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The efficacy of *Moringa oleifera* as a practical application for sustainable water treatment

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This thesis is submitted for examination for the degree of

Doctorate of Philosophy

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Declaration for a thesis that contains publications

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Executive Summary

The aim of this research was to determine if the seeds of *Moringa oleifera* were appropriate for use in potable water treatment. The seeds grow, and are generally used, in regions that lack basic water treatment infrastructure. In these regions, the seeds serve as a primary method of water treatment by removing pathogens and causing flocculation and coagulation of suspended solids. Significant information is available in the literature describing the use of the crushed seeds as an antibacterial agent. The compounds that cause the antibacterial effects have been isolated, identified and tested against a range of pathogenic bacteria. Of these compounds, the *M. oleifera* cationic proteins were chosen for further research with a focus on the attachment of these proteins to the surface of silica and sand, the inactivation of bacteria using these functionalised surfaces, and the regeneration of the functional surfaces.

This thesis outlines the results from studies where the use of sand and silicon dioxide that had been functionalised with $MO_{2\cdot1}$ was investigated for bacterial attachment and inactivation. A method of immobilising $MO_{2\cdot1}$ onto the surface of sand to produce the functional sand (f-sand) was experimentally optimised. The antibacterial effect of the f-sand on *Escherichia coli* suspended in water was examined by packing f-sand into columns. The columns were found to remove above 90% of the bacteria. Further experiments were conducted to determine whether the inhibited *E. coli* could be removed from the f-sand without causing the $MO_{2\cdot1}$ to separate from the sand surface. Two regeneration methods were successfully developed leading to the regeneration of the f-sand. In both cases the attachment of the $MO_{2\cdot1}$ was measured quantitatively by determining the amount of $MO_{2\cdot1}$ bound to the f-sand surface, and qualitatively by measuring the amount of *E. coli* that was removed by the f-sand after it had been regenerated. The first regeneration method used the non-ionic surfactant dodecyl glucoside. It was observed that after the initial exposure of f-sand to dodecyl glucoside, there was an increase in the bacterial removal by the f-sand on subsequent regenerations. The second method involved heating the used f-sand to between 50 ºC and 100 ºC for between 1 minute and 60 minutes. It was found that heating the f-sand to 60ºC allowed for repeated bacterial removal at a constant rate.

A laboratory scale-up trial of the f-sand was undertaken to determine the efficiency of the f-sand for removing *E. coli* from artificially contaminated water. The different experimental parameters that were investigated included varying the concentration of *E. coli*, operating three columns in series and reversing the direction of flow so that the filter behaved either as

a packed bed or a fluidised bed. In this series of experiments a 1.1 log reduction was the highest removal achieved from a 100 CFU/mL *E. coli* suspension through a fluidised f-sand bed.

In the final investigation, the attachment of $MO_{2.1}$ to silicon dioxide (f-SiO_2) and the effect of the f-SiO_2 on *E. coli* and *Micrococcus luteus* inactivation were quantified. Both bacteria were shown to attach to the f-SiO_2, and the effect of dodecyl glucoside on these bacteria was investigated. The *M. luteus* did not separate when exposed to the dodecyl glucoside, but remained attached to the f-SiO_2. The underlying mechanisms for removal were examined by measuring the zeta potential of each system and making physical observations with live cell microscope.

The thesis is divided into 8 chapters. These are comprised of the introduction and literature review; where all of the antibacterial components isolated from *M. oleifera* are discussed. Following the literature review, the methods chapter presents an outline of the general analytical methods used throughout all of the experiments. Chapters 4 to 7 provide the experimental, investigations that focussed on a laboratory-scale investigation using 100g f-sand, and the reusability of f-sand and f-SiO_2. The thesis is concluded in Chapter 8 where the applicability and relevance of f-sand and f-SiO_2 are discussed, followed by the identification of future areas of research.

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I have been helped by such a large group of people during my candidature that I could fill another thesis in naming them all. The most important people in this are my parents, who not only raised me to believe that I could do anything I set my mind to but actively supported me physically, emotionally and financially when I decided to undertake a PhD. This work would not exist without their support.

