonas fluorescens* biofilm (slime former) over a 28 day period (Bott & Tianqing, 2004). In addition, control of biofilm growth was achieved at a reduced concentration of 10 mg/L when combined with a physical agent (insert). This is a significant outcome compared to about 50 mg/L biocide concentration required for biofilm control without an insert (Wills, 2002).

2.4.2 Chemical

The most common method adopted to control biofilm development in cooling water systems is the use of chemical agents, referred to as biocides, which either inhibit or kill microorganisms formed in cooling water biofilms. These agents could be oxidizing (mainly chlorine, calcium hypochlorite, sodium hypochlorite, ozone, hydrogen peroxide, and bromine chloride) and/or nonoxidizing (principally heavy metal compounds, amines, aldehydes, thiocyanates, isothiazolone, and organo-bromine compounds) biocides (Di Pippo *et al*., 2018). Chlorine has been the primary biocide used for treatment in many industries for years. This results from its easy application, cost-effectiveness, availability, and accessibility. However, its carcinogenicity and detrimental effect on water quality discharged into the environment severely limit its consistent use (Di Pippo *et al*., 2018). Ozone is another chemical treatment that has presented a few limitations while being utilized in cooling water systems. Ozone reacts with natural substances to produce low molecular weight oxygenated by-products that further promote biological growth and limit ozone disinfection efficacy (Cloete *et al*., 1998).

2.4.2.1 Effects of surfactants

An extensive literature search has seen the use of synthetic surfactants in industrial treatment regimens, which further irritates the environment (Palmer & Hatley, 2018). Surfactant molecules often scatter uniformly through Brownian movement and unfavourably attach to the surface of substrates, resulting in even higher concentrations and can therefore lead to toxicity issues (Sun *et al*., 2018). The sequence of surfactant toxicity initializes from its very production, discharge, and consequent introduction to the ecosystem. Surfactants utilized in industrial cooling towers are often discharged to wastewater treatment facilities. The subsequent exposure of chemical surfactants to water bodies can create problems if they persist for long periods, prompting the bioaccumulation of possibly unsafe or otherwise toxic substances, and leading to severe environmental issues (Rebello *et al*., 2014). Surfactants are generally synthesised from both petrochemical and chemical materials and can result in solid wastes and atmospheric emissions capable of causing eutrophication of aquatic systems (Stalmans, 1995). Visible manifestations of surfactant toxicity are seen in microbes, aquatic systems, and animals. For instance, diverse biological effects on aquatic organisms occur when surfactants occur at relatively high concentrations (Romanelli *et al*., 2004). Also, the antibiofilm activity of surfactants is not biofilm specific and they incur deleterious effects to various other bacteria, thereby leading to DNA damage and cell lysis (Klebensberger *et al*., 2006; Rebello *et al*., 2014).

2.4.3 Biological

Quorum quenching is a biological mechanism that effectively reduces biofouling by inhibiting cell communication (Millanar-Marfa *et al*., 2020). In some instances, proteolytic enzymes and polysaccharide degrading enzymes such as proteinase K, subtilisin, glucanase, trypsin, and cellulase can destroy EPS and inhibit biofilm formation (Molobela *et al*., 2010; Pei *et al*., 2010). Cell wall hydrolases, such as lysozyme, function by hydrolysing cell walls and macromolecular EPS, causing cell lysis and consequently delaying biofouling by preventing bacterial accumulation on membrane surfaces (Bhagwat *et al*., 2020; Xiong & Liu, 2010).

Bacteriophages have been successfully utilized in water contamination control. For instance, the use of a phage cocktail inhibited the formation of biofilms induced by bacteria in membrane-based wastewater treatment systems (Aydin & Can, 2020). However, there are limitations associated with bacteriophage control methods, such as lack of sufficient information, development of resistance, and the destruction of other valuable bacteria (Cui *et al*., 2021).

The use of biological or natural flocculants such as chitosan, green bioflocculant, marine Arthrobacter cells, algae, and diatomite has also been developed with less adverse environmental impacts (Cui *et al*., 2021). Another group of natural products termed phytochemicals, which are classified as alkaloids, essential oils, phenolics, lectins, terpenoids, polypeptides, and polyacetylenes, possess high anti-biofilm properties (Yong *et al*., 2019). Some of these are also active in gene suppression and hindering bacterial adhesion that is often associated with biofilm formation (Adnan *et al*., 2020).

Antimicrobial peptides represent another promising approach for treating biofilms as they possess broad-spectrum activity in killing both Gram-positive and Gram-negative bacteria and fungi in biofilms (Pletzer *et al*., 2016). Anti-biofilm peptides show strong synergy with conventional antibiotics and act by targeting a universal stringent stress response. Peptides have successfully disrupted biofilms developed on surfaces by ESKAPE (nosocomial pathogens that exhibit multidrug resistance and virulence: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*) and non-ESKAPE pathogens (Rajput & Kumar, 2018). This occurs through electrostatic interaction with cell membrane phospholipids, followed by insertion, thereby leading to bacterial death (Shahrour *et al*., 2019). With regards to using antimicrobial peptides in biofouling control, the focus is now on narrowing the specificity of the activity to target the pathogenic organisms of the biofilm without affecting the normal microflora (Xu *et al*., 2020).

2.5 SURFACTANTS FOR THE CONTROL OF BIOFOULING

Surfactants are commonly used in many applications due to their surface, wetting, penetrating, and solubilizing properties (Simões *et al*., 2005). They are amphiphilic in nature as they possess dual features of hydrophilicity (polar end) and hydrophobicity (non-polar end). Surfactants are often characterized by their chemical structure, hydrophilic-lipophilic balance, charge, chemical structure, and source of origin. These chemical products are mainly effective as surface cleaning compounds, which predominantly act in the removal of biofilm and thus eradicating microbial fouling (Maria da Gloria & Sarubbo, 2021). Thus, the chemical nature of surfactants can alter the submerged and surface properties by enabling the detachment of microorganisms and thus, the inability to form biofilm and adhere to surfaces (Simões *et al*., 2006). The bacteria within biofilms are often well protected from many treatment routines. In contrast, exposure to chemical surfactants is more effective due to probable modifications in biofilm metabolism and it's structural composition (Simões, 2005; Simões *et al*., 2006). The response to the action of such agents is also influenced by environmental factors and intrinsic biological properties that greatly influence biofilm development, characteristics, and behaviour (Purevdorj *et al*., 2002; Simoes *et al*., 2003a; Simoes *et al*., 2003b).

Sulphonates, sulphates and quaternary ammonium compounds (QACs) represent the most used surfactants in industrial systems (Cloete *et al*., 1998). QACs are cationic surfactants with surface-bonding agents that initiate antifouling effects because of their cleaning, deodorization, and antimicrobial properties (Simoes *et al*., 2005). The mechanism of action of QACs revolves around the electrostatic interaction between their positive charge and the negatively charged sites on microbial cells, thereby leading to cell stresses, lysis, and death (McDonnell & Russell, 1999). For instance, the application of cetyltrimethylammonium bromide enhanced the reduction of biofilm cellular respiratory activity (Simoes *et al*., 2005). According to (Sprecher & Getsinger, 2000), a cationic surfactant named Bulab® 6086 Alkyl chloride (C12-16) dimethyl benzyl ammonium is applicable as a microbial fouling control in commercial and industrial recirculating cooling water towers. Also, cationic polymers viz., N-alkyl dimethylbenzyl ammonium chloride, Mexel® 432/0, H-130 Microbiocide, and Slimicide™ C-74 Dodecylguanidine hydrochloride are active microbiocides used to control microorganisms in commercial and industrial water systems (Sprecher & Getsinger, 2000). Anionic surfactants are another group that possess hydrophilic (negatively charged ion) and hydrophobic (positively charged ion) properties, making them dissociate when dissolved in aqueous solvents. Sodium dodecyl sulfate (SDS) is one of the most prominent anionic surfactants used in cleaning products that acts by solubilizing pollutants, forming micelles, and weakening the cohesive force on membrane surfaces (Simões *et al*., 2006). In a study conducted by (Azeredo *et al*., 2003), there was effective removal and detachment of *Pseudomonas fluorescens* biofilms when tested against SDS surfactants. Another group is the non-ionic surfactants which do not ionize in aqueous solution because their hydrophilic group is non-dissociable. They possess low irritation to microbial cells and include compounds such as polyalkylene glycols and poloxamers (Percival *et al*., 2019). Furthermore, biofilms treatment with chemical products and surfactants, including ortho-phthalaldehyde, cetyltrimethylammonium bromide, sodium hypochlorite, and sodium hydroxide, makes biofilm-framework less stable (Simoes *et al*., 2005).

2.6 BIOSURFACTANTS

As alluded to in the previous section, surfactants are synthesised chemically from petroleum products which are not readily biodegradable and are toxic to the environment (Henkel *et al*., 2012). There is therefore a global push for surfactants to be produced from renewable resources (Bhadani *et al*., 2020), and such “green”/“eco” or bio-based technologies are increasingly supported by governmental and industrial agencies (Gallezot, 2012). Biosurfactants are amphiphilic molecules of natural origin (bacteria, fungi, yeasts, plants, or animals) comprised of hydrophobic and hydrophilic ends (De *et al*., 2015; Henkel *et al*., 2012) that exhibit surface and interfacial tensio-active properties. They can offer many advantages over the synthetic counterparts including biodegradability, specificity, biocompatibility, digestibility, use of raw materials, acceptable production economics, usefulness in industrial and environmental biotechnology, and de-emulsification of industrial emulsions (Paraszkiewicz *et al*., 2021). Biosurfactants are sought-after as they possess valuable properties: hydrophilicity and hydrophobicity, emulsification, gelling, spreading, foaming, low critical micellar concentration, wetting, and penetrating activities (Paraszkiewicz *et al*., 2021). Biosurfactants are classified into four main types according to the chemical structure of the hydrophilic head group: (1) glycolipids, (2) fatty acid, (3) lipopeptide, and (4) polymer (Desai and Banat 1997). These classes constitute either low molecular weight compounds which can reduce surface and interfacial tension, or high molecular weight compounds/polymers which can serve as emulsifiers. **Table 2.1** summarises the classification of biosurfactants according to their molecular weight and structural groups, providing well-characterised examples and the main producer strains.

Table 2.1. Different biosurfactant classes, groups, and their respective producer microbial strains

Class	Groups	Biosurfactant within	Producer strains
Low molecular weight	Lipopeptides	Surfactin, iturin, subtilisin, vixcosin, serrawetin, polymyxin, fengycin, gramicidin, arthrofactin	*Bacillus subtilis, Bacillus polymyxia, Serratia marcenscens, Pseudomonas fluorescens, Arthrobacter* sp
	Glycolipids	Mannosylerythritol lipids, rhamnolipids, sophorolipids, xylolipid, cellobiose lipids, trehalose lipids	*Pseudomonas aeruginosa, Candida bombicola, Pseudozyma churashimaensis* sp. nov., *Rhodococcus erythropolis, Lactococcus lactis, Ustilago maydis*
	Lipoamino acids	*N*-acyl tyrosine, *N*-acyl asparagine, ornithine lipid	*Bacillus pumilus, Sinorhizobium meliloti, soil/sediment metagenomes*
	Lipids, fatty acids	Spiculisporic acids, corynomycolic acid, phosphatidylethanolamine	*Penicillium spiculisporum, Corynebacterium lupus, Acinetobacter* sp
	Flavolipids	Flavolipids	*Flavobacterium* sp.
	Neutral lipids	Neutral lipids	*Nocardia erythropolis*
	Phospholipids	Phospholipids	*Thiobacillus thiooxidans*
High molecular weight	Lipoproteins	Lipoproteins	*Pseudomonas gessardii*
	Polymeric	Liposan, alasan, emulsan, biodispersan, mannoprotein	*Acinetobacter radioresistens, Candida lipolytica, Acinetobacter radioresistens, Saccharomyces cerevisiae*
	Particulate	Vessicles, cells	*Acinetobacter* sp., Various bacteria
	Polyol lipids		*Rhodotorula glutinis*
	Polysaccharide-protein complexes		*Pseudomonas fluorescens*
	Lipopolysaccharides		*Klebsiella oxytoca*

Adapted from Disha & Sahasrabudhe (2018); Pacwa-Plociniczak *et al*. (2011); Sobrinho *et al*. (2013)

While numerous biosurfactants have been investigated for anti-biofilm properties, the most studied are glycolipids and lipopeptides. Thus, these two groups, as well as the biosurfactant group of interest for this project (amino lipids) are further detailed below.

2.6.1 Lipopeptides

Lipopeptide is a major biosurfactant group, with structural compositions of the hydrophobic end (C12–C18 fatty acid chain) and hydrophilic chain (peptide sequences of four to ten amino acids). The main producers are *Pseudomonas* or *Bacillus* species; however, yeast and fungi can also synthesize lipopeptides (Ndlovu *et al*., 2017; Vecino *et al*., 2021). The further classification of lipopeptides depends on their amino acid sequence, which could be short, linear, or cyclic structures. Thus, lipopeptides can be divided into several isoforms, such as surfactin, fengycin, iturin, arthrofactin, bacillomycin, plipastatin, viscosin, tensin, syrigomycin, pseudofactin, and so on (Sałek & Euston, 2019). One of the best known lipopeptides is surfactin which comprises a peptide loop of seven different amino acids and a hydrophobic fatty acid chain of 13 to 15 carbon length. Surfactin is of importance due to its interesting biological activities (Sałek & Euston, 2019). The other well-known LPs produced are iturin, fengycin, subtilisin, vixcosin, serrawetin, tensin, pseudofactin, and polymyxin (Sałek & Euston, 2019).

2.6.2 Glycolipids

Glycolipids are the largest group of biosurfactants with its prominent compounds being sophorolipids, rhamnolipids, trehalolipids, and mannosylerythritol lipids (**Table 3.1**). Compared to other BSs, glycolipids have interesting features such as high emulsifying, foaming, dispersing, solubilizing, dispersing, penetrating, and wetting abilities (Kitamoto *et al*., 2009). Their structural composition differs from mono- or disaccharide hydrophilic groups that can successively be joined to long-chain aliphatic- or hydroxyaliphatic acids (Jimoh & Lin, 2019). These structures could include spherical, oblate, and prolate micelles and other molecular assemblies such as cubic, sponge, hexagonal, and lamellar structures (Kitamoto *et al*., 2009). Two of the prominent glycolipids are further discussed below.

2.6.2.1 Sophorolipids

Sophorolipids are complex glycolipids, preferentially produced by the non-pathogenic yeast *Candida bombicola* which can yield over 400 g/L of sophorolipids (Van Bogaert, 2008; Van Bogaert *et al*., 2007). Sophorolipid comprises a hydrophilic sugar head called sophorose (an acetylated glucose disaccharide) and a hydrophobic fatty acid with a carbon chain length of 16 or 18 carbons hydroxylated on the subterminal or terminal carbon. The carboxylic end of sophorolipid could be either lactonized or acidic, while the fatty acid can have a single or multiple unsaturated bonds (Van Bogaert *et al*., 2007).

2.6.2.2 Rhamnolipids

Rhamnolipids were discovered initially as oily glycolipids and are commonly synthesised by *Pseudomonas aeruginosa*. They are structurally diverse compounds of approximately 57 homologues produced by different microbial strains (Abdel-Mawgoud *et al*., 2010). Rhamnolipids comprise one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose units connected by an O-glycosidic bond to a saturated or unsaturated β-hydroxy fatty acid of 8 – 22 carbon chain lengths. In uncommon cases, three β-hydroxy unsaturated fatty chains might be single, double, or triple bonded (Abdel-Mawgoud *et al*., 2010). Rhamnolipids have been shown to reduce the surface tension of water as low as 24 mN/m and possess superior emulsification abilities (Benincasa *et al*., 2004; Lovaglio *et al*., 2011). Other members of the low-molecular-weight biosurfactants include mannosylerythritol, trehalose lipids, succinoyl-trehalose lipid, cellobiose lipid, oligosaccharide lipids, and xylolipids (Kitamoto *et al*., 2009).

2.6.3 Amino lipids

Amino lipids are surfactant molecules of polar amino acids (hydrophilic moieties) and long-chain hydrophobic compounds that have built an amphipathic structure capable of producing surface activity (Infante *et al*., 2004). They possess extensive structural diversity and different physicochemical and biological properties. This is due to variation in the structure, length and number of fatty acid chains and their amino acid/peptide structures (Infante *et al*., 1997; Takehara, 1989; Xia, 2001). The distinctions in aggregation, adsorption and biological activity amongst different amino acid surfactants are majorly determined by amino acid or peptide moiety. Thus, three main structures, namely linear or single chain, dimeric and glycerolipid-like forms, can arise from the combination of amino acids and long aliphatic chains (Infante *et al*., 2004). Amino acid surfactants are classified as biosurfactants as they are made based on biomimetics – raw materials that mimic the chemical compounds of natural origin (Pinheiro & Faustino, 2017). A number of amino acid-based surfactants are established in the market, but the volume of such surfactants produced is still relatively small (Bordes and Holmberg, 2015). The lyso-ornithine lipids (LOL) and ornithine lipids (OL) are the surfactants of interest for this project, and their properties will be explained in the succeeding section.

2.6.3.1 Properties of lyso-ornithine and ornithine lipids as biosurfactants

Lyso-ornithine lipids and OL are amphiphilic molecules comprising polar and non-polar ends. These compounds are often classified as amino acid surfactants due to the presence of an amino acid (ornithine) and lipids (long-chain hydrophobic compounds). As shown in **Table 2.2**, LOL and OL biosurfactants compete well against other surface-active agents. Thus, they demonstrate performance properties that make them suitable for a variety of applications in personal care, cosmetics, detergent, water management, antimicrobials, medical, and bioremediation fields (Desai & Banat, 1997a; Maneerat *et al*., 2006; Williams *et al*., 2019).

LOLs, precursors of OLs, are the most common aminolipids and are typically located in bacterial membranes. Two novel isobranched LOLs, consisting of 3-hydroxy fatty acids (iso-C15:0 and iso-C16:0) joined to the ornithine α-amino group through an amide bond, were recently characterised (Kristoffersen *et al*., 2021). OLs are phosphorus-free membrane lipids that are found predominantly in eubacteria, with the ornithine joined by an amide bond to the 3-hydroxy fatty acyl group, which is further ester-linked to a second fatty acyl group. The capacity to synthesize OLs is found in about 25% of the bacterial species whose genomes have been sequenced (Vences-Guzman *et al*., 2012). Different genes that encode OL hydroxylases are known and can be hydroxylated as ornithine moiety, amide-linked fatty acid, and ester-linked fatty acid (Gonzalez-Silva *et al*., 2011; Rojas-Jimenez *et al*., 2005; Vences-Guzman *et al*., 2011). The genes that code for acyltransferase activities needed for OL biosynthesis (*olsB* and *olsA*) were first reported in *Sinorhizobium meliloti* (Gao *et al*., 2004; Weissenmayer *et al*., 2002).

The biosynthesis of the OL occurs in two different stages. For the first stage, catalysed by OlsB, LOL is formed by a 3-hydroxy fatty acyl group transfer from a 3-hydroxy fatty acyl-acyl carrier protein (ACP) to the α-amino group (Gao *et al*., 2004). In the second stage, catalysed by OlsA, OL is formed by an acyl group transfer from the acyl-acyl carrier protein to the 3-hydroxyl group of the LOL (Weissenmayer *et al*., 2002). Several studies have connected the increased synthesis of OLs to phosphate-limiting conditions. It was proposed that when there are low concentrations of phosphate, ornithine, amongst others, is used as replacement for phosphate on the lipids' polar end (Devers *et al*., 2011; Lejeune *et al*., 2021; Vences-Guzmán *et al*., 2013; Vences-Guzmán *et al*., 2015). The differences in gene organization amongst different genomic features might specify the modifications in the regulation of gene expression. This is shown when the induction of *olsB* expression is proportional to a limiting phosphate condition and *olsA* expression in *Sinorhizobium meliloti* (Gao *et al*., 2004), whereas phosphate limitation further increases *olsA* induction in *Pseudomonas aeruginosa* (Lewenza *et al*., 2011). Additionally, the frequency of OL hydroxylation seems to correlate in some cases with abiotic stress conditions such as the increase in temperatures and acidic conditions (Gonzalez-Silva *et al*., 2011; Vences-Guzman *et al*., 2011).

Table 2.2. Comparative analysis of the properties of biosurfactant groups to lyso-ornithine and ornithine lipids (Jimoh *et al.*, 2023)

Biosurfactant	Surface tension (mN/m)	Critical micelle concentration (mg/L)	Emulsification index (%)	References
Lyso-ornithine lipid (lipoamino acid)	36	86	78.3 (paraffin)	Williams *et al*., 2019
Ornithine lipid (lipoamino acid)	ND	40	Emulsification of weathered crude oil	Maneerat *et al*., 2006
Rhamnolipids (Glycolipid)	29	420	Emulsify kerosene with an emulsion index up to 40%	Radzuan *et al*., 2017
Trehalose lipids (Glycolipid)	29	250	46% emulsification against sunflower oil	White *et al*., 2013
Xylolipids (Glycolipid)	39.5	2.5	ND	Sharma *et al*., 2014
Putisolvin I and II (Lipopeptide)	30	ND	Emulsification was positive against Toluene	Kuiper *et al*., 2004
Flavolipid	26	300	100% against 10% (vol/vol) Pennzoil oil	Bodour *et al*., 2004
Lipoprotein	28	800	100% (waste motor oil)	Santos *et al*., 2018
Phosphatidylethanolamines (Phospholipid)	29	30	ND	Kretschner *et al*., 1982
Surfactin (Lipopeptide)	29.5	70µM	67.6% emulsification against crude oil	de Faria *et al*., 2011
Glycoprotein	42.5	7.5	60% emulsification against *n*-hexadecane	Gudiña *et al*., 2015

ND: Not Determined

Comparatively few studies are available describing the activity and biological properties of ornithine lipids. In a study by (Lohan *et al*., 2013), a range of OLs were produced between one and five ornithine groups, amide linked to fatty acids of C8-18 chain length. There was no antibacterial activity from the synthetic OLs, which contained one ornithine molecule, similar to that of LOLs (Lohan *et al*., 2013). It is important that antimicrobial action intensifies in the compounds with unsaturated fatty acid chain lengths in the range of C12 and 18 joined with two to five ornithine molecules (Lohan *et al*., 2013). In a recent study, two iso-branched LOLs, iso-C15:0 and iso-C16:0, were tested and showed no bioactivity against Gram-negative bacteria. However, there was antibacterial activity against Gram-positive *Streptococcus agalactiae* by the iso-C15:0 LOL. On the other hand, cytotoxicity profiling assays revealed that iso-C16:0 LOL was not active against MRC-5 cells but showed cytotoxic activity toward human melanoma cells (Kristoffersen *et al*., 2021). In another study, very high concentration of unbranched LOL compounds were required to show effect against Gram-negative bacteria, proving their low activity (Tahara *et al*., 1977). From these few studies, one gets the sense that OLs may play numerous roles in different organisms, and their application should be evaluated on a case-by-case basis.

2.7 BIOSURFACTANTS AS ANTI-BIOFILM, -FOULING AND -CORROSSION AGENTS

Biosurfactants harbour a range of properties that are suitable as anti-microbial, -biofilm, -adhesive, and -corrosion activities (**Figure 2.3, Table 2.3**) (Mishra *et al*., 2020). Biosurfactants have been found to inhibit the attachment of pathogenic organisms to solid surfaces due to prior absorption, which represents an effective means of combating colonization. The pathogenic *Listeria monocytogenes* LO28 was inhibited from attaching to polytetrafluoroethylene and stainless-steel surfaces by a synthesised biosurfactant from *Pseudomonas fluorescens* (Meylheuc *et al*., 2001). Similarly, (Diaz De Rienzo *et al*., 2015) reported that sophorolipids are efficient bactericidal agents which can target planktonic cell death. The sophorolipid disrupted the biofilm formation at a concentration of > 5% (v/v) in comparison to the conventional antimicrobial agents (Diaz De Rienzo *et al*., 2015). Anti-adhesion activity against biofilms by lipopeptides synthesised by *Bacillus subtilis* and *Bacillus licheniformis* has also been reported (Rivardo *et al*., 2009). Biosurfactants are also involved in the detachment and dispersion of biofilms by binding to the cell surface or its components, therefore altering the outer membrane's hydrophobicity (Kuiper *et al*., 2004; Neu, 1996). This mechanism has been displayed in surfactin's role in inhibiting *Salmonella enterica* from adhering to polyvinyl chloride and promoted biofilm dispersal rather than inhibition of the microbial growth (Mireles *et al*., 2001). Additionally, *Bacillus amyloliquefaciens* AR2 lipopeptide inhibited *Candida albicans* biofilms by affecting the cell surface hydrophobicity. The anti-adhesive and anti-biofilm capabilities of cell-bound biosurfactant synthesised by *Lactobacillus rhamnosus* and *Lactobacillus jensenii* against clinical strains of the ESKAPE pathogens (*Acinetobacter baumannii*, *Escherichia coli*, and *Staphylococcus aureus*) have also been demonstrated (Sambanthamoorthy *et al*., 2014). Another intriguing report from (Dusane *et al*., 2012) showed that rhamnolipid compounds display interruption movement of biofilms formed by the dimorphic ascomycete *Yarrowia lipolytica*.

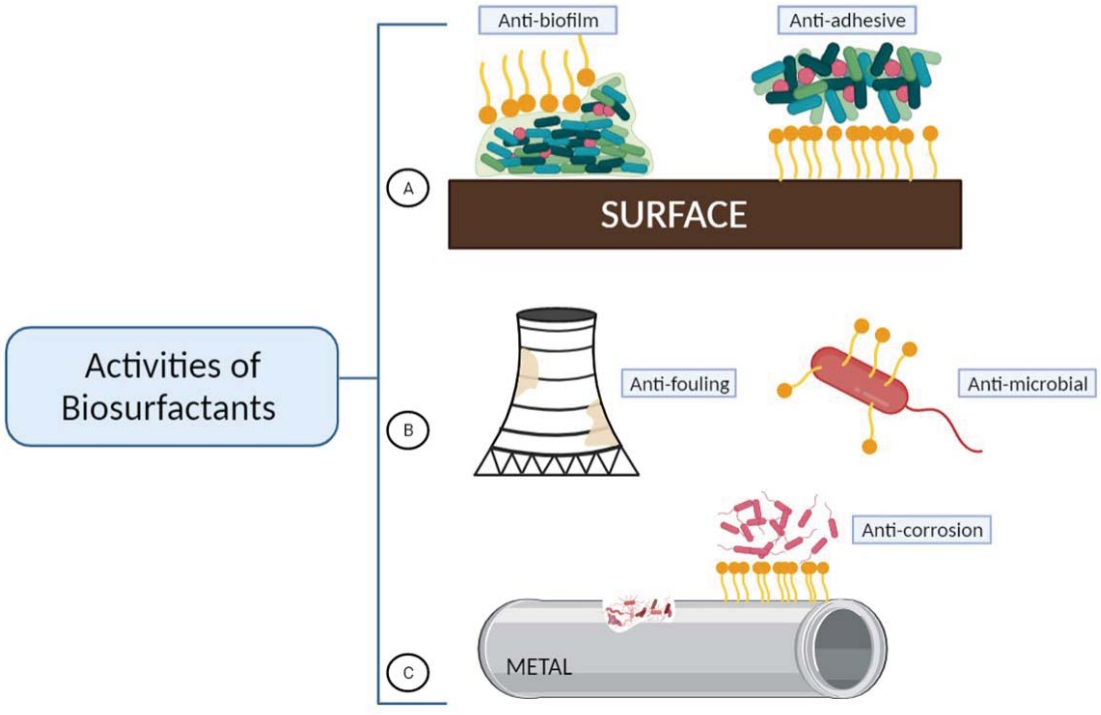


Figure 2.3. The roles played by biosurfactants in areas where fouling and corrosion commonly occur. (A) Anti-biofilm: Biosurfactants can prevent biofilm formation and further disrupt established biofilms. (B) Anti-adhesive: Biosurfactants can reduce contact or binding between bacteria and other microorganisms to abiotic surfaces. (C) Anti-fouling: Biosurfactants can prevent the accumulation of unwanted materials created by biofilm-fouled solid surfaces. (D) Anti-corrosion: Biosurfactants can prevent accelerated deterioration of metallic structures and other materials. (Taken from Jimoh *et al*., 2023).

Biosurfactants inhibit biofilm formation by modifying surface physicochemical properties, altering cell adhesion ability, membrane disruption, or inhibiting the electron transport chain, thus limiting energy demands. Other mechanisms that influence the formation of biofilms could include distorted protein structure, altered cell permeability, and interference in quorum sensing (Satpute *et al*., 2016). Biosurfactants also affect the biofilm network/framework by forming micelles within its centre (Paraszkiewicz *et al*., 2021). These micelles allow surfactants to improve the solubility and bioavailability of pollutants by decreasing the interfacial and surface tension (Varjani & Upasani, 2017). Several studies have reported that prior biosurfactant adsorption on surfaces (surface conditioning) efficiently reduced microbial cell adhesion and surface colonization (Araujo *et al*., 2016; Meylheuc *et al*., 2006; Zezzi do Valle Gomes & Nitschke, 2012).

There are adverse ecological effects generated over the years with the utilization of toxic substances in formulating antifouling mixtures used to prevent biofouling on submerged synthetic structures. Thus, innovative options with low environmental effects are desperately needed (Fay *et al*., 2013). As of late, biosurfactant research has expanded, with a particular focus on their effectiveness as biofilm control agents (Di Pippo *et al*., 2018). In this context, a study evaluated the properties of biosurfactants in cell-free supernatant towards antifouling in laboratory facilities and field trials (activity in a paint matrix) (Aleman-Vega *et al*., 2020). The study concluded that the *Bacillus niabensis* My-30 biosurfactant led to promising antifouling action by inhibiting the growth and adhesion of bacteria involved in the biofouling process compared to a synthetic agent (10% SDS). This hindered biofilm formation (up 50%) against all Gram-positive bacteria and most Gram-negative bacteria used in the experimental set-ups. Furthermore, this biosurfactant led to 30% less biofouling compared to the control in the field trials. There are other studies, though very limited, that have evaluated the effectiveness of biosurfactants as biofouling agents. These include the involvement of a rhamnolipid in cell adhesion and biofilms development (Nickzad & Déziel, 2014) and the antibiofilm activity of biosurfactants synthesised by coral-associated bacteria (*Bacillus anthracis* and *Psychrobacter* sp.) (Padmavathi & Pandian, 2014). Similarly, biosurfactant synthesised by the *Bacillus* strain AR2 maintained its antibiofilm activity at extreme conditions by dispersing 25 – 100% of *Candida* strains' preformed biofilms on polystyrene plates. Also, at varying concentrations of *Candida* strains inoculated, the biosurfactant inhibited biofilm formation within the range of 46 – 100% efficiency rate (Rautela *et al*., 2014).

There is also a case of microbially influenced corrosion (MIC) caused by microorganisms adhering to surfaces/interfaces. MIC is a significant phenomenon, increasingly acknowledged in the last decade as numerous metals, nickel-, and aluminium-based alloys are part of the steel used in structures such as industrial water-cooling circuits, water-cooling towers, vacuum pumps, and other related equipment such as submerged slight glass and sensors. Exposure to these substances can lead to biocorrosion through an electrochemical reaction between the interfaces influenced by microbes, water, carbon, and energy sources (Beech *et al*., 2000). Microbes are the first point of colonization for inanimate surfaces that coexist and form naturally occurring biofilms and complex consortia on corroding metal surfaces (Beech & Sunner, 2004). Therefore, combating MIC using surfactant is increasingly important. Surfactants inhibit metal corrosion due to their adsorption to metal surfaces and micelles aggregation (Malik *et al*., 2011). Several successful examples will be discussed briefly and are summarised in **Table 2.4**. The biosurfactant alkylpolyglucoside could inhibit the corrosion of 907 steel in static seawater, and varying inhibition efficiencies could be obtained by varying the length of the alkyl chain (Du *et al*., 2004). A biosurfactant complex containing rhamnolipids and an alginate-based biopolymer produced extracellularly by *Pseudomonas* sp. PS-17 was shown to successfully inhibit the corrosion of aluminium alloy D16T submerged in synthetic acid rainwater. This alloy is widely used in aerospace, transportation, and in building construction industries. Furthermore, a two- to four-fold increase in the repassivation kinetics was observed for the surfaces treated with the biosurfactant complex, and these effects were attributed mainly to the rhamnolipids in the complex (Zin *et al*., 2018).

Table 2.3. Different properties shown by different biosurfactant groups in the application areas relative to bioactivity against fouling

Producer	Biosurfactant class	Dosage	Biofilm-producing strains	Properties of interest	Reference
Bacillus licheniformis VS16, *Bacillus subtilis* VSG4	Lipopeptide	5 mg/mL	*Staphylococcus aureus* ATCC 29523, *Salmonella typhimurium* ATCC 19430, and *Bacillus cereus* ATCC 11778	There was 63.9 to 80.03% dispersal effect for VSG4 BS, and 61.1-68.4% for VS16 BS	Giri *et al*., 2019
Pandorea pnomenusa MS5	Exopolysaccharides	0.25 mg/mL	*Burkholderia cepacia*	*Burkholderia cepacia* biofilm was inhibited	Sacco *et al*., 2019
Candida bombicola ATCC22214	Sophorolipids	5% (v/v)	*S. aureus* ATCC 9144, *Bacillus subtilis* BBK006	Disrupt biofilms at concentrations more than 5% (v/v) by inducing the death of planktonic cells	Diaz De Rienzo *et al*., 2015
Bacillus circulans	Lipopeptide	10 g/L	*Escherichia coli, Micrococcus flavus, Serratia marcescens, Salmonella typhimurium, Proteus vulgaris, Citrobacter freundii, Alcaligenes faecalis, Klebsiella aerogenes*	Biofilm dislodging of 59 to 94% was achieved for the tested strains	Das *et al*., 2009
Burkholderia thailandensis E264	Rhamnolipids	0.39 - 12.5 mg/mL	*Streptococcus oralis, Actinomyces naeslundii, Neisseria mucosa, Streptococcus sanguinis*	50 to 90% inhibition of biofilms formed	Elshikh *et al*., 2017
Halomonas sp. (BOB-3)	Rhamnolipid	125 µg/mL	*Vibrio cholerae, Salmonella typhi*)	Anti-biofilm property with inhibition of 99.5% (*Vibrio cholerae*) and 99.8% (*Salmonella typhi*)	Kayanadath *et al*., 2019
Bacillus subtilis *Pseudomonas aeruginosa*	Surfactin Rhamnolipids	0.1% and 0.5% (w/v) concentration 0.25% and 1.0% (w/v) concentration	*Listeria monocytogenes, Staphylococcus aureus, Salmonella enteritidi*	Anti-adhesive and antibiofilm effects	Zezzi do Valle Gomes & Nitschke, 2012

Serratia marcescens GQ214001	Glycolipid	0.0125 – 25 mg/mL	*Candida albicans, Pseudomonas aeruginosa and Bacillus pumilus*	There was potent anti-adhesive and antibiofilm activity for the biofouling microbial strains tested.	Dusane *et al*., 2011
Lactobacillus pentosus, Lactobacillus paracasei	Glycolipopeptide	0.02 to 25 mg/mL	*Pseudomonas aeruginosa, Streptococcus agalactiae, Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Candida albicans*	There were significant anti-adhesive properties against all the microorganisms except for *E. coli* and *C. albicans*. (less than 30%).	Vecino *et al*., 2018
Acinetobacter indicus M6	Glycolipoprotein	500 µg/mL	MRSA	There was 82.5% biofilm removal form the surface	Peele Karlapudi *et al*., 2018
Bacillus safensis F4	Surfactin	6.25 mg/mL	*Staphylococcus epidermidis*	There was 80% anti-adhesive activity against the tested strain	Abdelli *et al*., 2019
Candida lipolytica UCP 0988	Rufisan	0.75 to 12 mg/L	*Streptococcus agalactiae, Streptococcus mutans, Streptococcus mutans* NS*, Streptococcus mutans* HG*, Streptococcus sanguis* 12, *Streptococcus oralis* J22	The BS showed anti-adhesive activity against most of the microorganisms tested	Rufino *et al*., 2011
Bacillus subtilis #309	Surfactin-C15	960 µg/mL	*Candida albicans*	Inhibition of approximately 85% of the biofilm formed	Janek *et al*., 2020
Lactobacillus agilis CCUG 31450	Glycoprotein	960 mg/L	*Staphylococcus aureus*	There was anti-adhesive activity against *Staphylococcus aureus*	Gudiña *et al*., 2015

Since microbial induced corrosion is a consequence of biofilm formation, all biofilm preventions will be effective towards preventing heavy microbial contamination of metallic materials and the development of biofouling, particularly when the surfaces are preconditioned with biosurfactant (Grasland *et al*., 2003; Silva *et al*., 2018). *Bacillus* species are particularly well endowed with biosurfactant producing capacity (**Table 2.1**) and the following examples demonstrate what an important source this genus could be towards combating biofilm-induced corrosion (Purwasena *et al*., 2019). Certain *Bacillus* spp. produced a variation of antimicrobial surfactants that inhibited the growth of different bacteria that induced metal surface corrosion (Jayaraman *et al*., 1999). The antibacterial peptides were shown to disrupt the outer and the cytoplasmic membranes as the mechanism of action. The lipopeptide from the endo-rhizospheric bacterium *Bacillus tequilensis* SDS21 showed bactericidal and biofilm dislodging activity against planktonic and sessile cells, removing more than 99% of bacterial biofilm on the surface of glass, polystyrene, and stainless steel. This biosurfactant was still viable when exposed to extreme conditions (high temperature, extreme pH, and in hard water containing Mg^{2+} and Ca^{2+} ions) and is therefore suitable as a disinfectant or in disinfectant-like formulations (Singh & Sharma, 2020). In another study, the cellular layer of two biofilm forming strains, *Pseudomonas aeruginosa* PAO1 and *Bacillus cereus* 1A06374, was disrupted by *Bacillus amyloliquefaciens* anti-CA lipopeptide. Moreover, the lipopeptide also killed the larvae of *Balanus amphitrite* and inhibited the germination of *Laminaria japonica* spore and growth of protozoa, all of which are fouling organisms in marine environments, therefore showing it's practical use against a range of biological agents (Song *et al*., 2016). Finally, in the oil and gas industry, where 30–40 % of the corrosion problems are attributed to microbial corrosion, the *Pseudomonas stutzeri* F01 biosurfactant has been proposed to be a an eco-friendly microbial inhibitor (biocide) to prevent the corrosion of carbon steel (API 5LX) (Parthipan *et al*., 2018). The authors confirmed that the BS compounds possess antibacterial properties against corrosive bacterial strains even at low concentration.

Table 2.4. Examples of biosurfactants used against biocorrosion

Producer strain	Class	Activity	References
Pseudomonas fluorescens	Biosurfactant	Inhibiting the corrosion of AISI 304 stainless steel.	Dagbert *et al*., 2006
Pseudomonas sp. PS-17	Rhamnolipid	Inhibiting the corrosion of alloy	Zin *et al*., 2018
Pseudomonas mosselii F01	Biosurfactant	Significant inhibition activity against corroding carbon steel (API 5LX) corrosive bacterial strains	Parthipan *et al*., 2018
Pseudoxanthomonas sp. F3	Rhamnolipid	Eliminate biofilms associated with biocorrosion	Astuti *et al*., 2018
Bacillus sp	Biosurfactant	Microbial-influenced corrosion on carbon steel ST37 was inhibited	Purwasena *et al*., 2019
Bacillus sp. H2O-1	Surfactin	Control of sulfate-reducing bacteria on examined surfaces such as carbon steel, stainless steel AISI 304;430, polystyrene, and galvanized steel	Korenblum *et al*., 2012
Pseudomonas sp. PS-17	Rhamnolipids biocomplex	The surface-active products were able to inhibit the corrosion of D16T aluminum alloy in distilled water	Pokhmurs'kyi *et al*., 2014

2.7.1 Shortcomings

Despite the many examples that point to biosurfactants as effective and eco-friendly antibiofilm agents, the hindrances associated with the use of these compounds should also be noted. These are outlined as areas of focused research that are required to bring these compounds to market and able to compete against their chemically synthesised counterparts (Olasanmi & Thring, 2018; Sobrinho *et al*., 2013).

- The large-scale production of biosurfactants is expensive. However, this problem may be overcome through the combination of cheap and renewable substrates.
- Complex culture medium and screening hurdles.
- Acquiring biosurfactants with high purity is difficult due to complex purification steps. Extraction and purification of biosurfactants involves using huge volumes of various expensive and hazardous organic solvents.
- Low production yields of biosurfactants. This problem can be solved through the development and engineering of hyper-producing microbial strains and also the optimization of fermentation conditions.
- The biosurfactant productivity is often diminished by high foam formation during large-scale fermentations, e.g., industrial bioreactors, semi-scale fermenters, and large volume flasks. In some instances, where the biosurfactants are trapped within these foams, this often requires extraction with a diluted medium that directly reduces biosurfactant concentration. In other cases, anti-foaming agents are applied which further contribute to the high production costs.
- Analysing the structural composition of biosurfactants requires a sequence of physio-chemical screening and the use of chromatographic and spectrometric instrumentations. These facilities are often expensive to acquire and maintain and are also not readily accessible to biosurfactant researchers.
- The commercial application of biosurfactants often undergoes rigorous processes as different organizations set out standard procedures to confirm its safety and toxicity profile before the introduction to high-end consumers.

2.8 CONCLUSION

There are many uses of biosurfactant molecules that are well explored in literature. However, studies in water management, specifically in areas of combating biofouling and biocorrosion, are scarce. Even from the limited studies conducted, biosurfactants have proven to be effective in industrial water systems and can be used simultaneously to protect surfaces as well as reduce the effect of microbially induced corrosion or fouling. By co-formulating biocides and biosurfactants one can also significantly increase the bioactivity of the biocide, ultimately decreasing the high concentrations of biocide required. Thus, co-formulation with biosurfactants represents a more ecological, cost-effective, and renewable solution with diminished impact when water is released into the environment. Given the number of novel biosurfactants yet to be described, there is enormous scope to introduce new compounds and methodologies to improve management programs for industrial wastewaters.

CHAPTER 3: PRODUCTION AND OPTIMISATION OF LYSO-ORNITHINE LIPIDS FROM *E. COLI* "B" STRAIN

3.1 INTRODUCTION

The novel, microbially produced lyso-ornithine lipid (LOL) biosurfactant, derived by the functional metagenomic screening of a South African aquatic environment has been patented by the Institute of Microbial Biotechnology and Metagenomics (Williams *et al*., 2019). The synthesis of C14 to C19 fatty acid chain length LOL has been observed through heterologous expression in *Escherichia coli*. These compounds possess properties suitable for application as emulsifiers such as maximum reduction of surface tension, good emulsification (EC24) index together with a hydrophilic-hydrophobic balance (HLB) of 6.4 (Williams *et al*., 2019). These properties allow scope for the investigation of LOL for enhanced reduction of microbial adhesion to surfaces exposed to various effluent waters. Based on these performance parameters, it can be expected--with a reasonable level of confidence--that the LOL biosurfactants will reduce microbial adhesion. Therefore, this offers the opportunity to investigate different biosurfactant mixtures for enhanced reduction of microbial adhesion and tailored towards different surfaces and water conditions. This is premised on studies showing that the maximal repulsive force is significantly higher for surfactant mixtures as compared with a single surfactant, owing to denser packing of micelles (Rabinovich *et al*., 2006).

This chapter aimed to further improve the yield of the LOL production through the heterologous expression of the L-ornithine N(alpha)-acyltransferase (OlsB) in *E. coli*. Two of the main strategies to achieve this goal were to manipulate growth conditions to favour biosurfactant production and to genetically engineer the bacterial strain to produce higher amounts of LOL. Both strategies were employed resulting in the "B" overproducing strain. We also developed a LOL purification strategy and a HPLC-based quantification method.

3.2 MATERIALS AND METHODS

3.2.1 Materials

All chemicals used in this study were supplied by Merck Chemicals and laboratory supplies (Darmstadt, Germany), Sigma Aldrich Chemical Company (Deissenhofen, Germany) and Lasec (Cape Town, South Africa).

3.2.2 Standard culturing and optimization of *E. coli* "B" strain for lyso-ornithine lipids production

The *Escherichia coli* BL21 (DE3) "B" strain (expressing *olsB* and *acpP*) was obtained from the culture collection at the Institute of Microbial Biotechnology and Metagenomics (IMBM), University of Western Cape, South Africa (Venter, 2022). The *E. coli* "B" strain was maintained on Luria–Bertani (LB) containing streptomycin (50 µg/mL) and ampicillin (100 µg/mL) for routine culturing. For biosurfactant production, the strain was cultured in mineral salt medium (referred to as mineral medium hereon) containing 42.26 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 18.69 mM NH_4Cl (pH 7.4), 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 22.2 mM glucose and the corresponding antibiotics.

To evaluate LOL yields, an inoculum was prepared by inoculating a single colony of the “B” strain into 20 mL LB broth supplemented with streptomycin (50 µg/mL) and ampicillin (100 µg/mL). The culture was incubated overnight (30^{o}C, 120 rpm), and 500 µL was inoculated into minimal media (100 mL) containing the respective antibiotics. The cultures were further incubated for six hours (30^{o}C, 120 rpm), after which expression was induced with 1 mM IPTG and supplemented with 20 mM L-ornithine. The cultures were further incubated for 72 hrs (30 ^{o}C, 120 rpm) for LOL production. For optimization, the following culturing conditions were varied: incubation temperature (25, 30, and 37°C), medium pH (pH 6, 7, and 8), L-ornithine supplementation (2, 20, and 200 mM), or IPTG concentration (0.1, 1, and 10 mM). At the end of the incubation periods the bacterial cells were harvested through centrifugation (4^{o}C, 11 000 x g for 30 min). The supernatant was recovered and passed through a 0.2 µm filter for subsequent testing. All evaluations were conducted in triplicate.

3.2.3 High-performance liquid chromatography analysis of culture supernatants for lyso-ornithine lipid production

A Thermo Fisher Scientific's Dionex UltiMate 3000 High-performance liquid chromatography (HPLC) fitted with a Luna 5 µm C18 liquid chromatography column (Phenomenex 250 mm x 4.6 mm, Torrance, USA) was used to quantify LOL production. To prepare the sample for HPLC analysis, 2 mL of the supernatant was clarified through centrifugation at 11 000 x g for 30 min and then passed through a 0.2 µm syringe filter. The clarified supernatant was transferred to 2 mL glass vials and 200 µL of sample was injected. The flow rate was 1 mL/min, and the following gradient and elution times were used: 40% B, 0 min; 40% B, 5 min; 70% B, 20 min; 95% B, 20.1 min; 95% B, 26 min; 40% B, 26.1 min; 40% B, 32 min; A: doubly deionized water; B: acetonitrile (Sigma, Saint Louis, USA). The column's temperature was maintained at 40°C.

3.2.4 Purification of the lyso-ornithine lipids

LOL was prepared from 8 × 3 L baffled flask cultures at the optimized parameters (25^{o}C, 1 mM IPTG, pH 7, 20 mM L-ornithine). The purification workflow is outlined in **Figure 4.1**. The culture supernatants (described earlier) were stored at -80 °C overnight followed by freeze-drying using a VirTis BenchTop Pro freeze dryer (SP Scientific, Warminster, Pennsylvania) until dry. The material was dissolved in as small a volume of sterile deionized water as possible and 0.2 µM filtered. The concentrate obtained was used to perform crude purification of the four dominant LOLs (C14-C18) produced as one fraction. A multi-step gradient using the Flash Pure Buchi EcoFlex C18 column (50 µm spherical, 12 g) was used to process 20 mL injections for 2 minutes with a flow rate of 10 mL/min to allow for the liquid sample to adhere to the beads. Thereafter, equilibration was done at 0.1 mL/min for 5 min and elution achieved at 30 mL/min for 4 min. Fractions were collected over a period of 10 min with a linear gradient of B (acetonitrile) from 0-95% at 10 mL/min. A decrease in flow rate allowed for constant separation and binding. The column was washed with 95% acetonitrile (isocratic), pushing 30 mL/min for 1 column volume (24 mL) for 1 min followed by equilibration for 5 min at 1 mL/min in 95% acetonitrile (B) to ensure all residual LOL compounds were washed off the column. The major LOL compounds C14:0, C16:1, C16:0, and C18:0 was fractionated and pooled together, and the acetonitrile was evaporated using a rotary evaporator. The sample was frozen overnight at -80^{o}C and freeze dried. The mass obtained was measured on an analytical balance and the purified materials were made available for subsequent applications.