I would also like to thank my supervisors, especially Professor David Lewis who accepted me as a PhD student and allowed me to undertake this thesis when all I had to go on was a vague idea that Moringa might be useful for water treatment. Dr Andrew Lee for his continuous help and careful editing of my work. His door was always open and I deeply appreciate the huge amount of time he spent on me. Dr Sanaz Orandi for stepping in and becoming my on campus supervisor. I could not have finished if it hadn't been for her patient editing and advice, especially when I was too stressed to think clearly and she told me to go home, it could wait till tomorrow.

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Combined traditional thesis and thesis by publication

This thesis is composed of the following manuscripts and accepted works

1. Williams F. E., Lee A. K., Orandi S., Sims S. K., Lewis D. M. (2017) *Moringa oleifera* functionalised sand – reuse with non-ionic surfactant dodecyl glucoside. *Journal of Water and Health* **15**(6), 863-872. **DOI: 10.2166/wh.2017.241**

2. Williams F. E., Lee A. K., Orandi S. &. Lewis D. M. (2018): Antibacterial action of functional silicon dioxide: an investigation of the attachment and separation of bacteria, *Environmental Technology*, In press. **DOI: 10.1080/09593330.2018.1509887**

Parts of this research were presented at the following conferences

1. Williams F., Lee A. K., Orandi S., Sims S. K., Lewis D. M. (2016) Reusable Water Treatment For *Escherichia coli* Removal Using *Moringa oleifera* Functional Sand. IChemE, CHEMECA conference, Adelaide, Australia (Oral presentation)

2. Williams F., Lee A. K., Orandi S., Lewis D. M. (2017) *Moringa oleifera* functional sand: a practical, reusable water treatment option. International Water Association S2Small Conference, Nantes, France (Oral presentation)

Additionally, an overview of this work was presented in the 2016 3 Minute Thesis competition at the University of Adelaide and at the Asia-Pacific Finals in Brisbane.

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Chapter 1. Introduction

1.1 Problem Statement

Providing potable water to many of the world's populations is a continuing challenge for governments and international bodies. It has been the focus of extensive global effort as exemplified by the United Nations Millennium Development Goals and more recently in the Sustainable Development Goals (United Nations, 2017). It is currently estimated that 2.1 billion people lack safely managed drinking water services, 159 million of whom rely solely on surface waters (WHO, 2017). These surface waters often contain pathogenic organisms that cause serious illness and death. These organisms include cholera, polio, dysentery and typhoid, and recent estimates by the World Health Organization state that contaminated water causes 502 000 diarrhoeal deaths each year (WHO, 2018).

There are many reasons why people do not have access to reliable, potable water, though in most cases it is a consequence of extreme poverty. The WHO has identified 22 countries where over 10% of the population relies on untreated surface waters (Figure 1). The majority of these 22 countries are in Sub-Saharan Africa where 10% of the population still rely on surface waters (WHO, 2017). In these areas where conventional water treatment options, such as chlorination, are not available or are too expensive, other traditional methods may be appropriate.