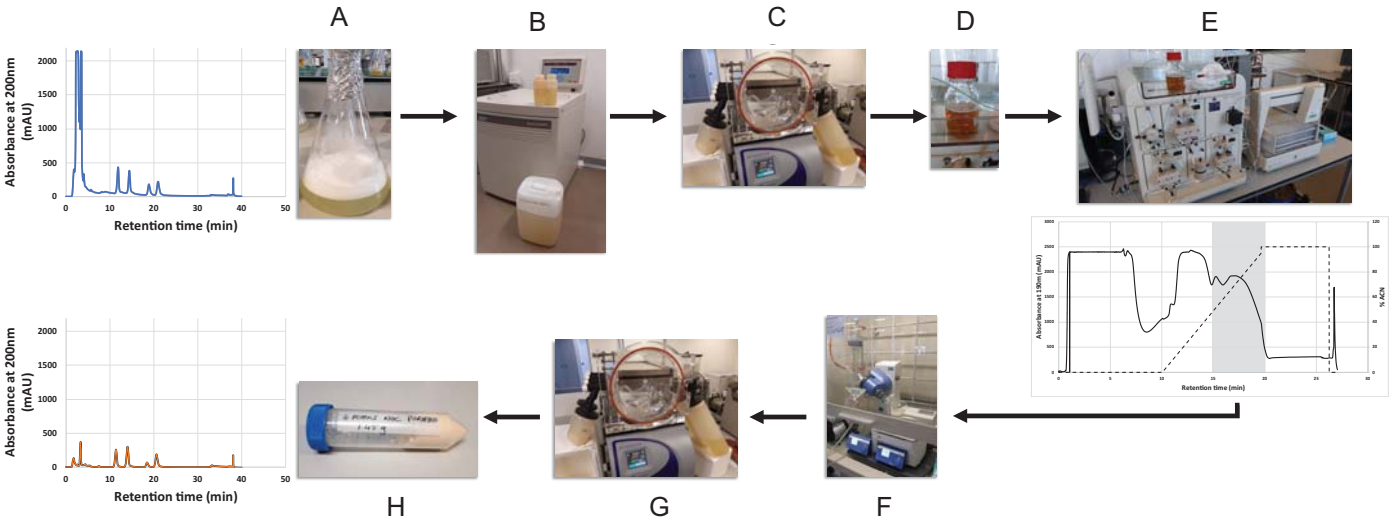


Figure 3.1 Workflow to purify the four combined peaks of lyso-ornithine lipids. (A) The "B"-strain is cultured in M9 minimal medium to produce LOLs. (B) The medium is clarified through centrifugation. (C) The clarified supernatant is concentrated by freeze drying. (D) The dried supernatant is resuspended in a minimal volume of water to produce a concentrate for fractionation. (E) The concentrate is fractionated using a Bio-Rad NGC chromatography system. (F) Acetonitrile is removed from the fractions obtained. (G) The remaining aqueous solution is freeze-dried. (H) The freeze-dried fraction is collected. The chromatogram shown next to the flask at step Ashows the contaminating water-soluble compounds in the supernatant that elute early (0-10 min). The chromatogram below the image of the Bio-Rad NGC at step E shows the fractions that are collected at this step (grey shaded area). The chromatogram next to the tube in step h shows that the contamination has been markedly reduced relative to the four LOLs.

3.3 RESULTS AND DISCUSSION

3.3.1 The identification of four major lyso-ornithine congeners by the *E. coli* "B" strain

The clarified culture supernatant of the "B" strain (expressing *olsB* and *acpP*) was analyzed for the presence of LOL using HPLC. Four dominant species of LOL (C14:0, C16:0, C16:1, C18:0) were produced, as shown in a representative chromatogram (**Figure 3.2**).

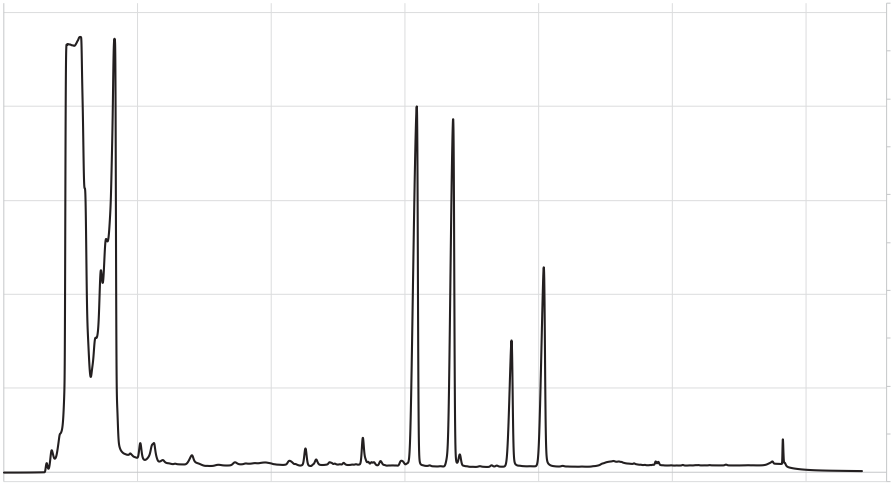


Figure 3.2. Representative HPLC chromatogram showing the four main LOL species produced by the *E. coli* "B" strain.

3.3.2 Culture optimization for lyso-ornithine lipids production

3.3.2.1 Growth Temperature

The first optimization parameter was temperature, where lower temperatures resulted in increased yields of LOL (**Figure 3.3**). At 25°C a 1.5-fold increase was obtained when considering the sum peak area of the four LOL species compared with expression at 37°C, coupled with an increase in two of the chain lengths (C16:1 and C18:1) (**Figure 3.3**). The increase in LOL production can be on account of increased expression and folding of the OlsB and AcpP at lower temperatures, leading to an increase in the active portion of the protein, resulting in more LOL production.

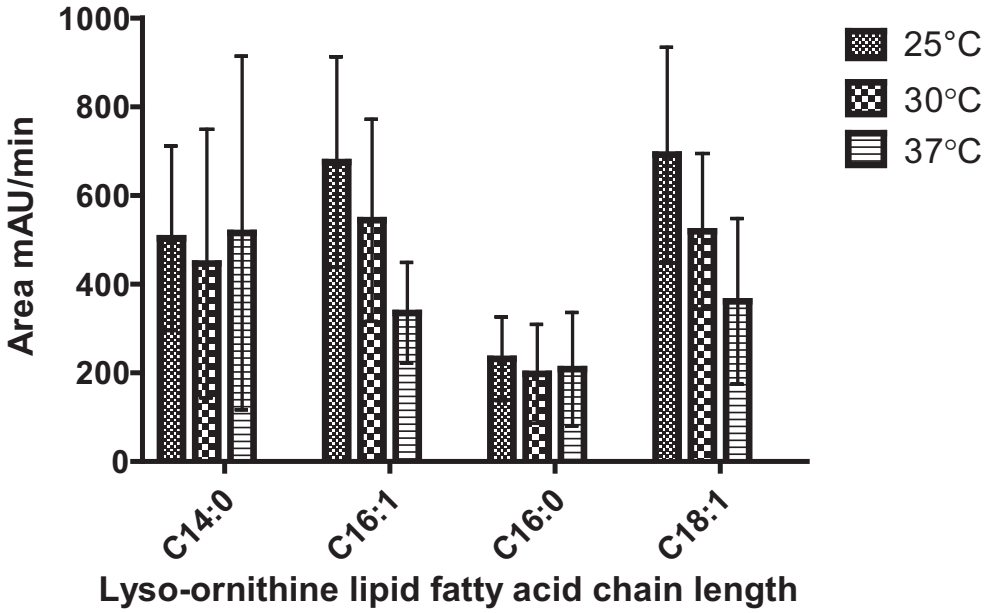


Figure 3.3. Peak area quantification of lyso-ornithine lipids produced in response to different growth temperatures.

3.3.2.2 IPTG Induction Concentration

The optimal inducer concentration was at 1 mM IPTG with a slight reduction in LOL production at 0.1 mM IPTG, and a noticeable decrease in LOL production at 10 mM IPTG induction (**Figure 3.4)**. A 1.3-fold increase was obtained for the sum peak area of the four LOL species induced with 1 mM IPTG compared with 10 mM IPTG induction. In addition, there is a trend towards an increase in two chain lengths (C16:1 and C18:1).

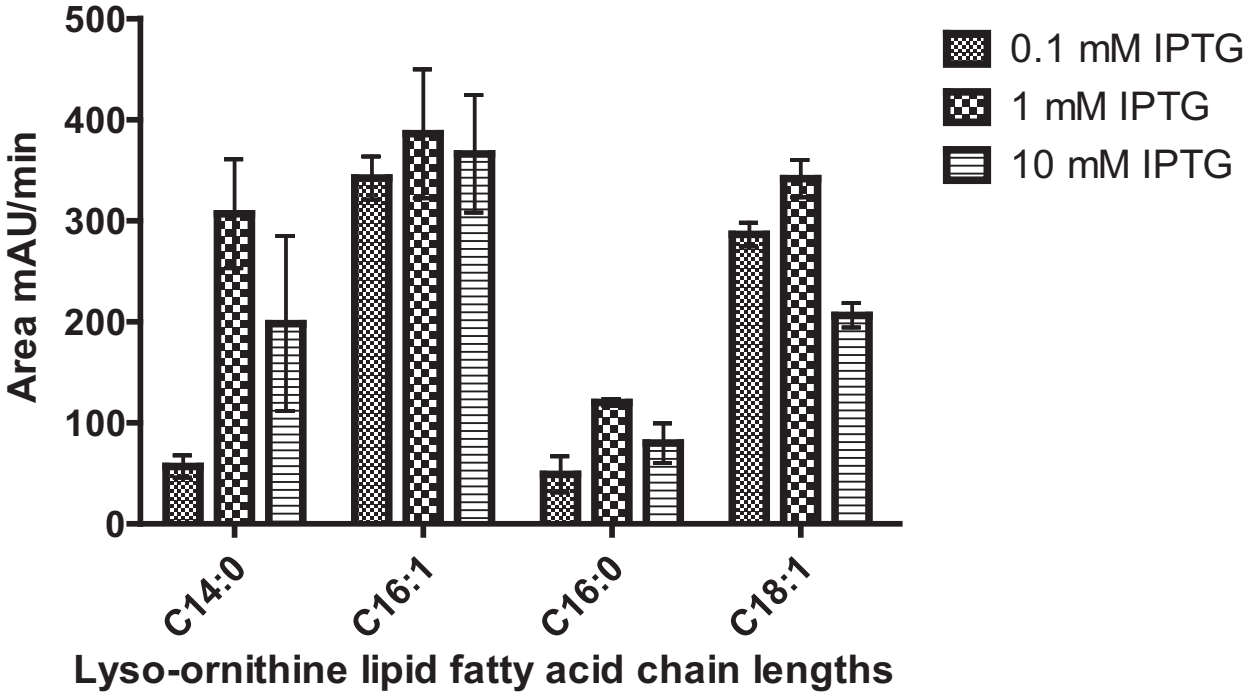


Figure 3.4. Peak area quantification of lyso-ornithine lipids produced in response to different IPTG concentrations.

3.3.2.3 L-Ornithine Supplementation

A 3.8-fold increase in LOL production was achieved with 2 mM ornithine supplementation compared with 200 mM L-ornithine supplementation **(Figure 3.5).** Furthermore, 2 mM L-ornithine resulted in an increase in C16:0 and C18:0 chain lengths, whereas the shorter C14:0 and C16:1 species were more abundant at 20 mM L-ornithine supplementation, suggesting a shift towards shorter chain profiles, while at 200 mM an appearance of even shorter carbon chain lengths (C12:0 and C14:1) is obtained. Therefore, ornithine supplementation could be a way to control the chain length profile and therefore, the hydrophilic-lipophilic balance (HLB) of compounds produced.

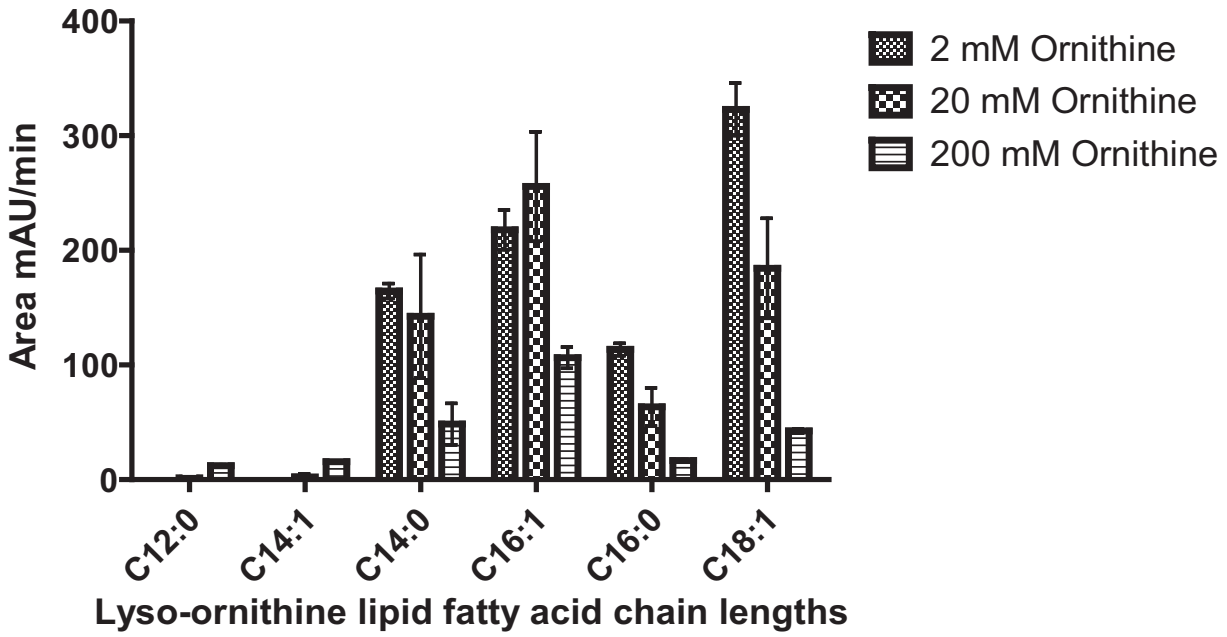


Figure 3.5. Peak area quantification of lyso-ornithine lipids produced in response to different L-ornithine concentrations.

3.3.2.4 Medium pH

The medium pH had a tremendous effect on LOL production **(Figure 3.6)** with highest yields at pH 8 and the complete disappearance of all LOL species at pH 6, despite comparable growth (data not shown). While we did observe high levels of both short and longer chains produced at pH 7 and 8 we observed a significant shift in production of the various chain lengths. For example, at pH 7 we observed an increase in the abundance of shorter chain lengths (C14:0 and C16:1) whereas at pH 8 an increase in the longer chain lengths (C16:0 and C18:1) was observed.

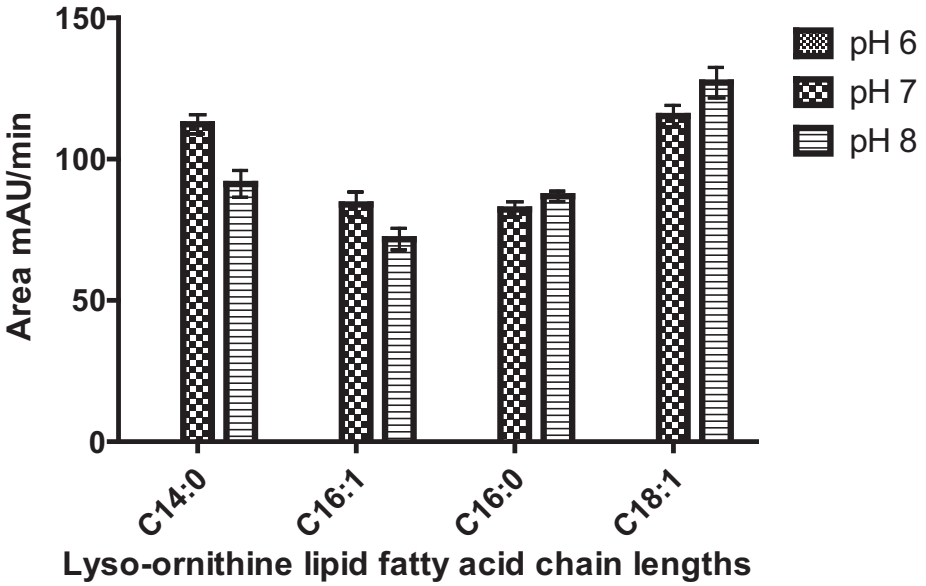


Figure 3.6. Peak area quantification of lyso-ornithine lipids produced in response to different medium pH values.

3.3.3 Purification of the major lyso-ornithine lipids

The column chromatography-based purification method developed in this study results in the removal of the bulk of the water-soluble components in the clarified culture supernatant **(Figure 3.7).** The major LOL species were collected as a single fraction and used as such for all the biofilm analyses performed in this study. A total of 50 mg LOL is purified per 1L of culture.

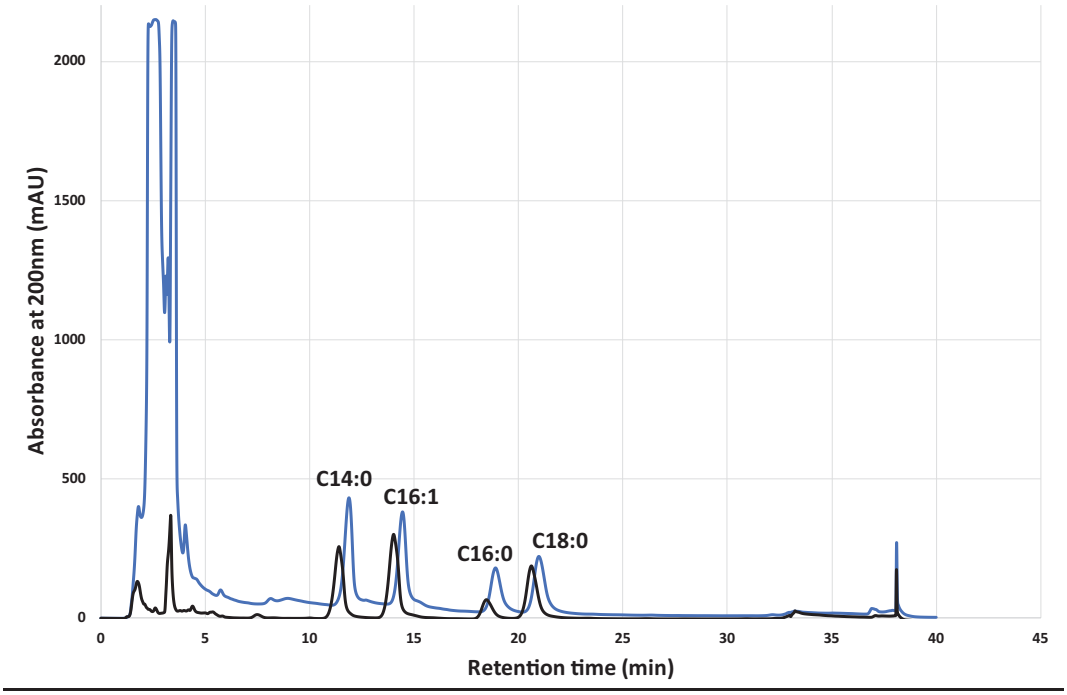


Figure 3.7. High-performance liquid chromatography analysis showing a representative fermentation supernatant (blue chromatogram) versus partially purified LOLs (black chromatogram) following fractionation on the Bio-Rad NGC system.

3.4 CONCLUSION

The parameters which resulted in maximized LOL titres yielded by the *E. coli* "B" strain were established; 25°C culture incubation with 1 mM IPTG, 20 mM L-ornithine supplementation, and the medium pH between 7 and 8. The LOL chain lengths C16:1 and C18:1 were produced in the greatest quantity assuming the same molar extinction coefficient for the four compounds at 190 nm. The results demonstrate that genetic engineering can improve biosurfactant yield, and further engineering strategies can be considered to optimise the strain to alleviate other metabolic bottlenecks. An exciting outcome was the effect that each parameter has on the ratio of the LOL species formed. This provides the opportunity to develop tailored LOL formulations with varied hydrophilic lipid balance and thus, varied performance properties which may expand the application of these compounds.

CHAPTER 4: HIGH THROUGHPUT BIOFILM EVALUATION

4.1 INTRODUCTION

Current methods used to prevent and control biofilm formation are mainly based on the treatment of industrial cooling water systems with biocides and/or chemical surfactants (Di Pippo *et al*., 2018). However, these products are highly toxic and further represent an ecological hazard. This calls for a bio-based approach involving novel natural-based biosurfactants for their ability to function as anti-biofilm agents. The concept is that developing a biocide with biosurfactant products may enhance biocide efficacy and thus reduce the quantity required to attain an acceptable level of control.

Biocides are chemical or naturally derived compounds that have the capacity to kill, destroy, inhibit, or control microorganisms (Cloete & Flemming, 2012; Płaza & Achal, 2020). BIODX Ltd (Pty), a South African biotechnology company, developed two green biocidal agents: DECONT-A and DECONT-M, specifically for use in the disinfection of industrial waters. The products contain a mixture of a quaternary ammonium compound and one of two fruit acids. In this report, we performed co-formulation of each biocide with the LOL preparation discussed in chapter three. This is premised on studies showing that the maximal repulsive force is significantly higher for surfactant mixtures as compared with a single surfactant, owing to denser packing of micelles (Rabinovich *et al*., 2006). A modified version of the high throughput biofilm assay described by Ceri *et al*. (1999) was used to evaluate the most effective biosurfactant/biocide combinations on biofilms formed by three industrial wastewater sources. It becomes necessary to use biofilms pre-formed with these industrial water sources at the initial stage to mimic the real-life conditions and factors encountered on extremely polluted sites of industrial cooling systems. Thus, the approach used in this report may give a clear method of assessing different proportional ratios/combinations of the formulation mix for full-scale evaluation in the CEMS (a continuous flow system that mimics industrial cooling systems) that allows the development of biofilms under shear and nutrient conditions (Klopper *et al*., 2020).

4.2 MATERIALS AND METHODS

4.2.1 Evaluating the microbial community present in industrial wastewater

Three sources of industrial wastewater were obtained from power generating facilities in South Africa. These are referred to as cooling tower pond 1, 2 or 3 (CTP1/2/3) water throughout the report. A sample from each one was spotted on LB containing 200 µg/mL ampicillin, 50 µg/mL streptomycin, 34 µg/mL chloramphenicol, and 50 µg/mL kanamycin. The plates were incubated at 37°C for 24 hours and colonies were recorded.

4.2.2 Evaluating the microbial community of industrial water for biofilm formation

The 96-well plate biofilm assay was adapted from Merritt *et al*. (2011). The three industrial wastewaters were tested for their biofilm forming abilities. The water sources were incubated at 30°C for four or seven days, after which the biofilm was stained with 1% crystal violet for 10 min followed by decanting the unbound crystal violet into a waste container. Any additional unbound crystal violet was washed by carefully submerging the plate in water and inverting the plate over the waste container. This was repeated three times. Plates were allowed to dry to remove any of the remaining water followed by solubilization of the crystal violet with 95% ethanol for 15 min. Solubilized crystal violet was transferred to a new 96-well plate and measured spectrophotometrically at 590 nm.

4.2.3 Determining the minimum inhibitory concentration of Decont-A, Decont-M and LOL

Industrial wastewater was aliquoted into sterile 96 well plates (90 µL) and 10 µL of various concentrations of Decont-A/M (10 ppm, 8 ppm, 4 ppm, and 2 ppm) and LOL (500 µg/mL, 250 µg/mL, 125 µg/mL, 60 µg/mL, 30 µg/mL, and 20 µg/mL) was added. The effect of Decont-A/M at 1 ppm, 0.5 ppm, and 0.25 ppm on industrial wastewater from the cooling tower pond 1 was also evaluated. The plates were incubated at 30°C for 4 days followed by staining with crystal violet as described in 4.2.2. All the experiments were performed with three biological repeats and 30 technical repeats.

4.2.4 Preparation of Decont-A/M and LOL formulations

Decont-A and M + LOL formulations were created in two ways (**Table 4.1**). Formulation A was prepared by preparing the Decont-A/M and LOL separately at 2 x the final concentration followed by mixing the two components 1:1. For formulation B, Decont-A/M and LOL were directly mixed. The final concentrations of both sets of formulations were used as follows: 0.5 ppm Decont-A/M + 60 µg/mL, 0.5 ppm Decont-A/M + 30 µg/mL, 0.5 ppm Decont-A/M + 20 µg/mL, 0.5 ppm Decont-A/M + 10 µg/mL, 0.25 ppm Decont-A/M + 60 µg/mL, 0.25 ppm Decont-A/M + 30 µg/mL, 0.25 ppm Decont-A/M + 20 µg/mL, and 0.25 ppm Decont-A/M + 10 µg/mL.