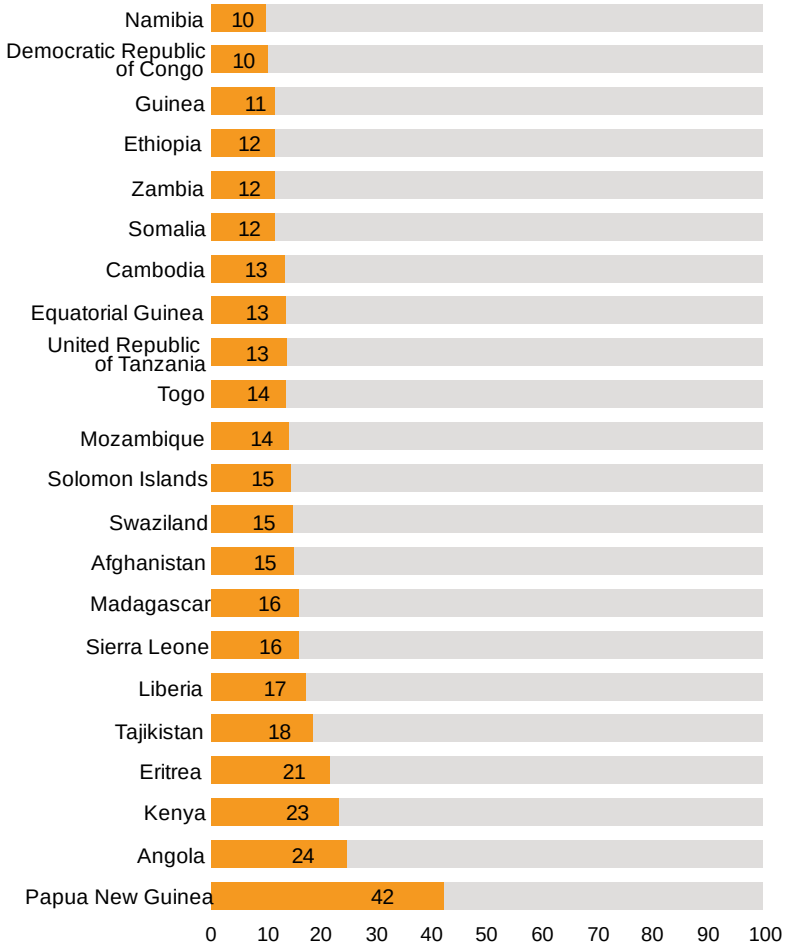

Figure 1: Percentage of the population without access to treated surface water listed by country, as identified by the WHO, (2017).

Plants have been used to treat water for thousands of years (Kansal & Kumari, 2014). Reviews into the use of plant material for coagulation and antibacterial action have identified a range of plant seeds, fruit waste and bark. Commonly-researched plant materials include the seeds of *Moringa oleifera* and *Strychnos potatorum*, and the leaves and sap of *Opuntia ficus indica* (Yin, 2010; Kansal & Kumari, 2014). All of these plant materials exhibit coagulant or antibacterial properties with *M. oleifera* seeds capable of achieving both (Choy, 2014).

M. oleifera is a tree that is native to the foothills of the Himalayas (Jahn, 1988). It has been naturalised across the globe in tropical and subtropical regions (Figure 2) including many of the countries listed by the WHO, where surface water is primarily relied upon for drinking water. The seeds have a long history of use in local foods and traditional medicines and were routinely used in the Sudan to clarify turbid water (Madsen *et al.*, 1987). The application of the seeds of *M. oleifera* for the removal of bacteria from water

was chosen as a topic for further investigation. This choice was driven by the global availability of the tree, its wide-spread cultural acceptance and because of its previous traditional use as both a coagulant and antibacterial agent.

Figure 2: Map of the world showing the regions where *M. oleifera* is reportedly grown. (Trees for Life, accessed 13/07/2017)

An extensive body of literature describes the use of the crushed seeds of *M. oleifera* as a primary method of water treatment to inactivate pathogenic organisms and reduce turbidity via flocculation and coagulation (Kansal & Kumari, 2014). Several review papers are available that discuss the use of crude *M. oleifera* seeds for potable water treatment (Anwar *et al*, 2007; Kansal and Kumari, 2014; Wang *et al*., 2016; Yin, 2010). Much of the antibacterial research is focused on the aqueous seed extract for household-scale inactivation of waterborne bacteria. The *M. oleifera* seeds contain several antibacterial agents with different mechanisms of action that are discussed in the literature review.