Table 4.1. Preparation of formulation A and B used in the biofilm inhibition assays

Concentrations	Formulation A*				Formulation B		
	Decont-A/M (µL)	H_2O (µL)	LOL (µL)	H_2O (µL)	Decont-A/M (µL)	LOL (µL)	H_2O (µL)
0.5 ppm + 60 µg/mL LOL	5	495	120	380	5	120	875
0.5 ppm + 30 µg/mL LOL	5	495	60	440	5	60	935
0.5 ppm + 20 µg/mL LOL	5	495	40	460	5	40	955
0.5 ppm + 10 µg/mL LOL	5	495	20	480	5	20	975
0.25 ppm + 60 µg/mL LOL	2.5	497.5	120	380	2.5	120	877.5
0.25 ppm + 30 µg/mL LOL	2.5	497.5	60	440	2.5	60	937.5
0.25 ppm + 20 µg/mL LOL	2.5	497.5	40	460	2.5	40	957.5
0.25 ppm + 10 µg/mL LOL	2.5	497.5	20	480	2.5	20	977.5

* Decont-A/M preparation mixed 1:1 with prepared LOL

4.2.5 Evaluating the effect of Decont/LOL formulations on the biofilm forming microbial community in CTP1 water

The effect of formulation A and B on the microbial community of wastewater from the CTP1 was performed as described in section 4.2.4. In short, 90 µL of industrial wastewater was aliquoted into a 96-well microtiter plate containing 10 µL of formulation A and B. The plates were incubated at 30°C for 4 days followed by staining with crystal violet as described. All experiments were performed with three biological repeats and 3 technical repeats. One-way ANOVA was used for statistical analysis.

4.2.6 Statistical analysis

GraphPad Prism (version 5.01) was used for all statistical analyses and data was expressed as average ±SEM. When necessary, one-way ANOVA and Turkey's *post hoc* test were used to analyse the data. When $P < 0.05$, the difference was considered statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Evaluating the microbial community present in industrial wastewater

The microbial community of industrial wastewater collected from the three CTPs is diverse **(Figure 4.1)**. Bacteria resistant to 50 µg/mL kanamycin is present in the CTP2 water **(Figure 4.1A)**, bacteria resistant to 200 µg/mL ampicillin is present in the CTP1 water **(Figure 4.1B)** and bacteria resistant to 50 µg/mL streptomycin is present in the CTP3 water **(Figure 4.1C)**.

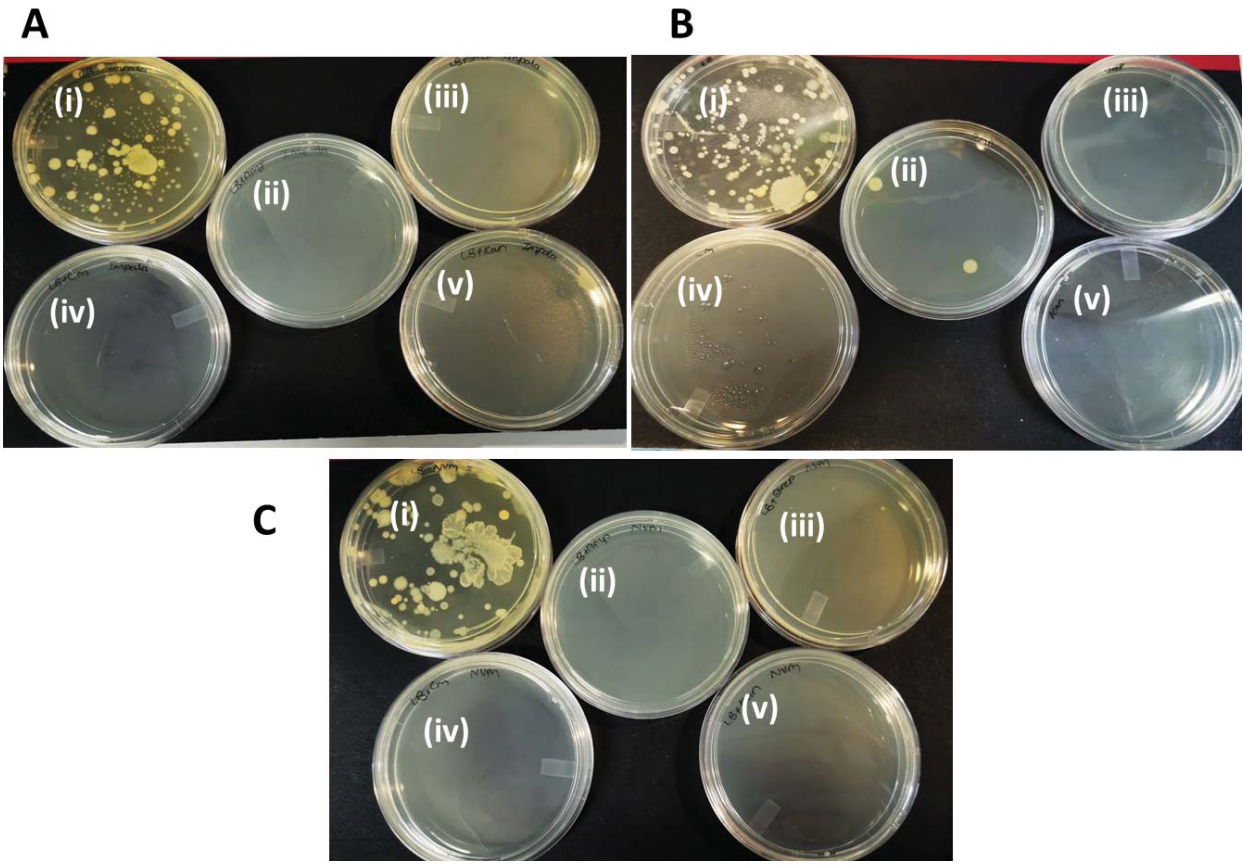


Figure 4.1. Morphological evaluation of the microbial community present in the water sampled from (A) CTP2, (B) CTP1, and (C) CTP3; plated onto (i) Luria agar only, or Luria agar containing (ii) 200 µg/mL ampicillin (iii), 50 µg/mL streptomycin (iv), 34 µg/mL chloramphenicol (v) and 50 µg/mL kanamycin.

4.3.2 Evaluating the microbial community of industrial water for biofilm formation

The three industrial wastewater sources were evaluated for biofilm formation. The water was incubated at 30°C for four and seven days followed by staining with crystal violet. Industrial wastewater from the CTPs 1 and 2 formed a biofilm within four days, while a biofilm was only detected after seven days for the CTP3 water **(Figure 4.2)**. The thickest biofilm was formed by the microbial community in the CTP1 wastewater. No significant improvement in biofilm formation was observed for the wastewater from CTP2 after 7-day incubation. Although the microbial community in the CTP3 water formed a biofilm after seven days, it was significantly less developed compared to the biofilms formed by the other two water sources.

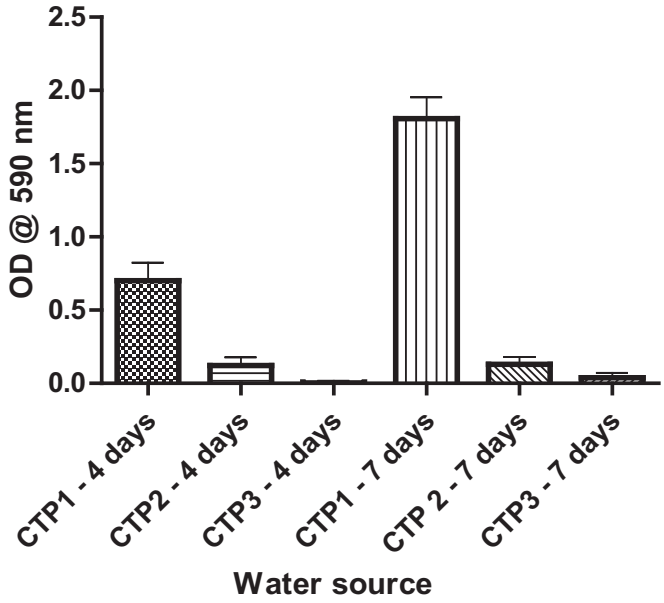


Figure 4.2. Biofilm evaluation in the 96-well format as a function of the optical density of solubilized crystal violet after four and seven days.

4.3.3 Determining the minimum inhibitory concentration of Decont-A, Decont-M and LOL

Biofilm formation by the microbial community in the CTP1 water was inhibited at 1 ppm Decont-A and M and 60 ug/mL (60 ppm) LOL **(Figure 4.3)**. Higher concentrations of Decont-A and M (8 -10 ppm) had the opposite effect and did not inhibit biofilm formation. Decont-A concentrations currently used by Biodx (2 – 4 ppm) in the field did inhibit biofilm formation. Decont-A performed better when compared to Decont-M. At low levels of LOL, biofilm formation was still reduced, but only by 20%.

Biofilm formation by the microbial community in the CTP2 water was more resilient to Decont-A, M and LOL treatment resulting in much higher MIC of Decont-A **(Figure 4.4A)**, Decont-M **(Figure 4.4B)** and LOL **(Figure 4.4C)** when compared to the CTP1 water. In contrast to the CTP1 water, Decont-M is more effective in preventing biofilm formation by the microbial community in the CTP2 water, although it is at higher concentrations than the concentration recommended by Biodx.

The same was observed for the microbial community in the CTP3 water. Decont-M is more effective in preventing biofilm formation when compared to Decont-A **(Figure 4.5A-B)**, while high concentrations (500 µg/mL) of LOL are needed to prevent biofilm formation by the microbial community present in the water from CTP3.

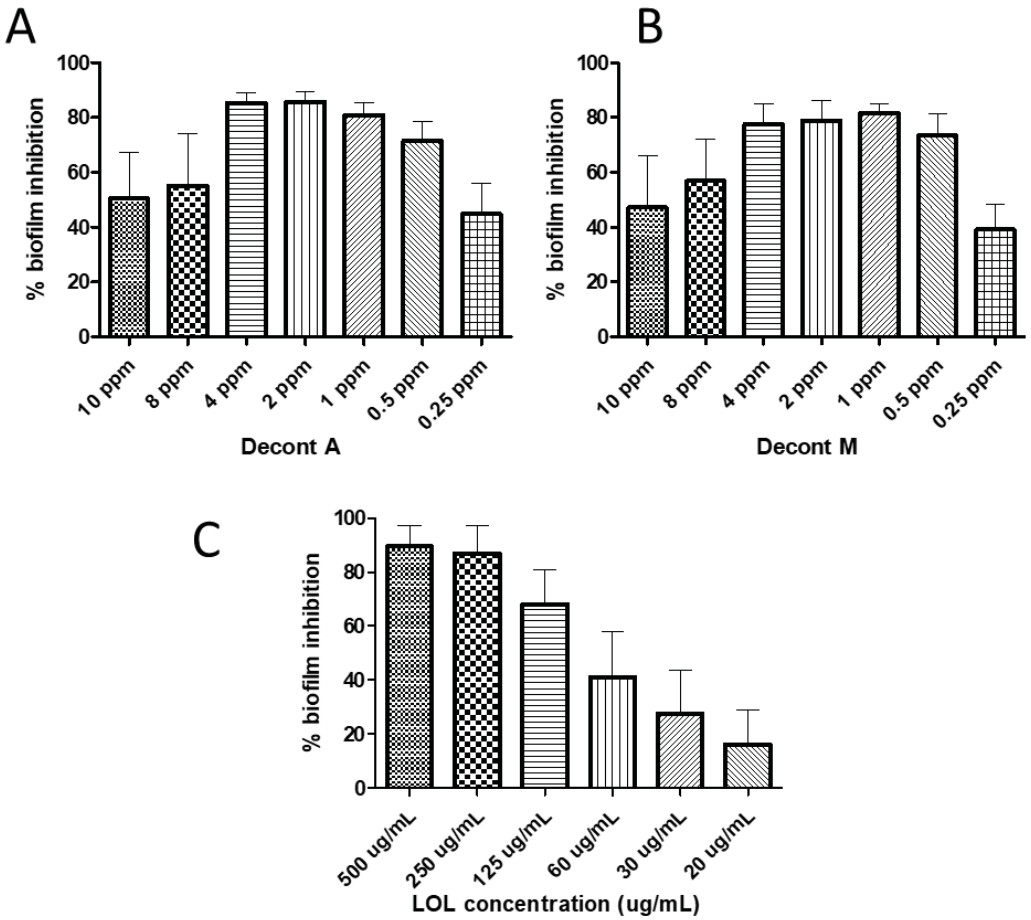


Figure 4.3. The Inhibition of the biofilm formed by the microbial community present in the CTP1 water treated with (A) Decont-A, (B) Decont-M, and (C) LOL.

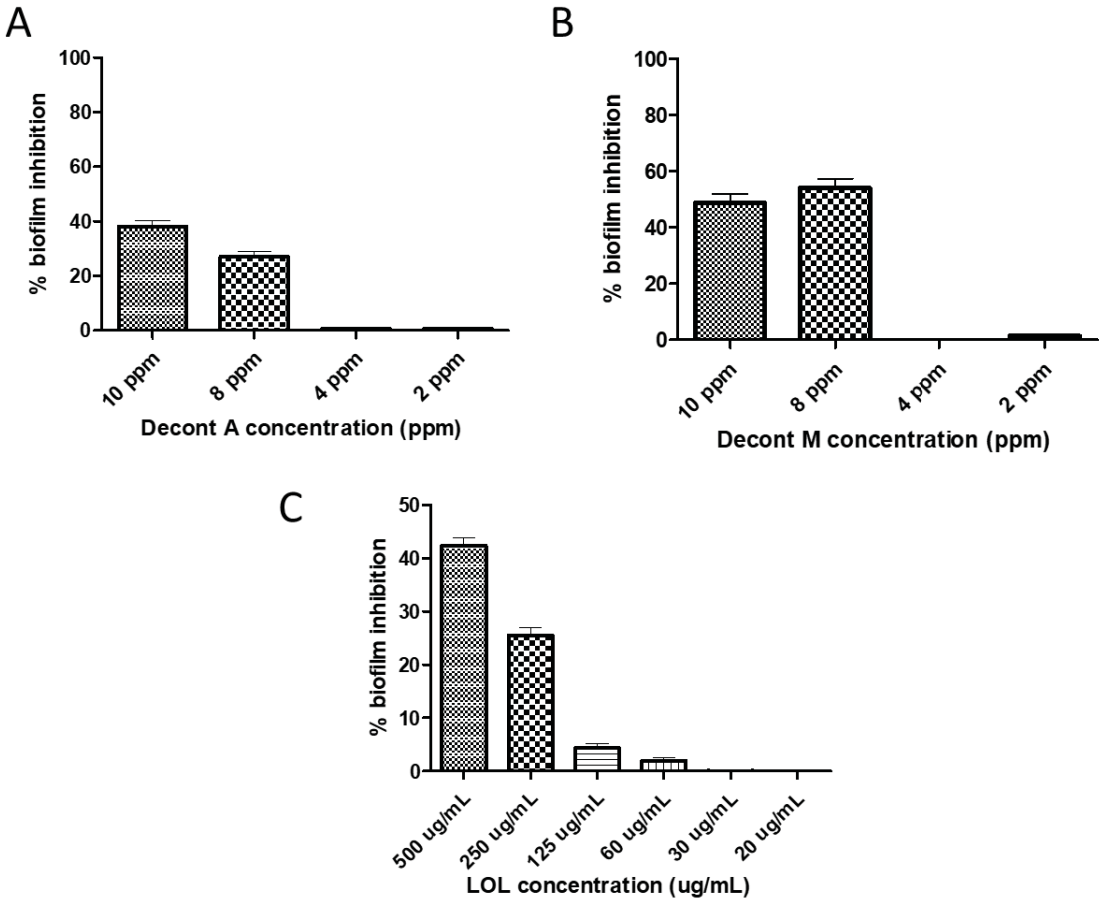


Figure 4.4. The Inhibition of the biofilm formed by the microbial community present in the CTP2 water treated with (A) Decont-A, (B) Decont-M, and (C) LOL.

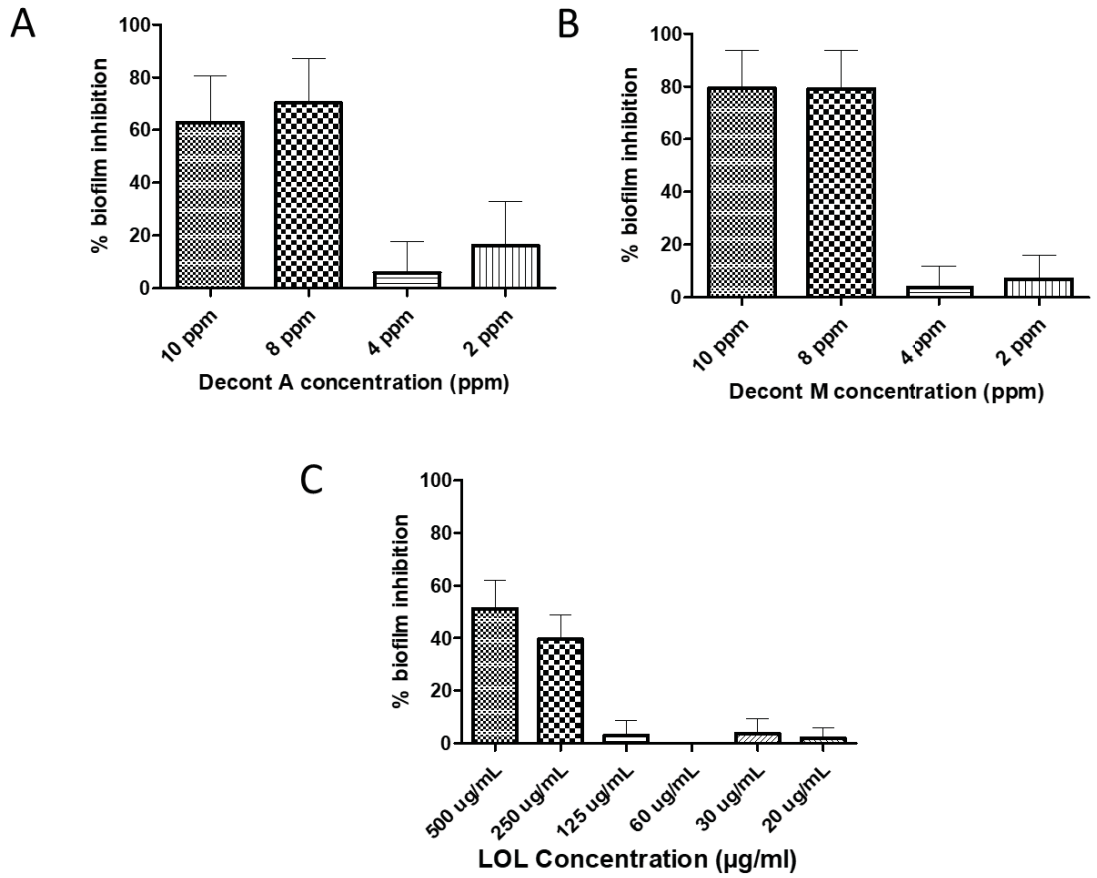


Figure 4.5. The Inhibition of the biofilm formed by the microbial community present in the CTP3 water treated with (A) Decont-A, (B) Decont-M, and (C) LOL.

4.3.4 Preparation of Decont-A/M and LOL formulations for testing against the biofilm forming microbial communities present in industrial wastewater

Differences in the turbidity of the two formulations were noticed **(Figure 4.6)**. Aliquots of LOL formulations A and B were injected on a HPLC. A significant shift in peak retention is observed when LOL (**Figure 4.6B**, pink chromatogram) is compared to formulation A (**Figure 4.6B**, blue chromatogram) and B (**Figure 4.6B**, black chromatogram). Significant differences in the area under the curve for the four peaks (peaks 4, 5, 6 and 7) representing the four LOL congeners were also observed for the 2 formulations. Peak tailing is also observed for peaks 4, 5, 6 and 7 indicating that the addition of Decont-A causes a change in LOL chemistry leading to precipitation of LOL. This was further confirmed when the formulations were centrifuged and a pellet formed for formulation 1. The intensity of peaks 8 and 9 was also decreased, but the peak intensity of peaks 1 and 2 increased.

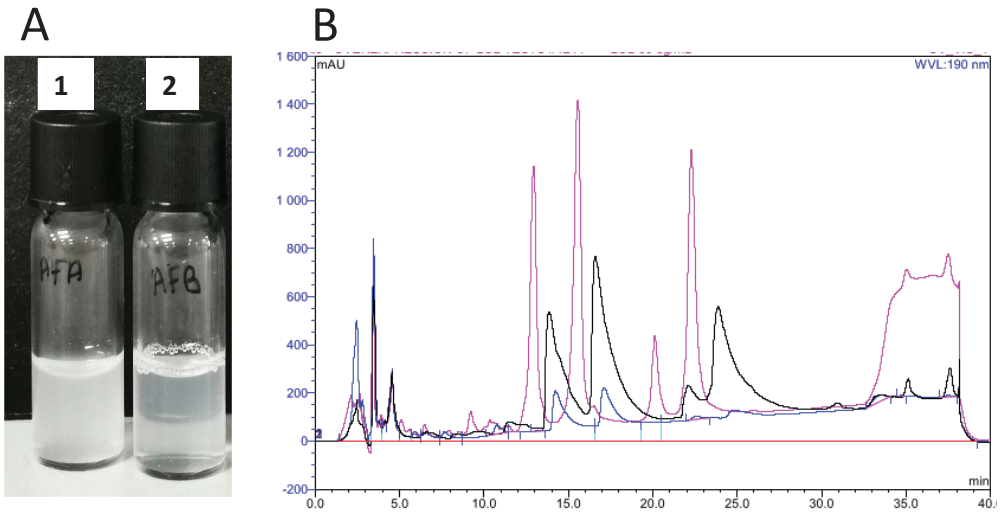


Figure 4.6. The preparation of Decont-A/M and LOL formulations. A) The image shows the difference in turbidity between formulation 1 and 2. B) HPLC chromatograms comparing the area under the curve of the four LOL compounds. Formulation 1 is represented by the blue chromatogram and formulation 2 is represented by black chromatogram. The pink chromatogram represented LOL biosurfactant.

4.3.5 Evaluating the effect of Decont/LOL formulations on the biofilm-forming microbial community in CTP1 water

Both formulations A and B were evaluated for biofilm inhibition. Decont-A used as a single compound inhibited biofilm formation by 63%, but when used in combination with LOL prepared according to formulation A, biofilm inhibition was significantly reduced and formation could only be inhibited by a maximum of 45% when LOL was used at low concentrations **(Figure 4.7A)**. The same effect was observed when Decont-M and LOL was prepared according to formulation A **(Figure 4.7B), i**ndicating that when Decont-A and LOL are mixed according to formulation A, the two compounds act antagonistically.

In contrast, when Decont-A and LOL formulation was prepared according to formulation B, biofilm inhibition was significantly improved **(Figure 4.8A)**. The same trend of antagonism was observed at higher concentrations of LOL, but once the concentration was reduced to 10 µg/mL biofilm formation was inhibited by 91%. The same synergistic effect was not observed for the Decont-M formulation and a decrease in efficacy was again observed **(Figure 4.8B)**. The results indicate that Decont-A and LOL act synergistically at low concentrations of LOL. Furthermore, when LOL is used in combination with Decont-A the MIC is reduced to 0.5 ppm.

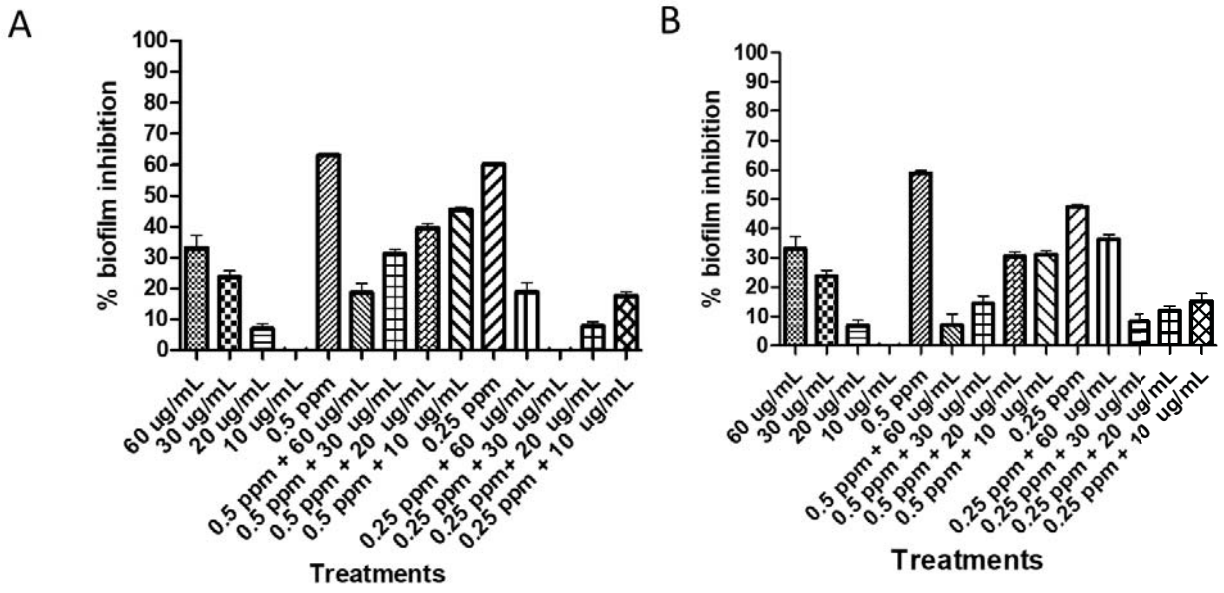


Figure 4.5. The effect of formulation A on the biofilm-forming microbial community present in the CTP1 water. (A) CTP1 water treated with the Decont-A/LOL formulation. (B) CTP1 water treated with the Decont-M/LOL formulation. The Decont concentration is given in ppm units and the LOL concentration in µg/mL.