Adding the crushed seeds of *M. oleifera* to bacterially contaminated water is an effective treatment in the short term; however, the increase in organic matter encourages bacterial regrowth and prevents storage of the water (Ali *et al.*, 2011). The degrading seed particles also release sulphurous compounds that give an unpleasant odour to the water (Ndabigengesere & Narasiah 1998). To overcome the issues currently preventing the wide-scale use of *M. oleifera* seeds, many of the compounds within the seeds have been isolated, identified, and tested for bacterial inactivation. Of these compounds, a group of cationic proteins were considered to be the most promising for further research. Proteins within this group have been shown to adsorb to the surface of sand. This functional sand

(f-sand) retains the antibacterial properties of the *M. oleifera* seed kernel and can be used to remove *Escherichia coli* from water (Jerri *et al.*, 2012).

1.2 Objective of this research

The focus was specifically on the use of the *M. oleifera* cationic protein as antibacterial agents by means of the attachment to sand and silicon dioxide (SiO_2). The natural progression of this research was to make the f-sand reusable by removing the inhibited bacteria from the proteins without separating the proteins from the sand.

The principal aims of this work were:

- To discover whether inactivated *E. coli* could be separated from the f-sand
    - By treating the used f-sand with surfactants,
    - By exposing the f-sand to water heated to different temperatures.
- To validate *E. coli* removal from water using a bench-scale study to determine the capacity of f-sand to treat bacterially contaminated water.
- To prepare functional SiO_2 (f- SiO_2) and then investigate:
    - The inactivation of *Micrococcus luteus* using the f-SiO_2,
    - Whether the *M. luteus* could be separated from the f- SiO_2 in the same manner as the *E. coli*.

The above objectives were achieved through a series of laboratory experiments, with the primary focus being the ability to separate the inactivated bacteria from the sand.

1.3 Thesis outline

This thesis is written in a combined publication-traditional format. It comprises a literature review, methods chapter and experimental chapters. Two of the experimental chapters have been published. The following is a description of the thesis chapters.

Chapter 2 contains a literature review of the research that has been undertaken about the antibacterial compounds isolatable from *M. oleifera* seeds. The literature review includes the structure and composition of the antibacterial compounds, the methods of extraction and production, an overview of the antibacterial action, and recommendations on future research.

Chapter 3 describes the general preparations and methods followed during the experimental research reported in Chapters 4 to 7.

Chapter 4 is a bench-scale study where the effect of altering different sand-bed configurations was examined.

Chapter 5 is a presentation of the experiments undertaken to separate *E. coli* from f-sand using the surfactants dodecyl glucoside and sodium dodecyl sulphate. This chapter is presented as a journal paper and has been published with the *Journal of Water and Health*.

Chapter 6 is a comparison of two methods for the separation of *E. coli* from f-sand using dodecyl glucoside and heat. This chapter was originally a manuscript presented at the International Water Association S2Small Conference, 2017.

Chapter 7 is an examination of the behaviour of *M. luteus* and *E. coli* in the presence of f-SiO_2, and dodecyl glucoside. This chapter has been published in the journal *Environmental Technology*.

Chapter 8 summarises the key results and findings of this thesis and includes recommendations for future areas of research.

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Chapter 2. Literature Review

This chapter is a literature review of the antibacterial compounds that can be found in the seeds of *M. oleifera.* It was conducted at the beginning of the research but has been kept up to date over the intervening years as new studies have been published. While a literature search will display hundreds of articles about the efficacy of *M. oleifera* seeds for the removal of bacteria from water most of these studies focus on the crude seed extract. While this research is extremely useful, and was the basis for most of the studies that elucidated the specific antibacterial compounds present in the seeds, it has been discussed in several other review papers that are referenced in Chapter 2. The aim of the following chapter was to clarify how much research had been undertaken to identify the specific antibacterial compounds of the seeds and to discover the extent of the knowledge gap that could be used to drive further research.

Statement of Authorship

Title of Paper	The role of *Moringa oleifera* in water disinfection: an up-to-date review of compounds responsible for antibacterial activity
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Contribution to the Paper	Performed literature review and drafted manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andrew K. Lee
Contribution to the Paper	Supervised development of work. Helped to evaluate and edit the manuscript.
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Signature	Date 26, 4, 19

Name of Co-Author	David M. Lewis		
Contribution to the Paper	Supervised development of work. Helped to evaluate and edit the manuscript.		
Signature		Date	26/4/19

The role of *Moringa oleifera* in water disinfection: an up-to-date review of compounds responsible for antibacterial activity.

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Key words

Moringa oleifera seeds; Antibacterial; 4-(α-$_L$-rhamnosyloxy) benzyl isothiocyanate; MOCP; $MO_{2.1}$; Potable water

Abstract

It has been known for hundreds of years that the seeds of *Moringa oleifera* can be used to improve contaminated water. Adding the seeds to turbid water improves the perceived cleanliness by reducing the amount of visible pollution through coagulation. The seeds also reduce the amount of pathogenic organisms in the water through bacterial inactivation. An extensive body of literature exists that examines the effect of the seeds when they are added either directly or as an aqueous extraction added to contaminated water. The compounds responsible for the coagulation and antibacterial activity of the *M. oleifera* seeds have been described in individual studies; however, there is currently no comparison of this research. The object of this review is to identify these compounds and to discuss their isolation and use in potable water treatment. A particular focus is on the antibacterial compounds, which can be isolated by liquid extraction and purified by ion exchange chromatography. The first group of compounds are isothiocyanates, specifically 4-(α-L-rhamnosyloxy) benzyl isothiocyanate, a potent antimicrobial compound that is the product of the enzymatic reduction of 4-(α-L-rhamnosyloxy)-benzyl glucosinolates. The second group is comprised of a 12-14 kDa coagulant protein that under reducing conditions can be isolated as a monomer of approximately 6.5 kDa. This monomer is a coagulant and an antimicrobial agent. A summary is provided of the toxicity studies that investigate the use of *M. oleifera* seeds in water treatment. The toxicity is generally attributed to the isothiocyanates and so far no studies have been conducted on the toxicity of the proteins. The toxicity studies primarily conclude that the seeds are not harmful at the concentrations currently recommended for water treatment, but caution that increasing this concentration could cause harm.

2.1 Introduction

One quarter of the global population sources their water from a contaminated water supply (WHO, 2017). Untreated surface waters contain a variety of particulate impurities including suspended inorganic particles such as clays and metal oxides, and organic particles such as decomposing plant and animal matter. Untreated water also contains a range of microorganisms including bacteria, viruses, protozoa, microalgae and cyanobacteria. The contamination of water by faecally transmitted pathogenic organisms, including *Salmonella typhi* and *Vibrio cholerae*, significantly contributes to morbidity in vulnerable population groups, especially very young children and the elderly. Conventional drinking water treatment involves coagulation, flocculation, sedimentation, media filtration and disinfection. Disinfection is usually the final step in water treatment with the aim of removing pathogenic microorganisms. Certain methods of disinfection, including treatment with chlorine, provide residual protection to the treated water by preventing pathogen regrowth and by inactivating pathogens which enter the water after the initial treatment. Accessible, small-scale and low-cost water treatment is required in many low socioeconomic regions of the world, and methods using commonly grown plant material with proven water purifying capabilities can help fill this requirement. One plant already in use is *Moringa oleifera*. The crushed seed kernels are a traditional Sudanese method of water treatment used on a small scale to clarify water before consumption (Madsen *et al.*, 1987). *M. oleifera* is native to the Himalayan foothills and has been naturalized in many tropical regions including South America, Africa and Asia (Jahn 1988). An extensive body of literature exists describing the use of the crushed seeds as a primary method of water treatment. When added to water, the crushed seeds visibly clarify turbid water via coagulation (Muyibi & Evison 1995; Muyibi & Alfugara 2003; Pritchard *et al.*, 2009; Pritchard *et al.*, 2010a, 2010b; Abatneh *et al.*, 2014). The seed extract inhibits numerous bacteria (Vieira *et al.*, 2010b) and parasites (Olsen 1987; Sengupta *et al.*, 2012). The extract is also reported to cause heavy metal removal, (Sharma *et al.*, 2006; Sharma *et al.*, 2007a; Sharma *et al.*, 2007b; Sharma 2008) and the reduction of micro-fauna such as mosquito larvae (Coelho *et al.*, 2009). The seeds are currently used to treat drinking water in Malawi (Al-Anizi *et al.*, 2009) and are recommended by a Non-Government Organisation in Brazil (Araújo *et al.*, 2013).