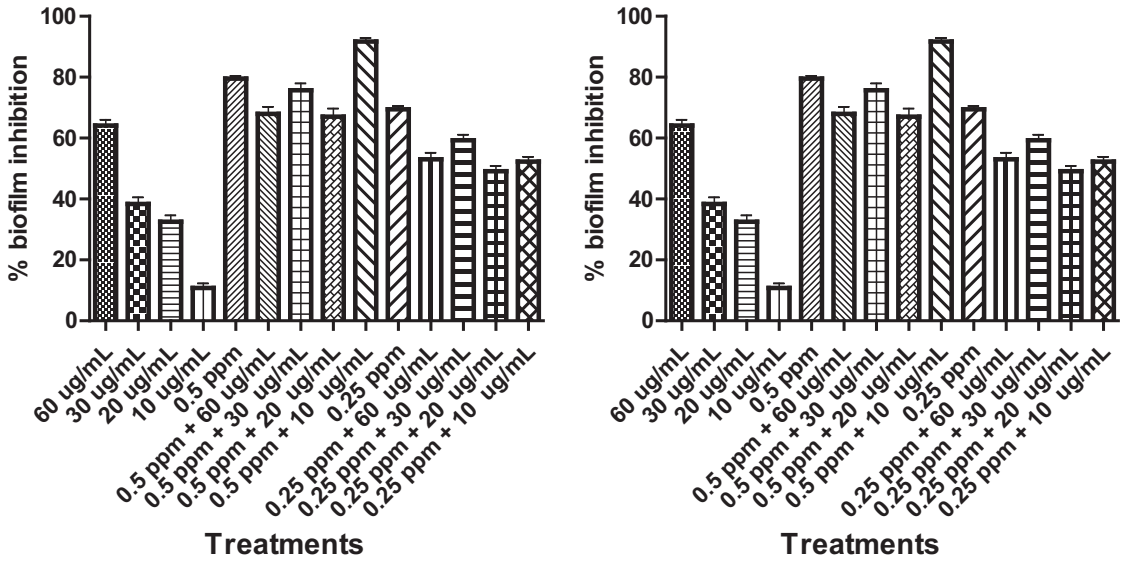


Figure 4.6. The effect of formulation B on the biofilm forming microbial community present in the water from CTP1. (A) CTP1 water treated with the Decont-A/LOL formulation. (B) CTP1 water treated with the Decont-M/LOL formulation. The Decont concentration is given in ppm units and the LOL concentration in µg/mL.

4.4 CONCLUSION

High throughput 96-well biofilm assays were used to assess the effect of LOL, Decont-A, Decont-M, and Decont-A/M co-formulated with LOL on the biofilm-forming community present in the selected industrial wastewater sources. The minimum biofilm inhibitory concentration of LOL is 60 µg/mL and 0.5 ppm for Decont-A and M. We also found that co-formulating Decont-A and LOL significantly improved inhibition of biofilm formation, while the biofilm inhibitory effects of Decont-M were not improved when co-formulated with LOL. The industrial wastewater sourced from CTP1 contained the highest population of biofilm-forming bacteria and was selected as the water source for future biofilm studies.

CHAPTER 5: BIOFILM DYNAMICS EVALUATION

5.1 INTRODUCTION

Biofilms are the predominant growth phase of bacteria – bacteria rarely exist as individual cells in natural habitats (Klopper *et al.,* 2020). This is also the case for microbial communities in cooling water systems used to dissipate heat generated by powerplants (Di Pippo *et al.,* 2018). Numerous model systems for studying biofilms *in vitro* have been developed (Blanco-Cabra *et al.,* 2021). The most common method used for screening vast amounts of compounds entails high-throughput 96-well assays and colorimetric measurements, as presented in chapter 4. Unfortunately, the majority of high-throughput assays use static devices with limited nutrients and are destructive endpoint assays (Christensen *et al.,* 1985; O'Toole, 2010). Thus, these biofilms do not resemble biofilms found in natural conditions. Biofilms cultivated under continuous flow systems are therefore more representative of biofilms found in nature (Sackmann *et al*., 2014), but generally these biofilms are analysed using advanced microscopy systems and endpoint assays. Although these studies did give researchers a lot of insight into biofilm formation, structure, and composition, real-time monitoring is needed to evaluate the effect of antibiotics or biocides on biofilm growth. In an article published by Kroukamp and co-authors (2009), a carbon dioxide evolution measurement system was proposed for measuring the metabolic activity of biofilms in real-time. Although metagenomic analysis of biofilm communities in industrial, marine, and medical environments are extremely common (Guzmán *et al.,* 2020; Pinnell and Turner 2019; Dagmar *et al.,* 2014; Xu and Gunsolley 2014), little research has been done on the changes of biofilm communities in laboratory setups (Bernardini *et al.,* 2022). In a study by Denvir and co-authors (2019), the importance of metagenomic analysis of biofilms in industrial water systems were highlighted which could give insight to the best treatment approach with regards to antibiofilm agents.

Biofilms are inherently more resistant to antibiotics and biocides due to multiple factors including reduced growth rate, persister cells, efflux pumps, and limited biocide/antibiotic penetration (Verderosa *et al.,* 2019). Due to the reduced sensitivity of biofilms to biocides, higher concentrations of biocide are needed to remove the biofilms in cooling towers. Alternatively, co-formulation of biosurfactants with biocides can greatly increase the biocides' antibiofilm activity.

In the previous chapters of this report, we reported on developing a co-formulation of the proprietary biocide Decont-A and biosurfactant lyso-ornithine lipid (LOL). The formulation was tested using 96-well high throughput assays and the most effective formulation was selected for the continuous flow biofilm system to better study the effect of biocides on biofilms. Metagenome analysis of the microbial community present in the effluent was also analysed to give more insight into the effect of the biocide and biocide-biosurfactant treatment on biofilm community.

5.2 MATERIALS AND METHODS

5.2.1 Biofilm metabolic activity monitoring under continuous flow

A carbon dioxide evolution measurement system (CEMS; **Figure 5.1**) was used to monitor biofilm growth. The CEMS consists of a silicone tube biofilm reactor (ID: 1.98 mm; OD: 3.16 mm) encased in a Tygon tube (ID: 4.8 mm; OD: 7.9 mm) with the annular space connected to a CO_2 analyser. Unless stated otherwise, the flow rate was set to 10 ml/hour using a Watson Marlow 120U/DM2 analogue control peristaltic pump and the flow of the sweeper gas was set to 1 mL/hour. The silicone tubing was sterilized using 10% bleach for 1 hour followed by rinsing with sterile distilled water for 8 hours. Industrial wastewater collected from the cooling tower pond 1 (hereon referred to as "industrial water") was sterilized by filtering through 0.45 µm cellulose acetate

membrane filters and transferred to sterile flasks containing sterile Tygon tubing (ID: 1.98mm; OD: 3.16mm). The biofilm reactor was flushed with the sterile industrial water for 1 hour followed by inoculating the biofilm reactor with a 1 mL culture from the industrial water. This culture was prepared by incubating 100 mL of the industrial water at 30°C for 96 hours. The culture was concentrated by centrifugation (5000 x g for 10 min) and resuspended to an OD of 1 using sterile industrial water containing 5% Tryptic Soy Broth (TSB). After inoculation the flow rate of the system was set to 0 mL/hour for one hour followed by increasing the flow to 10 ml/hour. The temperature of the biofilm reactor was set to 25°C using a heating-cooling water bath. The effluent was plated every 24 hours on water from the Lethabo power station containing 1.5% agar. Although doses were determined using high throughput screening evaluations (presented in Chapter 3), preliminary studies using the continuous flow system indicated that higher concentrations of Decont-A were needed to disrupt an already formed biofilm (**Figure S1-3**). Thus 60 ppm Decont-A was used for the continuous flow systems. Once the biofilm reached steady state (~2 weeks – 328 hours) the biofilm was either left untreated or treated with 60 ppm Decont-A, 60 ppm Decont-A + 10 µg/mL LOL, or 10 µg/mL LOL for 4 hours. The biofilm was monitored for four days thereafter.

Biofilm dispersal and the concentration of planktonic cells derived from the biofilm was also monitored with colony counts. The protocol by Klopper *et al.,* (2023) was adapted. The effluent was collected from the CEMS system and serially diluted in sterile industrial water and plated on agar-solidified industrial water (15 g/L agar). Effluent was collected every 24 hours throughout the entire experimental period, as well as 30 min, 1, 2, 4, 24 and 48 hours after treatment. Colonies were incubated at 30°C and counted after 96 hours.

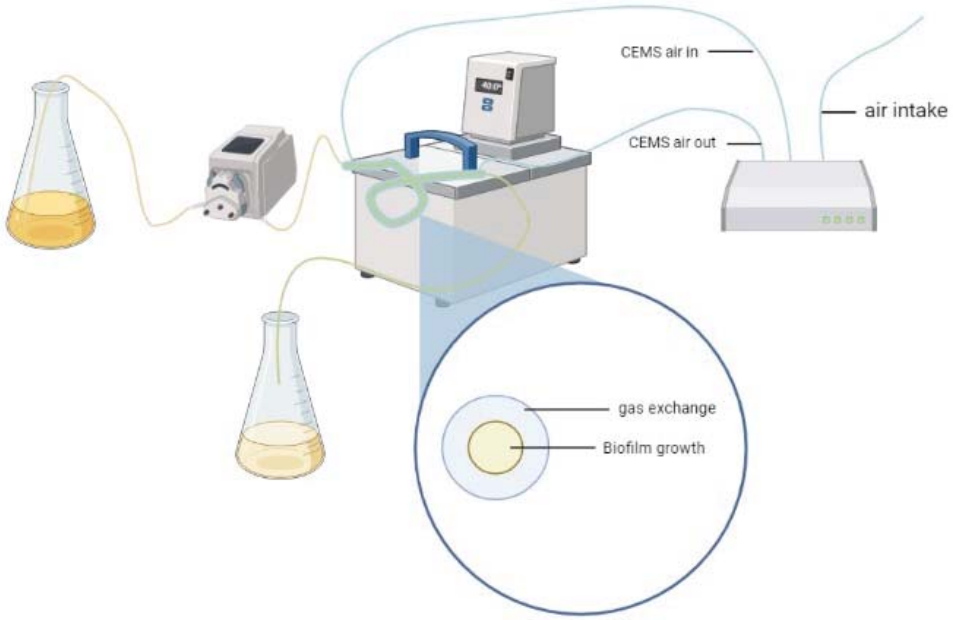


Figure 5.1. Schematic of the carbon dioxide evolution measurement system (CEMS) used for the real-time monitoring of biofilm under continuous flow conditions. The media was introduced using a Watson Marlow 120U/DM2 peristaltic pump and the sweeper gas is introduced into the annular space (blue area) of CEMS using a built-in pump in the CO_2 analyser. The biofilm reactor consists of 1 m of silicon tubing within 1 m of Tygon tubing and the temperature is controlled at 25°C by placing the biofilm reactor in a temperature-controlled water bath.

5.2.2 Metagenomic analysis of biofilm

Metagenomic DNA of the established bacterial community was isolated using the Machery-Nagel Nucleospin Microbial DNA kit. The microbial cells were collected by centrifuging (10 000 x g for 30 min) the biofilm effluent and the microbial DNA was extracted with the kit. After isolation the microbial DNA was amplified with the EquiPhi29 DNA polymerase using an amended version of the protocol by Stepanauskas *et al.* (2017). The amended 20 µL reaction contained 2.5 µL denatured DNA, 0.8 µL of dNTP's (Nuew England BioLabs) 1.5 µL EquiPhi29 reaction buffer (Thermo Fisher Scientific), 2 µL dithiothreitol (Thermo Fisher Scientific), 6 µL 40 µM random heptamers with two 3'-terminal phosphorothioated nucleotide bonds (Integrated DNA Technologies), and 1 µL Equiphi29 polymerase (Thermo Fisher Scientific). The amplification was done at 45°C for 16 hours followed by inactivation at 75°C for 15 min. After amplification the DNA was precipitated with 95% ethanol and resuspended in sterile nuclease and RNase free double distilled water. Metagenomic analysis was done using a MinION nanopore sequencer. Base calling was done with Guppy Basecalling Software, (C) Oxford Nanopore Technologies, Limited. Version 3.1.5+781ed57, followed by running Pore chop, nanofilt, and flye for quality control. For metagenomic analysis the assembled genomes were subjected to kraken2 and kaiju. The ResFinder platform on the Center for Genomic Epidemiology website was used to identify antibiotic resistance genes in the metagenomic data sets (https://genomicepidemiology.org/services/). The metagenomic data was also analysed to establish the sequencing coverage of the metagenomes (http://enve-omics.ce.gatech.edu/nonpareil/newjob).

5.2.3 Extraction of genomic DNA of the most common bacteria identified on agar

After counting the colonies on the solidified industrial water, the plates were incubated at 30 °C for an additional 96 hours. After incubation, the unique colonies were picked and streaked until they were pure. Single colonies were picked and inoculated in sterilised industrial water spiked with 5% glucose and incubated at 30°C for 96 hours. After incubation, the genomic DNA was isolated using the Machery-Nagel Nucleospin Microbial DNA kit and amplified, sequenced, and analysed as described in the metagenomic section. The assembled genomes were used to interrogate the NCBI non-redundant database.

5.3 RESULTS AND DISCUSSION

5.3.1 Biofilm metabolic activity monitoring under continuous flow

Biofilm dispersal was monitored through colony counts, while the metabolic activity was monitored with a CO_2 analyser. Stabilization of the biofilm was only achieved after 288 hours (12 days); this is significantly slower when compared to biofilm formation and stabilization of homogenous biofilms in the reference studies used for the planning of this project (Klopper *et al.,* 2023; Klopper *et al*., 2020; Klopper *et al*., 2019; Jackson *et al*., 2015). For example, *P. aeruginosa* PAO1 and *Listeria monocytogenes* biofilms reached a steady state with respect to metabolic activity after 75 h (3 days) and 100 h (4 days), respectively, although this was highly dependent on several factors (Klopper *et al*., 2020; 2023).

Once CO_2 production stabilized, the biofilm was either left untreated or dosed with 60 ppm Decont-A, 60 ppm Decont-A + 10 µg/mL LOL, or 10 µg/mL LOL. Although doses were determined using high-throughput screening evaluations (presented in Chapter 3), preliminary studies using the continuous flow system indicated that higher concentrations of Decont-A are needed to disrupt an already formed biofilm (**Figure S1-3**). For both the 60 ppm Decont-A and 60 ppm Decont-A + 10 µg/mL treated biofilms, a significant increase in CO_2 production was observed after treatment, indicating an increase in metabolic activity (**Figure 5.2**). The increase in metabolic activity might be due to an increase in expression of efflux pumps. This increase, although short lived, was also observed when *P. aeruginosa* PAO1 biofilms were treated with 4000 mg/L streptomycin

(Jackson *et al.,* 2015). The same increase was not observed for the untreated biofilm and the biofilm treated with 10 µg/mL LOL, further indicating that the increase might be due to stress. Shortly after treatment, a 4% decrease in measured CO_2 was observed in the biofilms treated with 60 ppm Decont-A, an 8% decrease for the biofilms treated with 10 µg/mL LOL, and a 23% decrease for the treatment with 60 ppm Decont-A + 10 µg/mL. A difference in the colonies after treatment was also observed (**Figure 5.3**). Unfortunately, complete biofilm eradication was not observed, since the CO_2 levels recorded did not return to the baseline (400 ppm).

A significant difference in the total colony counts between the control (green) and Decont-A + LOL (purple) and LOL (black) was observed after 4 and 24 hours, as indicated by the asterisks in **Figure 5.3E**. A more significant difference between the treatment groups and the control groups was observed when the individual colonies were counted, as seen in **Figure 5.3 A-D**. The most significant difference is the appearance of the translucent flat colonies (blue) after the biofilms were treated with LOL, Decont-A, and Decont-A + LOL (**Figure 5.3 B-D**). This could be an indication that LOL, Decont-A, and Decont-A + LOL caused biofilm dispersal. The translucent flat colonies were also observed after 264 hours in the Decont-A + LOL treatment group. This could be from biofilm sloughing that occurs when a biofilm has reached a critical mass (Kaplan, 2010). The significant decrease in CO_2 when Decont-A is co-formulated with LOL indicated that LOL improved the activity of Decont-A.

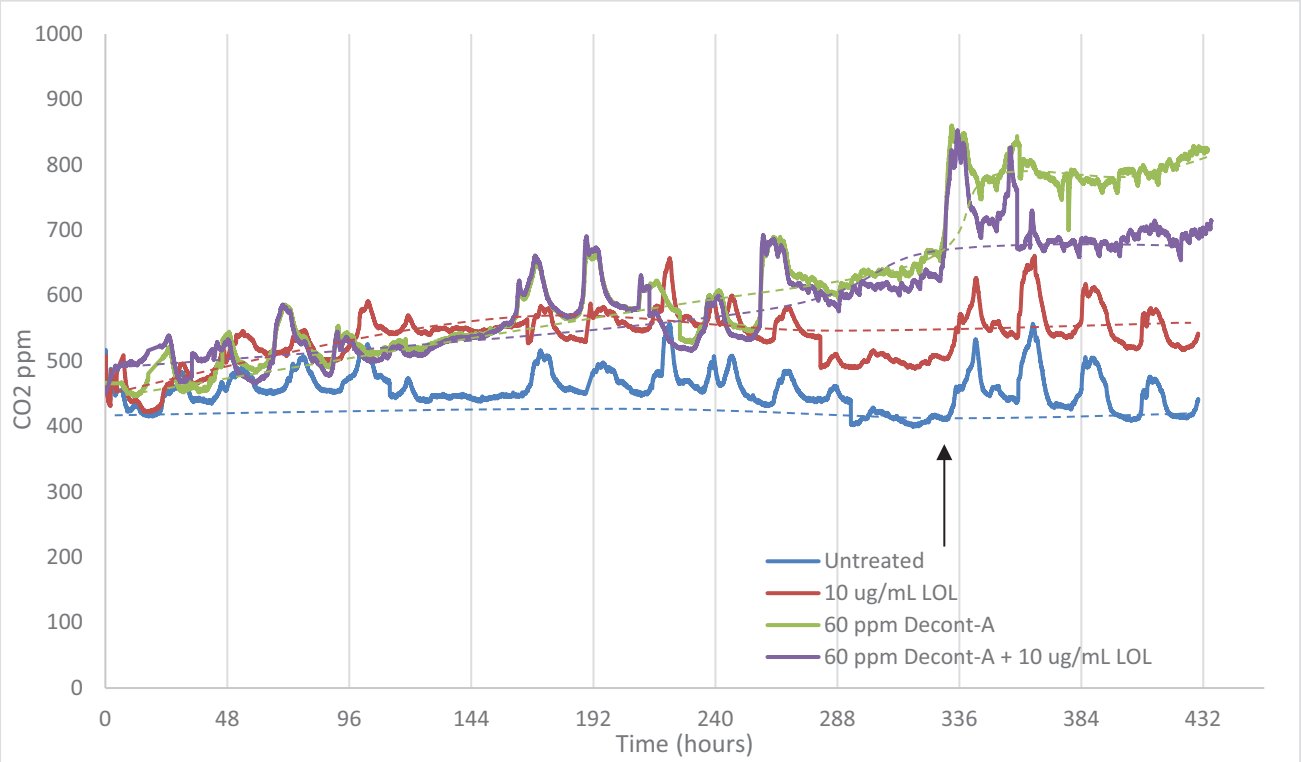


Figure 5.2. Biofilm growth at 25 °C of the microbial community present in industrial wastewater collected from cooling tower pond 1. The biofilms were cultivated in the CEMS system on sterilized industrial wastewater. After the biofilms were established (2 weeks - 328 hours), the biofilms were either left untreated (blue), treated with 10 µg/mL LOL (red), 60 ppm Decont-A (green), or 60 ppm Decont-A and 10 µg/mL LOL (purple). Treatment is indicated with a black arrow. The biofilm was monitored for an additional 72 hours after treatment. Changes in the metabolic activity of the biofilm were measured in real-time with a CO_2 analyser. The dashed blue, red, green, and purple lines represent the trendlines.

5.3.2 Metagenomic analysis of biofilm

Although biofilm eradication was not observed after biocide treatments, the profiles of the culturable community indicated that a shift in the community occurred in response to these treatments. Therefore, evaluating the microbial community pre- and post-treatments could represent an additional level of analysis to describe the effect that the treatments have on the biofilms. The microbial communities pre- and post-treatment of the four experimental treatment groups were analysed through metagenome sequencing. The most dominant order of bacteria present in the biofilms pre-treatment (**Figure 5.4**) was Caulobacteraceae (pink) Sphingomonadaceae (orange), Rhodobacteraceae (blue), Rhizobiaceae (light green), Comamonadaceae (dark green), Hyphomonadaceae (red) Burkholderiaceae (purple), and Pseudomonadaceae (dark blue). Bacterial pathogens belonging to the family Legionellaceae were also present in the microbial community, with the highest concentration found in the LOL treated effluent (0.1%). Similar metagenomic profiles were observed for both treatment groups before treatment indicating that the extraction and amplification methods are reproducible between different biological repeats. After treatment with LOL, a significant increase was seen in the concentration of Streptomycetaceae and Burkholderiaceae in the effluent and a significant decrease in Sphingomonadaceae. After treatment with Decont-A, a significant increase of the following micro-organisms was observed in the effluent: Rhizobiaceae, Comamonadaceae, Pseudomonadaceae and Streptomycetaceae; while a significant decrease of the following micro-organisms was observed: Sphingomonadaceae and Bradyrhizobiaceae. After treatment with the Decont-A + LOL co-formulation a significant increase of the following micro-organisms was observed in the effluent: Rhodobacteraceae and Pseudomonadaceae; while a significant decrease of the following micro-organisms was observed: Sphingomonadaceae, Caulobacterales and Burkholderiaceae.

Numerous genes related to antibiotic resistance were identified in the metagenomic data sets from the effluent pre-treatment, specifically related to resistance against ciprofloxacin, nalidixic acid, beta-lactam antibiotics, trimethoprim, and chloramphenicol. This indicates that the bacteria that sloughed from the biofilm during the initial attachment and maturation phases are resistant to a wide range of antibiotics. In addition to the previously mentioned genes, genes related to tetracycline, doxycycline, minocycline, and tigecycline resistance were also identified in the effluent after treating with LOL and Decont-A + LOL (**Table 5.1**), once again confirming our earlier statement that treating with LOL and Decont-A + LOL significantly changed the microbial community in the biofilm. Furthermore, no genes related to antibiotic resistance were identified in the metagenomic data generated from the control and Decont-A treatments, indicating that once the biofilm matured, the bacteria responsible for the antibiotic resistance phenotype of the biofilm were not sloughed off during the control treatment and were not disturbed when the biofilm was treated with Decont-A.

The biofilm community that established using the cooling tower pond 1 wastewater is similar when compared to other metagenomic studies of cooling towers (Tsao *et. al.,* 2019; Pinel *et. al.,* 2020; Pereira *et. al.,* 2017). However, the nonpareil curve (**Figure 5.5**) indicates that the metagenome is not fully covered, and far deeper sequencing is required to obtain the full community profile before any detailed analysis can be concluded.

5.3.3 Extraction of genomic DNA of the most common culturable bacteria identified on agar

Six unique colonies were identified on the industrial water agar plates after incubating the plates for 8 days at 30°C. The colonies were picked based on shape, size, colour, and edge. The first colony type was big white colonies with a smooth edge. According to the isolated DNA, these colonies were identified as *Pasteurella* spp (**Figure 5.3 A-D**, **grey bars**). From the metagenomic data, on average, 0.03% of all the bacteria in the 5 samples belonged to the family Pasteurellaceae. The second colony type was extremely small cream colonies and according to the isolated DNA, these colonies were identified as *Pseudomonas* spp. (**Figure 5.3 A-D, red bars**). From the metagenomic data, on average, 5% of all the bacteria in the five samples belonged to the family *Pseudomonadaceae.* The third colony type was bright yellow colonies with a smooth edge. The colonies were identified as *Shinella* spp. *Shinella* spp belong to the family Rhizobiaceae and on average, 7% of the

population in the five samples belonged to the Rhizobiaceae family. The fourth colony type was a flat translucent colony with an irregular edge. These bacteria were also identified as *Pseudomonas* spp. (**Figure 5.3 A-D, blue bars**). These were also the flat translucent colonies that only appeared after treatment. The fifth colony type was a white irregular colony while the sixth colony was a pale-yellow with a smooth edge and both were identified as *Pseudomonas* spp.

5.4 CONCLUSION

In the high-throughput screening work presented in the previous chapter, Decont-A exhibited antibiofilm activity at 0.5 ppm and improved antibiofilm activity was observed with the addition of 10 µg/mL LOL biosurfactant in industrial wastewater. Those results informed the parameters used for continuous biofilm assessment performed in the CEMS system in this reporting period. We first conducted preliminary tests with 2 different conditions: Decont-A biocide (0.5, 2 ppm) with and without 10 µg/mL LOL biosurfactant. Unfortunately, this did not translate to the CEMS system and higher concentrations of Decont-A were needed to disperse the biofilm. This suggests that the biofilms formed in the 96-well plates versus that formed in the CEMS system are not entirely comparable, and although high-throughput assays can be a valuable tool, there are well-known limitations associated with the assay (Haney *et al.,* 2018). For instance, this assay does not take into account the complexity of biofilms formed under sheer stress. Furthermore, biofilms formed in continuous flow systems are more robust and thus, can be more resistant to antibiofilm treatments (Rath *et al.,* 2017).

When using a higher concentration of Decont-A (60 ppm), a slight (8%) drop was seen in the CO_2 produced by the biofilm, although no significant difference was observed in the colony counts indicating that the biofilm was only slightly dispersed. When Decont-A was co-formulated with LOL, a significant (23%) decrease in the CO_2 produced by the biofilm was measured, but once again the colony counts did not disappear, indicating that the Decont-A-LOL co-formulation also caused dispersion of the biofilm. The significant increase in activity observed indicated that the dispersal activity of Decont-A was improved by the addition of LOL. A spike in metabolic activity was seen when the biofilms were treated with the co-formulation and Decont-A--this increase was likely due to stress (Jackson *et al.,* 2015). Overall, there is sufficient evidence to conclude that the LOL improved the activity of Decont-A by 19%, although more repeats and optimization is needed to give recommendations regarding the application of the Decont-A and LOL co-formulation.

Although not performed quantitatively, the metagenomic analyses highlight the level of antibiotic resistance that has evolved in these biofilms. Therefore, the targeted effect of the LOL on the antimicrobial-resistant members of the community, which are typically the most difficult to eradicate and why biofilms are recalcitrant to antibiotic treatments (Trubenová *et al*., 2022), is highly significant. Multifaceted resistance and tolerance mechanisms contribute to the overall recalcitrance of biofilms to antimicrobial treatment, not only the presence of antimicrobial resistance genes (Orazi *et al*., 2019). However, this study lays the groundwork for further studies to elucidate the multiple mechanisms that contribute to drug susceptibility of polymicrobial biofilms in industrial wastewater systems. The ability to track the biofilm response to these treatments at the genome level may enable us to uncover novel ways in which polymicrobial interactions can impact microbial physiology, while also devising novel strategies to manage these problem biofilms in industry settings. This highlights the importance of continuing to investigate mixed-species communities rather than in isolation which will bring greater understanding of how interspecies interactions alter the efficacy of agents used to manage biofilm-related problems in various industries.

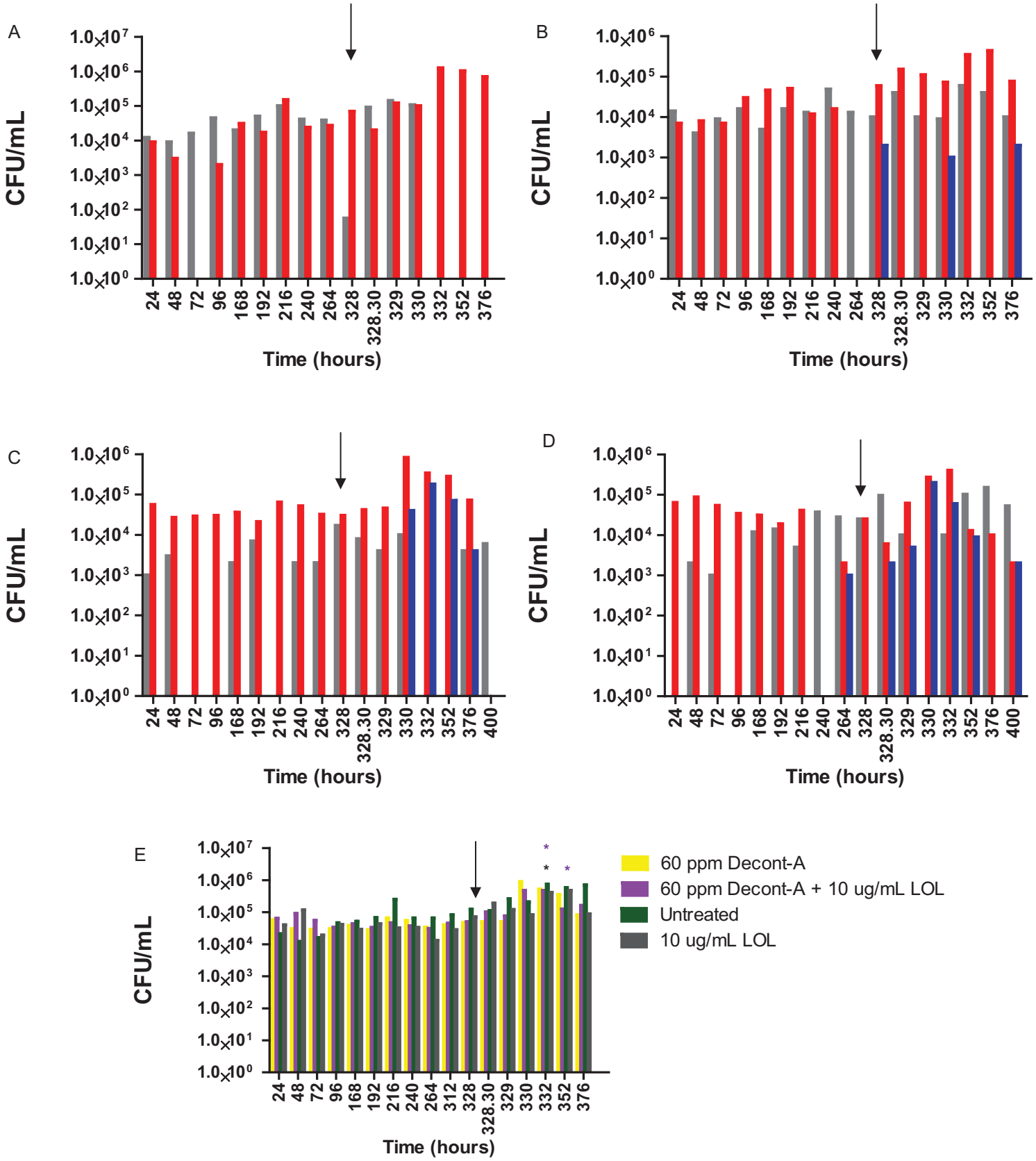


Figure 5.3. Planktonic cell yields from the biofilm communities before and after treating with (A) sterile wastewater, (B) 10 µg/mL LOL, (C) 60 ppm Decont-A, and (D) 60 ppm Decont-A and 10 µg/mL LOL. The red bars represent the small colonies (*Pseudomonas* spp.), grey bars the big colonies (*Pasteurella* spp.), and the blue bars represent the flat translucent colonies (*Pseudomonas* spp.). Graphs (A) to (D) represent the counts of the different colonies identified on the agar plates while (E) represents the total

number of colonies on the agar plates. In graph (E) the yellow bars represent the total number of colonies after treating with 60 ppm Decont-A, purple bars represent the total number of colonies after treating with 60 ppm Decont-A and 10 µg/mL LOL, green bars represent the total number of colonies of an untreated biofilm, and black bars represent the total number of colonies after treating with 10 µg/mL LOL.

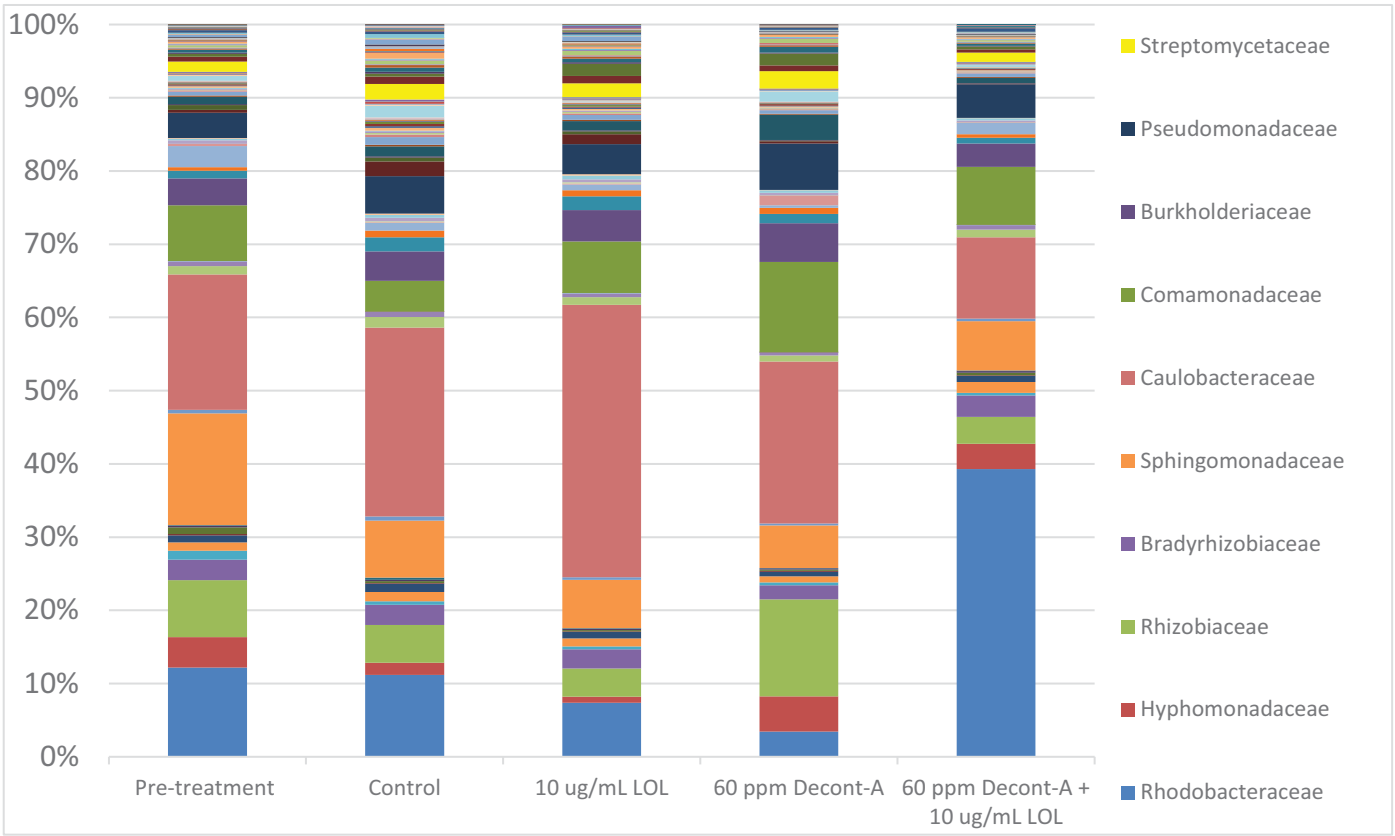


Figure 5.4. Stacked bar graph depicting the family-level analysis of the microbial community composition following treatment of biofilms with 10 µg/mL lyso-ornithine lipid (LOL), 60 ppm Decont-A, or 60 ppm Decont+ 10 µg/mL LOL. An untreated biofilm was used as a control. Families with significant differences between pre- and post-treatment is indicated in the legend.

Table 5.1 Antibiotic resistance genes identified in the 5 metagenomic datasets generated from the DNA isolated from the biofilm effluent. Genes identified as responsible for antibiotic resistance are indicated in brackets

Pre- Control	Post- Control	Post- Decont-A	Post- LOL	Post- Decont-A + LOL
Ciprofloxacin (qepA4)	-	-	-	Ciprofloxacin (OqxB)
nalidixic acid (OqxB)	-	-	-	nalidixic acid (OqxB)
unknown beta-lactam (blaSGM)	-	-	-	-
Trimethoprim (OqxB)	-	-	-	Trimethoprim (OqxB)
-	-	-	Tetracycline (tmexD2)	Tetracycline (tmexD4)
-	-	-	Doxycycline (tmexD2)	Doxycycline (tmexD4)
-	-	-	Minocycline (tmexD2)	Minocycline (tmexD4)
-	-	-	Tigecycline (tmexD2)	Tigecycline (tmexD4)
Chloramphenicol (OqxB)	-	-	-	Florfenicol (OqxB)

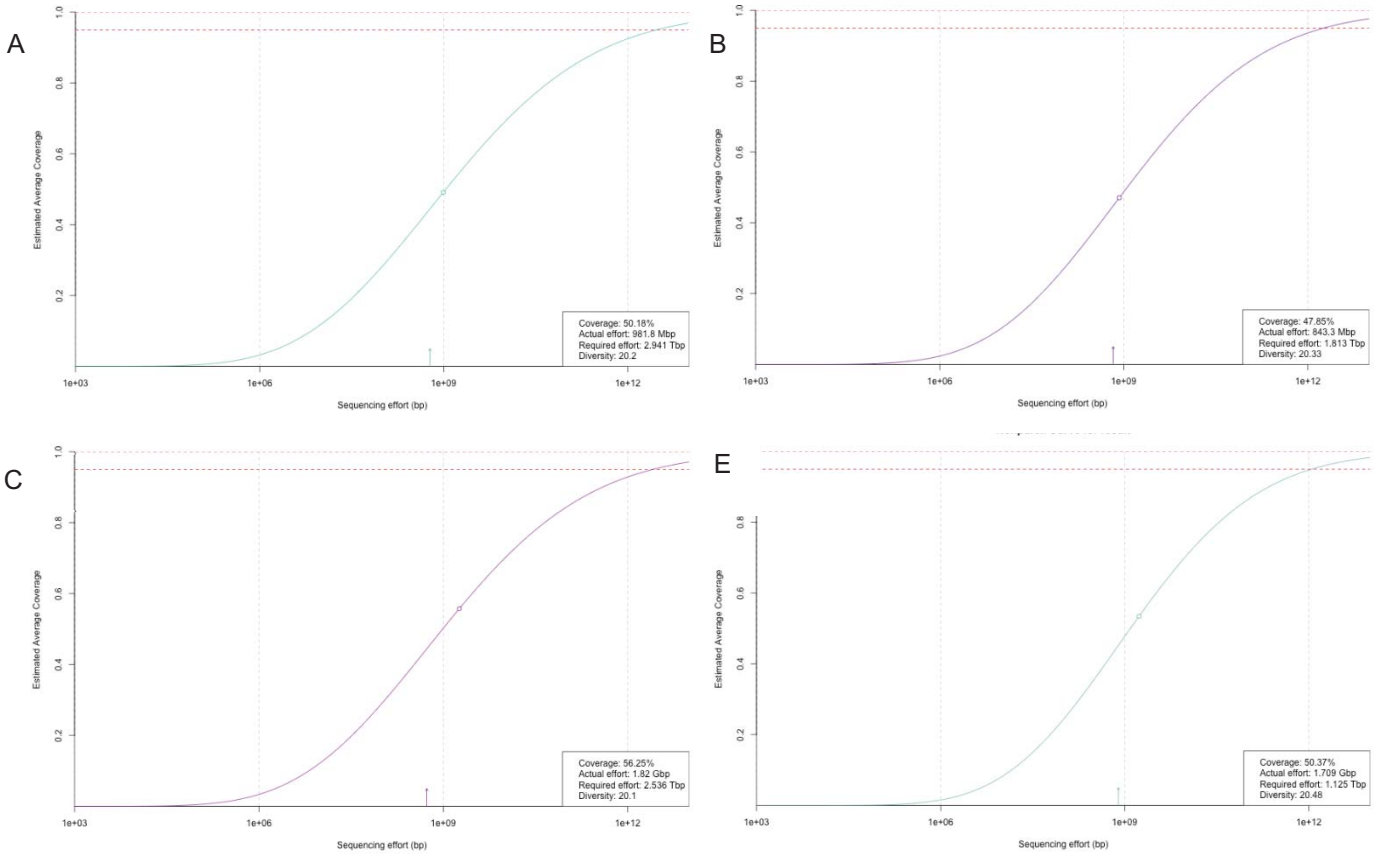


Figure 5.5. Nonpareil curves (Rodriquez-R *et al.,* 2018) of the industrial wastewater metagenomic sequence datasets showing estimated coverage. (A) Decont-A pre-treatment, (B) Decont-A + LOL co-formulation pre-treatment, (C) Decont-A post-treatment, and (D) Decont-A + LOL co-formulation post-treatment.

CHAPTER 6: CONCLUSIONS & RECOMMENDATIONS

6.1 CONCLUSIONS

Surfactants have long been used in combination with biocides to improve antibiofilm activity, but the increase in environmental legislation necessitates the introduction of more environmentally friendly antibiofilm agents. In this report we reported on the co-formulation of a biocide, Decont-A, and a recombinantly expressed biosurfactant (lysine-ornithine lipid, LOL). Optimisation studies proved that expressing LOL in *E. coli* BL21 at 25°C with 1 mM IPTG, 20 mM L-ornithine, and at a medium pH between 7 and 8 resulted in the highest concentration of produced and purified LOL.

The Decont-A and LOL co-formulation was tested for antibiofilm activity in 96-well high-throughput assays, these assays indicated that 0.5 ppm Decont-A + 10 µg/mL LOL was able to effectively prevent biofilm formation in three different sources of industrial water. This dose (0.5 ppm Decont-A + 10 µg/mL LOL) was not an effective antibiofilm agent. According to the proprietors, Decont-A is used at a maintenance concentration range of 2 - 4 ppm to manage biofilms in industrial wastewater. However, under certain conditions, and especially in power station setups, a slug dosing of Decont-A is also used where the initial treatment concentration can vary between 18 and 60 ppm. During the trial runs of Decont-A at the Lethabo power station, the in-flow of water was 90 megaliters of water per hour. To this 1 ton of Decont-A was added to the system at a flow of 45 megaliters of water per hour, achieving a final concentration of 18 ppm. Thus, the dose of Decont-A was increased to 60 ppm in this study and was found to be more effective. Furthermore, co-formulation with LOL significantly improved the antibiofilm activity of Decont-A. Of course, the CEMS system does not fully replicate a cooling tower, but there is now a precedent for the requirement of varied and significantly higher concentrations of biocide for the effective treatment of the biofilms in industrial wastewaters.

The metagenomic analysis identified the dominant taxa in the cooling tower pond 1 water to belong to the families Caulobacteraceae, Sphingomonadaceae, Rhodobacteraceae, Rhizobiaceae, Comamonadaceae, Hyphomonadaceae, Burkholderiaceae, and Pseudomonadaceae. Similar microbial profiles were identified in other industrial waters. Significant changes were observed between the different treatment groups indicating that treating the biofilm with Decont-A and LOL had significant influences on the microbial community present in water. This forms the foundation for future studies to determine the mode of action of the developed formulation and to perform more detailed assessment of the community evolution, especially with respect to developing resistance to the applied treatment.

6.2 RECOMMENDATIONS

Due to the dynamic nature of microbial biofilms, even for a homogenous population, significant optimisation and fine tuning for each system (experimental conditions and microorganism(s)) was required. The biofilm we established in this project is heterogenous and has never been assessed in a CEMS system before. The data suggests that this complex community structure is vastly different to reference studies, where up to 328 hours (~2 weeks) is required for the biofilms to establish before any treatments can be conducted. This results in each experiment requiring 1 month to complete, and not 1 week as anticipated based on the reference studies for homogenous biofilms. For future studies we recommend taking into account that biofilm communities from industrial environments might take significantly longer to establish.

We also recommend that more time is allocated to optimising the continuous flow systems. As shown in this report, the data from the 96-well high-throughput assays did not correlate to the performance in the continuous

flow system setup. The optimization had to be repeated using the continuous flow system, which took significantly longer than expected.

REFERENCES

ABDEL-MAWGOUD, AM, LEPINE, F, DEZIEL, E (2010) Rhamnolipids: diversity of structures, microbial origins and roles. *Appl. Microbiol. Biotechnol*, **86 (5)** 1323-36.

ABDELLI, F, JARDAK, M, ELLOUMI, J, STIEN, D, CHERIF, S, MNIF, S, AIFA, S (2019) Antibacterial, anti-adherent and cytotoxic activities of surfactin(s) from a lipolytic strain *Bacillus safensis* F4. *Biodegradation*, **30 (4)** 287-300.

ADNAN, M, PATEL, M, DESHPANDE, S, ALRESHIDI, M, SIDDIQUI, AJ, REDDY, MN, EMIRA, N, DE FEO, V (2020) Effect of adiantum philippense extract on biofilm formation, adhesion with its antibacterial activities against foodborne pathogens, and characterization of bioactive metabolites: An in vitro-in silico approach. *Front.Microbiol.*, **11** 823.

ALEMAN-VEGA, M, SANCHEZ-LOZANO, I, HERNANDEZ-GUERRERO, CJ, HELLIO, C, QUINTANA, ET (2020) Exploring antifouling activity of biosurfactants producing marine bacteria isolated from Gulf of California. *Int. J. Mol. Sci.*, **21 (17)** 6068.

AQUATECH (2019) Industrial water: Our essential guide to pollution, treatment & solutions.

ARAUJO, LVd, GUIMARÃES, CR, MARQUITA, RLdS, SANTIAGO, VMJ, DE SOUZA, MP, NITSCHKE, M, FREIRE, DMG (2016) Rhamnolipid and surfactin: Anti-adhesion/antibiofilm and antimicrobial effects. *Food Control*, **63** 171-178.

AYDIN, S, CAN, K (2020) Pyophage cocktail for the biocontrol of membrane fouling and its effect in aerobic microbial biofilm community during the treatment of antibiotics. *Bioresource Technology*, **318** 123965.

AZEREDO, J, PACHECO, AP, LOPES, I, OLIVEIRA, R, VIEIRA, MJ (2003) Monitoring cell detachment by surfactants in a parallel plate flow chamber. *Water Sci. Technol.*, **47 (5)** 77-82.

BEECH, I, BERGEL, A, MOLLICA, A, FLEMMING, H, SCOTTO, V, SAND, W (2000) Microbially influenced corrosion of industrial materials. *Recent Advances in the Study of Bio-corrosion*, **2** 00-02.

BEECH, IB, SUNNER, J (2004) Biocorrosion: towards understanding interactions between biofilms and metals. *Curr.Opin.Biotechnol.*, **15 (3)** 181-6.

BENINCASA, M, ABALOS, A, OLIVEIRA, I, MANRESA, A (2004) Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. *Antonie Van Leeuwenhoek*, **85 (1)** 1-8.

BERESCHENKO, LA, PRUMMEL, H, EUVERINK, GJ, STAMS, AJ, VAN LOOSDRECHT, MC (2011) Effect of conventional chemical treatment on the microbial population in a biofouling layer of reverse osmosis systems. *Water Research*, **45 (2)** 405-16.

BHADANI, A, KAFLE, A, OGURA, T, AKAMATSU, M, SAKAI, K, SAKAI, H, ABE, M (2020) Current perspective of sustainable surfactants based on renewable building blocks.COCIS*COCIS*, **45** 124-135.

BHAGWAT, A, MIXON, M, COLLINS, CH, DORDICK, JS (2020) Opportunities for broadening the application of cell wall lytic enzymes. *Appl.Microbiol.Biotechnol.*, **104 (21)** 9019-9040.

BODOUR, AA, GUERRERO-BARAJAS, C, JIORLE, BV, MALCOMSON, ME, PAULL, AK, SOMOGYI, A, TRINH, LN, BATES, RB, MAIER, RM (2004) Structure and characterization of flavolipids, a novel class of biosurfactants produced by *Flavobacterium* sp. strain MTN11. *Appl. Environ.Microbiol.*, **70 (1)** 114-20.

BOTT, TR (2009) Biofouling Control in Cooling Water. *Int. J. Chem. Eng.*, **2009** 1-4.

BOTT, TR, TIANQING, L (2004) Ultrasound enhancement of biocide efficiency. *Ultrasonics Sonochemistry*, **11 (5)** 323-326.

CERI, H, OLSON, ME, STREMICK, C, READ, R, MORCK, D, BURET, A (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. microbiol.*, **37 (6)** 1771-1776.

CLOETE, E, FLEMMING, H-C (2012) Environmental impact of cooling water treatment for biofouling and biocorrosion control. in: *Operational and environmental consequences of large industrial cooling water systems*, Springer. Boston, MA, pp. 303-314.

CLOETE, TE, BRÖZEL, VS, VON HOLY, A (1992) Practical aspects of biofouling control in industrial water systems. *Int. Biodeterior. Biodegradation*, **29 (3-4)** 299-341.

CLOETE, TE, JACOBS, L, BRÖZEL, VS (1998) The chemical control of biofouling in industrial water systems. *Biodegradation*, **9 (1)** 23-37.

COSGROVE, WJ, LOUCKS, DP (2015) Water management: Current and future challenges and research directions. *Water Resources Research*, **51 (6)** 4823-4839.

COSTERTON, JW, CHENG, K, GEESEY, GG, LADD, TI, NICKEL, JC, DASGUPTA, M, MARRIE, TJ (1987) Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.*, **41 (1)** 435-464.

CUI, Y, GAO, H, YU, R, GAO, L, ZHAN, M (2021) Biological-based control strategies for MBR membrane biofouling: a review. *Water Sci. Technol.*, **83 (11)** 2597-2614.

DAS, P, MUKHERJEE, S, SEN, R (2009) Antiadhesive action of a marine microbial surfactant. *Colloids Surf. B..*, **71 (2)** 183-6.

DE FARIA, AF, TEODORO-MARTINEZ, DS, DE OLIVEIRA BARBOSA, GN, GONTIJO VAZ, B, SERRANO SILVA, Í, GARCIA, JS, TÓTOLA, MR, EBERLIN, MN, GROSSMAN, M, ALVES, OL, REGINA DURRANT, L (2011) Production and structural characterization of surfactin (C14/Leu7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. *Process Biochemistry*, **46 (10)** 1951-1957.

DE, S, MALIK, S, GHOSH, A, SAHA, R, SAHA, B (2015) A review on natural surfactants. *RSC advances*, **5 (81)** 65757-65767.

DESAI, JD, BANAT, IM (1997a) Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.*, **61 (1)** 47-64.

DESAI, JD, BANAT, IM (1997b) Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev*, **61 (1)** 47-64.

DEVERS, EA, WEWER, V, DOMBRINK, I, DORMANN, P, HOLZL, G (2011) A processive glycosyltransferase involved in glycolipid synthesis during phosphate deprivation in *Mesorhizobium loti*. *J Bacteriol*, **193 (6)** 1377-84.

DENVIR, A, VELA, D, DELEGARD, A (2019) Biofilms in industrial water systems: Metagenomic insight on biofilm populations from a new real-time biomonitoring system. *Corrosion*, NACE-2019-13504

DI PIPPO, F, DE TORA, F, DI GREGORIO, L, BUCCOLINI, M, CAPOCECERA, R, ROSSETTI, S, TANDOI, V (2017) Green bio-dispersant removal efficacy estimation for controlling biofilms in cooling towers. *Ann. Microbiol.*, **67 (11)** 779-784.

DI PIPPO, F, DI GREGORIO, L, CONGESTRI, R, TANDOI, V, ROSSETTI, S (2018) Biofilm growth and control in cooling water industrial systems. *FEMS Microbiol. Ecol.*, **94 (5)** fiy044.

DIAZ DE RIENZO, MA, BANAT, IM, DOLMAN, B, WINTERBURN, J, MARTIN, PJ (2015) Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. *Nat. Biotechnol.*, **32 (6)** 720-6.

DIEDEREN, BM (2008) Legionella spp. and Legionnaires' disease. *J. Infect.*, **56 (1)** 1-12.

DISHA, SS, SAHASRABUDHE, S (2018) Biosurfactant: Classification, properties and recent application in cosmetic. *J. Emerg. Technol. Innov. Res.*, **5 (10)** 160-167.

DONLAN, RM (2000) Biofilm control in industrial water systems: approaching an old problem in new ways. *Biofilms: recent advances in their study and control* 333-359.

DU, M, GAO, RJ, GONG, P, WANG, QZ (2004) The new-generation of green surfactant (alkylpolyglucoside) as an inhibitor to the corrosion of 907 steel in seawater. *Chin. Chem. Lett.*, **15 (8)** 985-988.

DUSANE, DH, DAM, S, NANCHARAIAH, YV, KUMAR, AR, VENUGOPALAN, VP, ZINJARDE, SS (2012) Disruption of *Yarrowia lipolytica* biofilms by rhamnolipid biosurfactant. *Aquatic Biosystems*, **8 (1)** 1-7.

DUSANE, DH, PAWAR, VS, NANCHARAIAH, YV, VENUGOPALAN, VP, KUMAR, AR, ZINJARDE, SS (2011) Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens*. *Biofouling*, **27 (6)** 645-54.

ELSHIKH, M, FUNSTON, S, CHEBBI, A, AHMED, S, MARCHANT, R, BANAT, IM (2017) Rhamnolipids from non-pathogenic *Burkholderia thailandensis* E264: Physicochemical characterization, antimicrobial and antibiofilm efficacy against oral hygiene related pathogens. *N. Biotechnol*, **36** 26-36.

FAY, F, CARTEAU, D, LINOSSIER, I, DELBURY, M, VALLEE-REHEL, K (2013) Joint-action of antifouling substances in copper-free paints. *Colloids Surf. B.*, **102** 569-77.

FLEMMING, H-C, NEU, TR, WINGENDER, J (2016a) *The perfect slime: microbial extracellular polymeric substances (EPS)*. IWA publishing.

FLEMMING, HC, WINGENDER, J (2010) The biofilm matrix. *Nat. Rev. Microbiol.*, **8 (9)** 623-33.

FLEMMING, HC, WINGENDER, J, SZEWZYK, U, STEINBERG, P, RICE, SA, KJELLEBERG, S (2016b) Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.*, **14 (9)** 563-75.

FLEMMING, HC, WUERTZ, S (2019) Bacteria and archaea on Earth and their abundance in biofilms. *Nat. Rev. Microbiol.*, **17 (4)** 247-260.

FOLEY, P, KERMANSHAHI POUR, A, BEACH, ES, ZIMMERMAN, JB (2012) Derivation and synthesis of renewable surfactants. *Chem. Soc. Rev.*, **41 (4)** 1499-1518.

GALLEZOT, P (2012) Conversion of biomass to selected chemical products. *Chem. Soc. Rev.*, **41 (4)** 1538-58.

GAO, JL, WEISSENMAYER, B, TAYLOR, AM, THOMAS-OATES, J, LÓPEZ-LARA, IM, GEIGER, O (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Mol. Microbiol.*, **53 (6)** 1757-1770.

GIRI, SS, RYU, EC, SUKUMARAN, V, PARK, SC (2019) Antioxidant, antibacterial, and anti-adhesive activities of biosurfactants isolated from *Bacillus* strains. *Microb. Pathog.*, **132** 66-72.

GONZALEZ-SILVA, N, LOPEZ-LARA, IM, REYES-LAMOTHE, R, TAYLOR, AM, SUMPTON, D, THOMAS-OATES, J, GEIGER, O (2011) The dioxygenase-encoding olsD gene from *Burkholderia cenocepacia* causes the hydroxylation of the amide-linked fatty acyl moiety of ornithine-containing membrane lipids. *Biochemistry*, **50 (29)** 6396-408.

GRASLAND, B, MITALANE, J, BRIANDET, R, QUEMENER, E, MEYLHEUC, T, LINOSSIER, I, VALLEE-REHEL, K, HARAS, D (2003) Bacterial biofilm in seawater: cell surface properties of early-attached marine bacteria. *Biofouling*, **19 (5)** 307-13.

GUDIÑA, EJ, FERNANDES, EC, TEIXEIRA, JA, RODRIGUES, LR (2015) Antimicrobial and anti-adhesive activities of cell-bound biosurfactant from *Lactobacillus agilis* CCUG31450. *RSC Adv.*, **5 (110)** 90960-90968.

HENKEL, M, MÜLLER, MM, KÜGLER, JH, LOVAGLIO, RB, CONTIERO, J, SYLDATK, C, HAUSMANN, R (2012) Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochemistry*, **47 (8)** 1207-1219.

INFANTE, M, PINAZO, A, SEGUER, J (1997) Non-conventional surfactants from amino acids and glycolipids: structure, preparation and properties. *Colloids Surf.Colloids Surf. A: Physicochem. Eng.*, **123** 49-70.

INFANTE, MR, PÉREZ, L, PINAZO, A, CLAPÉS, P, MORÁN, MC, ANGELET, M, GARCÍA, MT, VINARDELL, MP (2004) Amino acid-based surfactants. *Comptes Rendus Chimie*, **7 (6-7)** 583-592.

JANEK, T, DRZYMALA, K, DOBROWOLSKI, A (2020) In vitro efficacy of the lipopeptide biosurfactant surfactin-C15 and its complexes with divalent counterions to inhibit *Candida albicans* biofilm and hyphal formation. *Biofouling*, **36 (2)** 210-221.

JAYARAMAN, A, HALLOCK, P, CARSON, R, LEE, C-C, MANSFELD, F, WOOD, T (1999) Inhibiting sulfate-reducing bacteria in biofilms on steel with antimicrobial peptides generated in situ. *Appl. Microbiol. Biotechnol.* , **52 (2)** 267-275.

JIMOH, AA, LIN, J (2019) Biosurfactant: A new frontier for greener technology and environmental sustainability. *Ecotoxicol. Environ. Saf.*, **184** 109607.

JIMOH, AA, BOOYSEN, E, VAN ZYL, L, TRINDADE, M (2023) Do biosurfactants as anti-biofilm agents have a future in industrial water systems? *Front. Bioeng. Biotechnol.*, **11** 1244595.

KAYANADATH, S, NATHAN, VK, AMMINI, P (2019) Anti-biofilm activity of biosurfactant derived from *Halomonas* sp., a Lipolytic marine bacterium from the Bay of Bengal. *Microbiology*, **88 (5)** 585-599.

KIROV, SM (2003) Bacteria that express lateral flagella enable dissection of the multifunctional roles of flagella in pathogenesis.*FEMS Microbiol. Lett.*, **224 (2)** 151-159.

KITAMOTO, D, MORITA, T, FUKUOKA, T, KONISHI, M-a, IMURA, T (2009) Self-assembling properties of glycolipid biosurfactants and their potential applications. *COCIS*, **14 (5)** 315-328.

KLEBENSBERGER, J, RUI, O, FRITZ, E, SCHINK, B, PHILIPP, B (2006) Cell aggregation of *Pseudomonas aeruginosa* strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate. *Archives of Microbiology*, **185 (6)** 417-27.

KLOPPER, KB, DE WITT, RN, BESTER, E, DICKS, LM, WOLFAARDT, GM (2020) Biofilm dynamics: linking in situ biofilm biomass and metabolic activity measurements in real-time under continuous flow conditions. *NPJ biofilms and microbiomes*, **6 (1)** 1-10.

KRETSCHNER, A, BOCK, H, WAGNEE, F (1982) Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on n-alkane. *Applied and Environmental Microbioliogy*, **44** 864-870.

KRISTOFFERSEN, V, JENSSEN, M, JAWAD, HR, ISAKSSON, J, HANSEN, EH, RAMA, T, HANSEN, KO, ANDERSEN, JH (2021) Two novel lyso-ornithine lipids isolated from an arctic marine *Lacinutrix* sp. bacterium. *Molecules*, **26 (17)** 5295.

KUIPER, I, LAGENDIJK, EL, PICKFORD, R, DERRICK, JP, LAMERS, GE, THOMAS-OATES, JE, LUGTENBERG, BJ, BLOEMBERG, GV (2004) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol. Microbiol.*, **51 (1)** 97-113.

LEJEUNE, C, ABREU, S, CHAMINADE, P, DULERMO, T, DAVID, M, WERTEN, S, VIROLLE, MJ (2021) Impact of phosphate availability on membrane lipid content of the model strains, *Streptomyces lividans* and *Streptomyces coelicolor*. *Front. microbiol.*, **12** 623919.

LEWENZA, S, FALSAFI, R, BAINS, M, ROHS, P, STUPAK, J, SPROTT, GD, HANCOCK, RE (2011) The *olsA* gene mediates the synthesis of an ornithine lipid in *Pseudomonas aeruginosa* during growth under phosphate-limiting conditions, but is not involved in antimicrobial peptide susceptibility.*FEMS Microbiol. Lett.*, **320 (2)** 95-102.

LI, C, LIANG, J, YANG, Y, PU, J, HOU, L-a (2018) Novel insights into the role of *Pseudomonas* quinolone signal in the control of reverse osmosis membrane biofouling. *J. Membr. Sci.*, **563** 505-512.

LIU, Y, ZHANG, W, SILEIKA, T, WARTA, R, CIANCIOTTO, NP, PACKMAN, A (2009) Role of bacterial adhesion in the microbial ecology of biofilms in cooling tower systems. *Biofouling*, **25 (3)** 241-53.

LIU, Y, ZHANG, W, SILEIKA, T, WARTA, R, CIANCIOTTO, NP, PACKMAN, AI (2011) Disinfection of bacterial biofilms in pilot-scale cooling tower systems. *Biofouling*, **27 (4)** 393-402.

LOHAN, S, CAMEOTRA, SS, BISHT, GS (2013) Systematic study of non-natural short cationic lipopeptides as novel broad-spectrum antimicrobial agents. *Chem. Biol. Drug. Des.*, **82 (5)** 557-566.

LOVAGLIO, RB, DOS SANTOS, FJ, JUNIOR, MJ, CONTIERO, J (2011) Rhamnolipid emulsifying activity and emulsion stability: pH rules. *Colloids Surf.Colloids Surf. B.*, **85 (2)** 301-305.

LUDENSKY, M (2003) Control and monitoring of biofilms in industrial applications. *Int. Biodeterior. Biodegradation* **51 (4)** 255-263.

LUTTERBACH, M, DE FRANCA, F (1996) Biofilm formation in water cooling systems. *World J. Microbiol. Biotechnol.*, **12 (4)** 391-394.

MALIK, MA, HASHIM, MA, NABI, F, AL-THABAITI, SA, KHAN, Z (2011) Anti-corrosion ability of surfactants: a review. *Int. J. Electrochem. Sci.*, **6 (6)** 1927-1948.

MANEERAT, S, BAMBA, T, HARADA, K, KOBAYASHI, A, YAMADA, H, KAWAI, F (2006) A novel crude oil emulsifier excreted in the culture supernatant of a marine bacterium, *Myroides* sp. strain SM1. *Appl. Microbiol. Biotechnol.*, **70 (2)** 254-9.

MARIA DA GLORIA, C, SARUBBO, LA (2021) Synthetic and biological surfactants used to mitigate biofouling on industrial facilities surfaces. *Biointerface Res. Appl. Chem.*, **12 (2)** 2560-2585.

MCDONNELL, G, RUSSELL, AD (1999) Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev.*, **12 (1)** 147-179.

MEYLHEUC, T, RENAULT, M, BELLON-FONTAINE, MN (2006) Adsorption of a biosurfactant on surfaces to enhance the disinfection of surfaces contaminated with *Listeria monocytogenes*. *Int. J. Food Microbiol.*, **109 (1-2)** 71-8.

MEYLHEUC, T, VAN OSS, C, BELLON-FONTAINE, MN (2001) Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes* LO28. *J. Appl. Microbiol.*, **91 (5)** 822-832.

MILLANAR-MARFA, JMJ, BOREA, L, HASAN, SW, DE LUNA, MDG, BELGIORNO, V, NADDEO, V (2020) Advanced membrane bioreactors for emerging contaminant removal and quorum sensing control. in: *Current Developments in Biotechnology and Bioengineering*, Elsevier, pp. 117-147.

MIRELES, JR, 2nd, TOGUCHI, A, HARSHEY, RM (2001) *Salmonella enterica* serovar typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. *J. Bacteriol.*, **183 (20)** 5848-54.

MISHRA, R, PANDA, AK, DE MANDAL, S, SHAKEEL, M, BISHT, SS, KHAN, J (2020) Natural anti-biofilm agents: Strategies to control biofilm-forming pathogens. *Front. microbiol.*, **11** 566325.

MOLOBELA, IP, CLOETE, TE, BEUKES, M (2010) Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria. *Afr. J. Microbiol. Res.*, **4 (14)** 1515-1524.

NDLOVU, T, RAUTENBACH, M, VOSLOO, JA, KHAN, S, KHAN, W (2017) Characterisation and antimicrobial activity of biosurfactant extracts produced by Bacillus amyloliquefaciens and *Pseudomonas aeruginosa* isolated from a wastewater treatment plant. *AMB Express*, **7 (1)** 108.

NEU, TR (1996) Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol. Rev.*, **60 (1)** 151-166.

NICKZAD, A, DÉZIEL, É (2014) The involvement of rhamnolipids in microbial cell adhesion and biofilm development–an approach for control? *Lett. Appl. Microbiol.*, **58 (5)** 447-453.

OLASANMI, I, THRING, R (2018) The role of biosurfactants in the continued drive for environmental sustainability. *Sustainability*, **10 (12)**.

ORAZI, G, O'TOOLE, GA (2019) "It Takes a Village": Mechanisms Underlying Antimicrobial Recalcitrance of Polymicrobial Biofilms. *J Bacteriol*. **202 (1)** e00530-19.

PACWA-PLOCINICZAK, M, PLAZA, GA, PIOTROWSKA-SEGET, Z, CAMEOTRA, SS (2011) Environmental applications of biosurfactants: recent advances. *Int. J. Mol. Sci.*, **12 (1)** 633-54.

PADMAVATHI, AR, PANDIAN, SK (2014) Antibiofilm activity of biosurfactant producing coral associated bacteria isolated from gulf of mannar. *Indian J. Microbiol.*, **54 (4)** 376-82.

PALMER, M, HATLEY, H (2018) The role of surfactants in wastewater treatment: Impact, removal and future techniques: A critical review. *Water Res.*, **147** 60-72.

PARASZKIEWICZ, K, MORYL, M, PLAZA, G, BHAGAT, D, S, KS, BERNAT, P (2021) Surfactants of microbial origin as antibiofilm agents. *Int. J. Environ. Health Res.*, **31 (4)** 401-420.

PARTHIPAN, P, SABARINATHAN, D, ANGAIAH, S, RAJASEKAR, A (2018) Glycolipid biosurfactant as an eco-friendly microbial inhibitor for the corrosion of carbon steel in vulnerable corrosive bacterial strains. *J. Mol. Liq.* **(261)** 473-479.

PEELE KARLAPUDI, A, VENKATESWARULU, TC, SRIRAMA, K, KOTA, RK, MIKKILI, I, KODALI, VP (2018) Evaluation of anti-cancer, anti-microbial and anti-biofilm potential of biosurfactant extracted from an *Acinetobacter* M6 strain. *J. King Saud Univ. Sci.*, **32 (1)** 223-227.

PEI, HY, HU, WR, LIU, QH (2010) Effect of protease and cellulase on the characteristic of activated sludge. *J. Hazard. Mater.* **178 (1-3)** 397-403.

PERCIVAL, SL, MAYER, D, KIRSNER, RS, SCHULTZ, G, WEIR, D, ROY, S, ALAVI, A, ROMANELLI, M (2019) Surfactants: Role in biofilm management and cellular behaviour. *Int. Wound J.*, **16 (3)** 753-760.

PEREIRA, RPA, PEPLIES, J, HOFLE, MG, BRETTAR, I (2017) Bacterial community dynamics in a cooling tower with emphasis on pathogenic bacteria and *Legionella* species using universal and genus-specific deep sequencing. *Water Research*, **122** 363-376.

PINHEIRO, L, FAUSTINO, C (2017) Amino acid-based surfactants for biomedical applications. in: *Application and characterization of surfactants*, InTech.

PŁAZA, G, ACHAL, V (2020) Biosurfactants: Eco-friendly and innovative biocides against biocorrosion. *Int. J. Mol. Sci.*, **21 (6)**.

PLETZER, D, COLEMAN, SR, HANCOCK, RE (2016) Anti-biofilm peptides as a new weapon in antimicrobial warfare. *Curr. Opin. Microbiol.*, **33** 35-40.

PUREVDORJ, B, COSTERTON, JW, STOODLEY, P (2002) Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.Appl. Environ. Microbiol.*, **68 (9)** 4457-64.

PURWASENA, IA, ASTUTI, DI, FAUZIYYAH, NA, PUTRI, DAS, SUGAI, Y (2019) Inhibition of microbial influenced corrosion on carbon steel ST37 using biosurfactant produced by *Bacillus* sp. *Mater. Res. Express*, **6 (11)** 115405.

RABINOVICH, YI, PANDEY, S, SHAH, DO, MOUDGIL, BM (2006) Effect of the chain-length compatibility of surfactants and mechanical properties of mixed micelles on surfaces. *Langmuir*, **22 (16)** 6858-6862.

RADZUAN, MN, BANAT, IM, WINTERBURN, J (2017) Production and characterization of rhamnolipid using palm oil agricultural refinery waste. *Bioresource Technology*, **225** 99-105.

RAJPUT, A, KUMAR, M (2018) Anti-biofilm peptides: a new class of quorum quenchers and their prospective therapeutic applications. in: *Biotechnological Applications of Quorum Sensing Inhibitors*, Springer, pp. 87-110.

RAO, TS (2012) Microbial fouling and corrosion: Fundamentals and mechanisms. in: *Operational and environmental consequences of large industrial cooling water systems*, Springer. Boston, MA, pp. 95-126.

RAUTELA, R, SINGH, AK, SHUKLA, A, CAMEOTRA, SS (2014) Lipopeptides from *Bacillus* strain AR2 inhibits biofilm formation by *Candida albicans*. *Antonie Van Leeuwenhoek*, **105 (5)** 809-21.

REBELLO, S, ASOK, AK, MUNDAYOOR, S, JISHA, MS (2014) Surfactants: toxicity, remediation and green surfactants. *Environ. Chem. Lett.*, **12 (2)** 275-287.

RIVARDO, F, TURNER, RJ, ALLEGRONE, G, CERI, H, MARTINOTTI, MG (2009) Anti-adhesion activity of two biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens. *Appl. Microbiol. Biotechnol.*, **83 (3)** 541-53.

ROJAS-JIMENEZ, K, SOHLENKAMP, C, GEIGER, O, MARTINEZ-ROMERO, E, WERNER, D, VINUESA, P (2005) A CIC chloride channel homolog and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance. *Mol. Plant Microbe*, **18 (11)** 1175-85.

ROMANELLI, MF, MORAES, MCF, VILLAVICENCIO, ALCH, BORRELY, SI (2004) Evaluation of toxicity reduction of sodium dodecyl sulfate submitted to electron beam radiation. *Radiat. Phys. Chem.*, **71 (1-2)** 411-413.

RUFINO, RD, LUNA, JM, SARUBBO, LA, RODRIGUES, LR, TEIXEIRA, JA, CAMPOS-TAKAKI, GM (2011) Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988. *Colloids Surf. B.*, **84 (1)** 1-5.

SACCO, LP, CASTELLANE, TCL, POLACHINI, TC, DE MACEDO LEMOS, EG, ALVES, LMC (2019) Exopolysaccharides produced by *Pandoraea* shows emulsifying and anti-biofilm activities. *J. Polym. Res.*, **26 (4)** 91.

SAŁEK, K, EUSTON, SR (2019) Sustainable microbial biosurfactants and bioemulsifiers for commercial exploitation. *Process Biochem*, **85** 143-155.

SAMBANTHAMOORTHY, K, FENG, X, PATEL, R, PATEL, S, PARANAVITANA, C (2014) Antimicrobial and antibiofilm potential of biosurfactants isolated from *Lactobacilli* against multi-drug-resistant pathogens. *BMC Microbiology*, **14 (1)** 1-9.

SANTOS, E, TEIXEIRA, M, CONVERTI, A, PORTO, A, SARUBBO, L (2018) Production of a new lipoprotein biosurfactant by *Streptomyces* sp. DPUA1566 isolated from lichens collected in the Brazilian Amazon using agroindustry wastes. *Biocatal. Agric. Biotechnol.* , **17** 142-150.

SATPUTE, S, BANPURKAR G, A, BANAT M, I, SANGSHETTI N, J, PATIL H, R, GADE N, W (2016) Multiple roles of biosurfactants in biofilms. *Curr. Pharm. Des.*, **22 (11)** 1429-1448.

SHAHROUR, H, FERRER-ESPADA, R, DANDACHE, I, BÁRCENA-VARELA, S, SÁNCHEZ-GÓMEZ, S, CHOKR, A, MARTINEZ-DE-TEJADA, G (2019) AMPs as anti-biofilm agents for human therapy and prophylaxis. *Antimicrobial Peptides* 257-279.

SHARMA, D, SAHARAN, BS, CHAUHAN, N, BANSAL, A, PROCHA, S (2014) Production and structural characterization of *Lactobacillus helveticus* derived biosurfactant. *Sci. World J.*, **2014** 493548.

SHARMA, J, SUNDAR, D, SRIVASTAVA, P (2021) Biosurfactants: Potential agents for controlling cellular communication, motility, and antagonism. *Front. Mol. Biosci.*, **8** 727070.

SHUKLA, SK, KHAN, A, RAO, TS (2021) Microbial fouling in water treatment plants. in: *Microbial and Natural Macromolecules*, Elsevier, pp. 589-622.

SILVA, M, ALMEIDA, D, DA SILVA, RS, MEIRA, H, ALMEIDA, F, FERNANDES, M, SILVA, A, SARUBBO, L (2018) Chemically modified natural substances screening for biofilms inhibition and biofouling control. *Chem. Eng. Trans.*, **64** 655-660.

SIMÕES, M (2005a) Use of biocides and surfactants to control *Pseudomonas fluorescens* biofilms: Role of the hydrodynamic conditions, Vol. **Ph.D**, University of Minho.

SIMÕES, M, PEREIRA, MO, VIEIRA, M (2003a) Monitoring the effects of biocide treatment of *Pseudomonas fluorescens* biofilms formed under different flow regimes. *Water Sci. Technol.*, **47 (5)** 217-223.

SIMÕES, M, PEREIRA, MO, VIEIRA, MJ (2005b) Action of a cationic surfactant on the activity and removal of bacterial biofilms formed under different flow regimes. *Water Res*, **39 (2-3)** 478-86.

SIMÕES, M, PEREIRA, MO, VIEIRA, MJ (2003b) Effect of different concentrations of ortho-phthalaldehyde on biofilms formed by *Pseudomonas fluorescens* under different flow conditions. *Biofouling*, **19 (5)** 287-95.

SIMÕES, M, PEREIRA, MO, VIEIRA, MJ (2005c) Control of biofilms using surfactants: persistence and regrowth. in: *Biofilms : Persistence and ubiquity*, Biofilm Club. Manchester, pp. 369-38.

SIMÕES, M, SIMÕES, LC, MACHADO, I, PEREIRA, MO, VIEIRA, MJ (2006) Control of flow-generated biofilms with surfactants. *Food Bioprod. Process.*, **84 (4)** 338-345.

SINGH, AK, SHARMA, P (2020) Disinfectant-like activity of lipopeptide biosurfactant produced by *Bacillus tequilensis* strain SDS21. *Colloids Surf. B.*, **185** 110514.

SOBRINHO, HB, LUNA, JM, RUFINO, RD, PORTO, A, SARUBBO, LA (2013) Biosurfactants: Classification, properties and environmental applications. *Recent Developments in Biotechnology*, **11 (14)** 1-29.

SONG, B, WANG, YZ, WANG, GY, LIU, GL, LI, WZ, YAN, F (2016) The lipopeptide 6-2 produced by *Bacillus amyloliquefaciens* anti-CA has potent activity against the biofilm-forming organisms. *Mar. Pollut. Bull.*, **108 (1-2)** 62-9.

SPRECHER, SL,GETSINGER, KD (2000). Zebra mussel chemical control guide.

STALMANS, M (1995) European life-cycle inventory for detergent surfactants production. *Tenside, Surfactants, Deterg.*, **32 (2)** 84-10.

SUN, W, WANG, Y, ZHANG, W, YING, H, WANG, P (2018) Novel surfactant peptide for removal of biofilms. *Colloids Surf. B.*, **172** 180-186.

TAHARA, Y, YAMADA, Y, KONDO, K (1977) Antimicrobial activity of the ornithine-containing lipid isolated from *Gluconobacter cerinus*. *Agric. Biol. Chem.*, **41 (2)** 417-418.

TAKEHARA, M (1989) Properties and applications of amino acid based surfactants. *Colloids Surf.*, **38 (1)** 149-167.

TRUBENOVÁ, B, ROIZMAN, D, MOTER A, ROLFF, J, REGOES, RR (2022) Population genetics, biofilm recalcitrance, and antibiotic resistance evolution. *Trends Microbiol,* **30 (9)** 841-852.

VAN BOGAERT, I (2008) Microbial synthesis of sophorolipids by the yeast *Candida bombicola*. Ghent University.

VAN BOGAERT, IN, SAERENS, K, DE MUYNCK, C, DEVELTER, D, SOETAERT, W, VANDAMME, EJ (2007) Microbial production and application of sophorolipids. *Appl. Microbiol. Biotechnol.*, **76 (1)** 23-34.

VARJANI, SJ, UPASANI, VN (2017) Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. *Bioresour. Technol.*, **232** 389-397.

VASILESCU, S (2017) The science behind biofilms and their impact on cooling towers. *Water Technology* https://www.watertechonline.com/process-water/article/16210901/the-science-behind-biofilms-and-their-impact-on-cooling-towers.

VECINO, X, RODRIGUEZ-LOPEZ, L, FERREIRA, D, CRUZ, JM, MOLDES, AB, RODRIGUES, LR (2018) Bioactivity of glycolipopeptide cell-bound biosurfactants against skin pathogens. *Int. J. Biol. Macromol.*, **109** 971-979.

VECINO, X, RODRÍGUEZ-LÓPEZ, L, RINCÓN-FONTÁN, M, CRUZ, JM, MOLDES, AB (2021) Nanomaterials synthesised by biosurfactants. in: *Comprehensive analytical chemistry*, Vol. 94, Elsevier, pp. 267-301.

VENCES-GUZMAN, MA, GEIGER, O, SOHLENKAMP, C (2012) Ornithine lipids and their structural modifications: from A to E and beyond.*FEMS Microbiol. Lett.*, **335 (1)** 1-10.

VENCES-GUZMAN, MA, GUAN, Z, ORMENO-ORRILLO, E, GONZALEZ-SILVA, N, LOPEZ-LARA, IM, MARTINEZ-ROMERO, E, GEIGER, O, SOHLENKAMP, C (2011) Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899. *Mol. Microbiol.*, **79 (6)** 1496-514.

VENCES-GUZMÁN, MÁ, GUAN, Z, BERMÚDEZ-BARRIENTOS, JR, GEIGER, O, SOHLENKAMP, C (2013) Agrobacteria lacking ornithine lipids induce more rapid tumour formation. *Environ. Microbiol.*, **15 (3)** 895-906.

VENCES-GUZMÁN, MÁ, GUAN, Z, ESCOBEDO-HINOJOSA, WI, BERMÚDEZ-BARRIENTOS, JR, GEIGER, O, SOHLENKAMP, C (2015) Discovery of a bifunctional acyltransferase responsible for ornithine lipid synthesis in *Serratia proteamaculans*. *Environ. Microbiol.*, **17 (5)** 1487-1496.

VENTER, P SEWELL, BT, TRINDADE, M (2022) Structural and enzymatic characterization of a novel ornithine acyl-acp n-acyltransferase and overexpression of its biosurfactant product. 52-64

WALSER, SM, GERSTNER, DG, BRENNER, B, HOLLER, C, LIEBL, B, HERR, CE (2014) Assessing the environmental health relevance of cooling towers--a systematic review of legionellosis outbreaks. *International Int. J. Hyg. Environ. Health*, **217 (2-3)** 145-54.

WEISSENMAYER, B, GAO, JL, LÓPEZ-LARA, IM, GEIGER, O (2002) Identification of a gene required for the biosynthesis of ornithine-derived lipids. *Mol. Microbiol.*, **45 (3)** 721-733.

WERY, N, BRU-ADAN, V, MINERVINI, C, DELGENES, JP, GARRELLY, L, GODON, JJ (2008) Dynamics of *Legionella* spp. and bacterial populations during the proliferation of *L. pneumophila* in a cooling tower facility. *Environ. Microbiol.Appl. Environ. Microbiol.*, **74 (10)** 3030-7.

WHITE, DA, HIRD, LC, ALI, ST (2013) Production and characterization of a trehalolipid biosurfactant produced by the novel marine bacterium *Rhodococcus* sp., strain PML026. *J. Appl. Microbiol.*, **115 (3)** 744-55.

WILLIAMS, W, KUNOROZVA, L, KLAIBER, I, HENKEL, M, PFANNSTIEL, J, GUNTHER, J, CONRAD, J, VAN ZYL, LJ, HAUSMANN, R, BURGER, A, TRINDADE, M (2019) Novel metagenome-derived ornithine lipids identified by functional screening for biosurfactants. *Appl. Microbiol. Biotechnol.*, **103 (11)** 4429-4441.

WILLS, A, BOTT, TR, GIBBARD, I. (2002) The effect of tube inserts on biocide efficacy, In Heat Exchanger Fouling Fundamental Approaches and Technical Solutions*,* Müller-Steinhagen, Ed., Publico, Essen,. Germany, pp. 341-346.

XIA, J (2001) Protein-based surfactants: Synthesis: Physicochemical Properties, and applications. *CRC Press*.

XIONG, Y, LIU, Y (2010) Biological control of microbial attachment: a promising alternative for mitigating membrane biofouling. *Appl. Microbiol. Biotechnol.*, **86 (3)** 825-837.

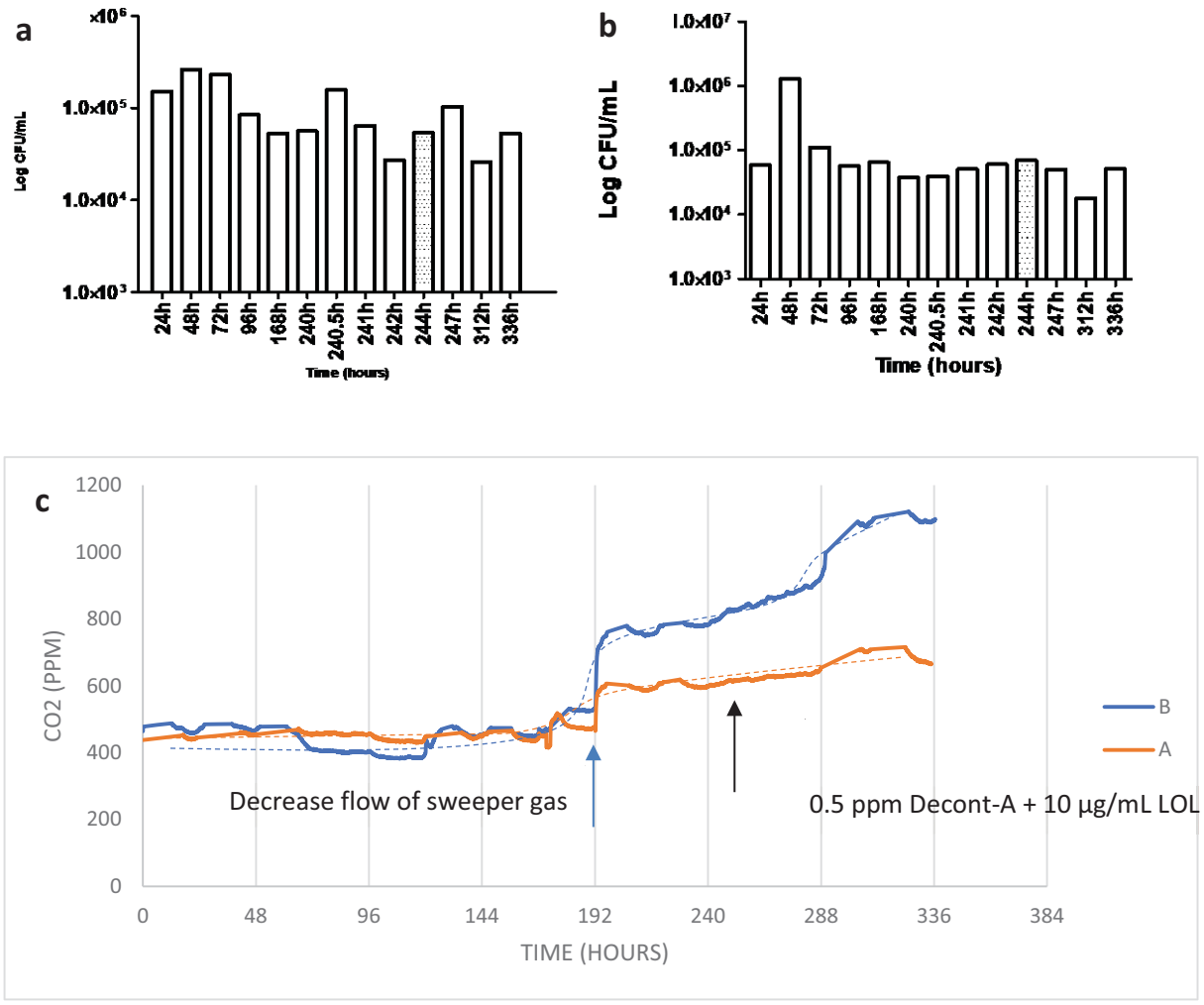
XU, L, SHAO, C, LI, G, SHAN, A, CHOU, S, WANG, J, MA, Q, DONG, N (2020) Conversion of broad-spectrum antimicrobial peptides into species-specific antimicrobials capable of precisely targeting pathogenic bacteria. *Sci. Rep.*, **10 (1)** 1-9.

YONG, YY, DYKES, GA, CHOO, WS (2019) Biofilm formation by staphylococci in health-related environments and recent reports on their control using natural compounds. *Crit. Rev. Microbiol.*, **45 (2)** 201-222.

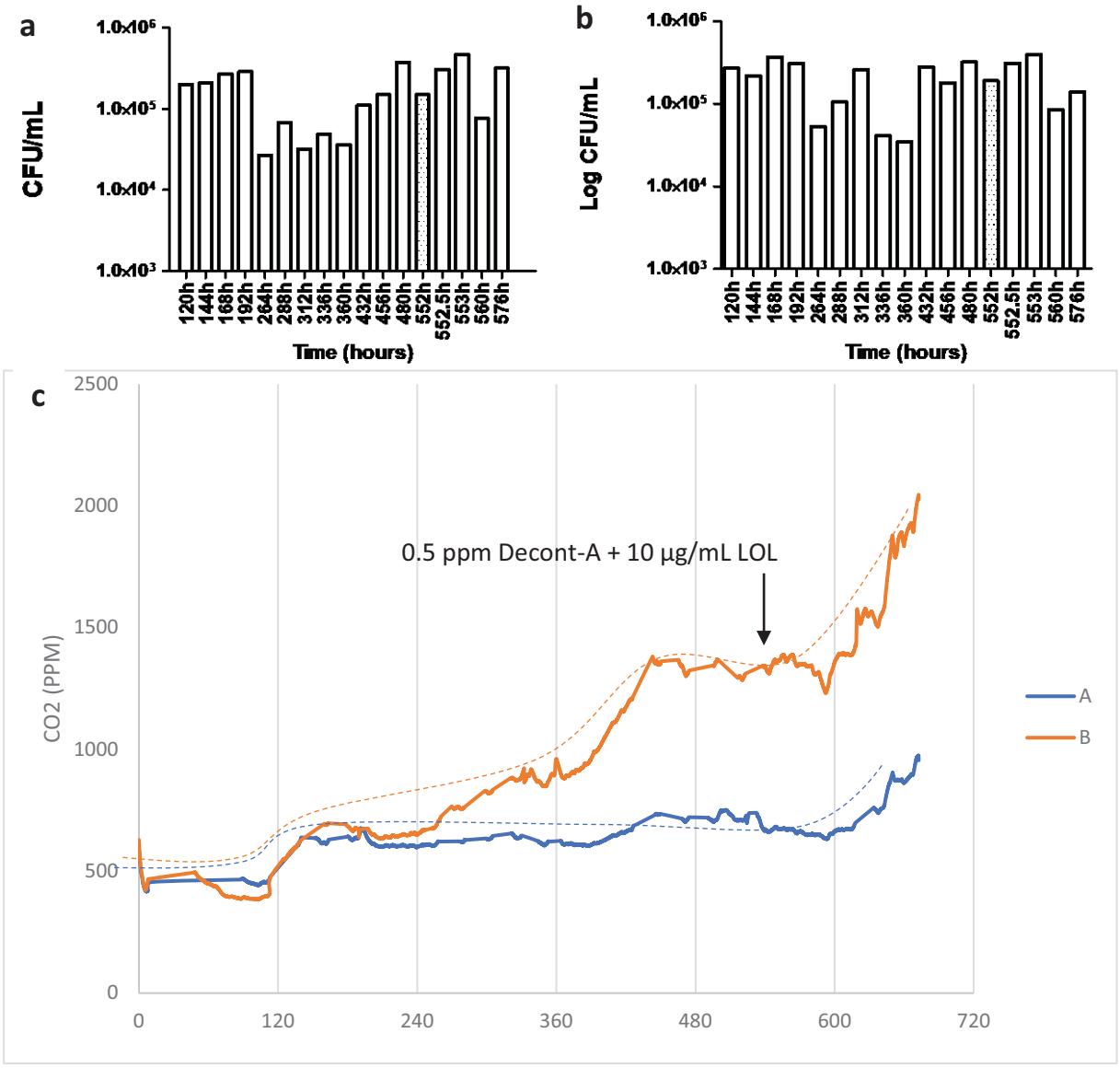
ZEZZI DO VALLE GOMES, M, NITSCHKE, M (2012) Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. *Food Control*, **25 (2)** 441-447.

ZIN, IM, POKHMURSKII, VI, KORNIY, SA, KARPENKO, OV, LYON, SB, KHLOPYK, OP, TYMUS, MB (2018) Corrosion inhibition of aluminium alloy by rhamnolipid biosurfactant derived from *Pseudomonas* sp. PS-17. *Anti-Corros. Methods Mater.*, **65 (6)** 517–527.

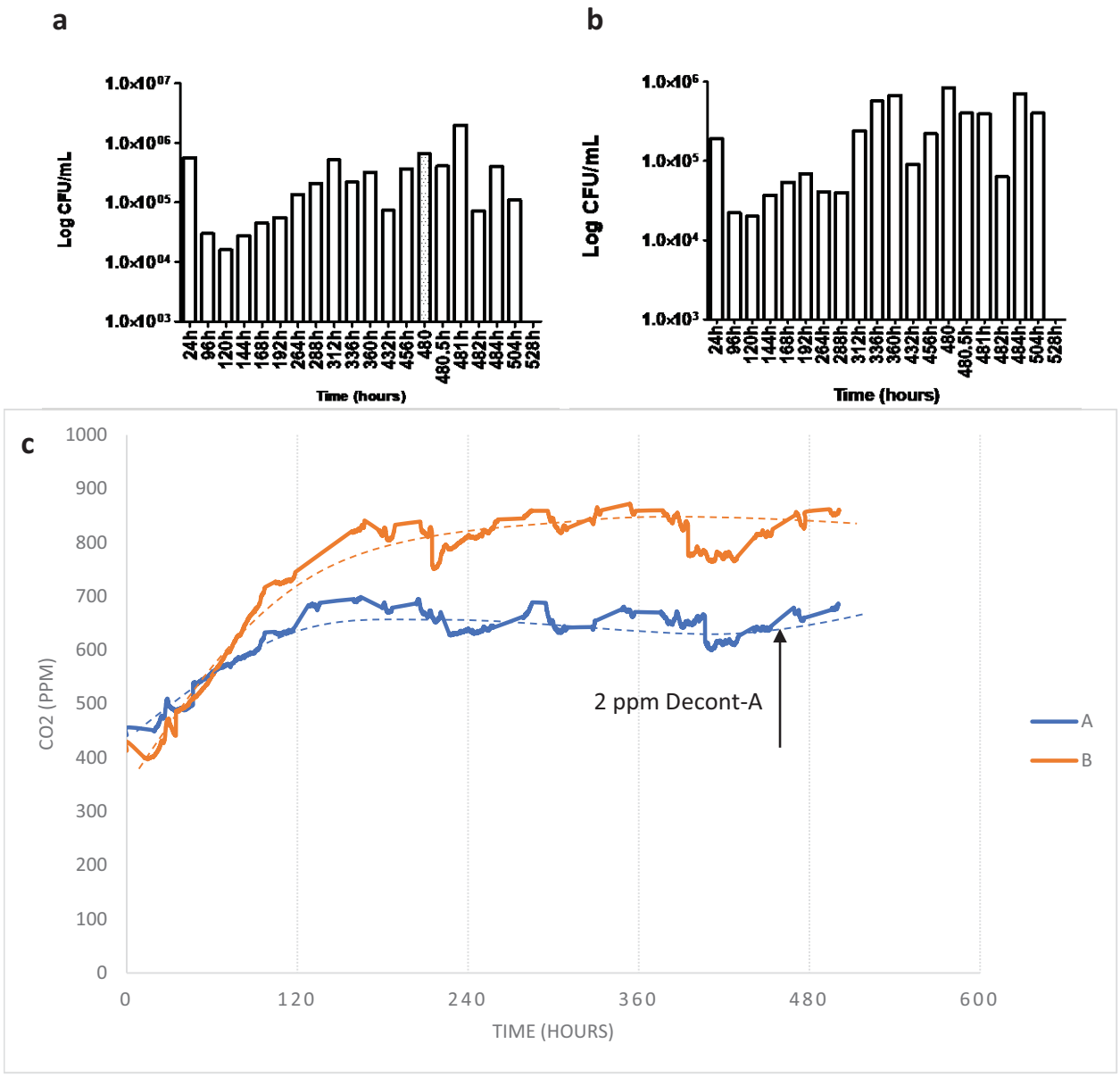
Appendix A: Supplementary Tables & Figures



Supplementary Figure 1. Biofilm growth at 25°C of the microbial community present in industrial wastewater collected from cooling tower 1 pond at the Lethabo power station. The biofilms were cultivated in the CEMS system on sterilized industrial wastewater. Planktonic cell yields from the biofilm community in system A (a) and B (b) were calculated every 24 hours before and after dosing (288 hours, indicated by black arrow) at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours and 48 hours. (c) Both system A (blue line) and B (orange line) were treated with 0.5 ppm Decont-A and 10 µg/mL LOL indicated by black arrow (244 hours) for a period of 48 hours under continuous flow conditions. Changes in the metabolic activity of the biofilm were measured in real-time with a CO_2 analyser. The dashed blue and orange lines represent trendlines for system A and B. After 192 hours the flow of the sweeper gas was decreased (indicated by blue arrow).



Supplementary Figure 2. Biofilm growth at 25 °C of the microbial community present in industrial wastewater collected from cooling tower 1 pond at the Lethabo power station. The biofilms were cultivated in the CEMS system on sterilised industrial water. Planktonic cell yields from the biofilm community in system A (a) and B (b) were calculated every 24 hours before dosing and after dosing at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours and 48 hours. (c) Both system A (blue line) and B (orange line) were treated with 0.5 ppm Decont-A+10 µg/mL LOL indicated by black arrow for a period of 48 hours under continuous flow conditions. Changes in the metabolic activity of the biofilm were measured in real-time with a CO_2 analyser. The dashed blue and orange lines represents trendlines for system A and B.



Supplementary Figure 3. Sessile growth at 25°C of microbial community present in industrial wastewater collected from cooling tower 1 pond at the Lethabo power station. The biofilms were cultivated in the CEMS system on sterilised industrial wastewater. Planktonic cell yields from the biofilm community in system A (a) and B (b) were calculated every 24 hours before dosing and after dosing at 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours and 48 hours after dosing. (c) System A (blue line) was exposed to 2 ppm Decont-A for a period of 24 hours under continuous flow conditions. System B (orange line) was left untreated and used as a control. Changes in the metabolic activity of the biofilm was measured in real-time with a CO_2 analyser. The dashed blue and orange lines represents trendlines for system A and B. The induction of Decont-A at 480 hours is indicated with a black arrow.