The general toxicity towards bacteria, parasites and micro-fauna raises the issue of the extract being a health risk to humans. There is a general assumption in the literature that

the seeds of *M. oleifera* are non-toxic and safe to use in water treatment (Muyibi & Alfugara 2003; Vieira *et al.,* 2010a). This assumption is usually based on the plant's long use as an ingredient in food and indigenous medicines (Thurber & Fahey 2009), and not from the result of any trials into the long- or short-term effect of treating water destined for human consumption. A range of compounds can be extracted from the *M. oleifera* seeds, though not all are water soluble and where any potential toxicity is mentioned, it is generally attributed to the mustard seed oils, with the most research performed using 4(α-$_{L}$-rhamnosyloxy) benzyl isothiocyanate (Grabow *et al.,* 1985; Araújo *et al.,* 2013; Al-Anizi *et al.,* 2014). Interestingly, the therapeutic effect of isothiocyanates from cruciferous vegetables is well researched and their consumption is recommended (Waterman *et al.,* 2014). A review of the safety and efficacy of the *M. oleifera* leaves, which contain higher levels of isothiocyanate than the seeds, reported no adverse effects in any of the human studies so far conducted (Stohs & Hartman, 2015). In a review on the genotoxic potential of isothiocyanates, Fimognari, *et al*., (2012) concluded that dietary consumption of isothiocyanates was generally several orders of magnitude lower than the amounts used in toxicity studies. This reflects the conclusions of Grabow *et al.,* (1985) regarding the use of the crude *M. oleifera* extract in water treatment.

The genotoxicity and cytotoxicity of the *M. oleifera* crude extract have been examined in several studies. In the *Salmonella typhimurium* strains TA97, TA98, TA100 and TA102 the aqueous extract is reported to be genotoxic above 0.6 µg/µL, an effect that did not occur when the purified protein was used (Rolim *et al.,* 2011). In a second genotoxicity assay performed on cell-free plasmid DNA the aqueous extract concentration of 0.8 µg/µL showed signs of interaction and again, this was not observed with the purified protein (Rolim *et al.,* 2011). In both cases the effect occurred at a higher dose than is recommended to treat water and the genotoxicity was not observed in this lower concentration of 0.2 µg/µL (Rolim *et al.,* 2011). Al-Anizi *et al.,* (2014) examined the cytotoxicity of the *M. oleifera* seed extract on the bacterium *Acinetobacter baylyi* ADPI host, fused with the luxCDABE gene. They determined that the lethal concentration for 50% of the population (LC_{50}) was 8.5 mg/L. The authors reported that the cause of the cytotoxic effect was primarily from the insoluble fatty acid, not the water soluble compounds, but concluded that *M. oleifera* seeds were not suitable for use in water treatment. An argument against this conclusion is that using *A. baylyi* as a model to test

the cytotoxic effect of an extract with proven antibacterial efficacy would give a false positive result.

The toxicity of a concentrated extract (50 µg/mL) and an aqueous seed extract, in the concentrations used to treat drinking water (20 µg/mL), has been evaluated by Araújo *et al.,* (2013) for several indicators: the cytotoxicity on peripheral blood mononuclear cells collected from human volunteers, the haemolytic activity on mouse erythrocytes, the acute and systemic toxicity (2000 mg extract /kg), and *in-vivo* anti-inflammation activity on Swiss albino mice. A substance is considered non-toxic if the half maximal inhibitory concentration (IC_{50}) for peripheral blood mononuclear cells is above 100 µg/mL For extract concentrations between 6.25 mg/L to 400 mg/L an IC_{50} of 144 µg/mL was recorded (Araújo *et al.,* 2013 Neither the 6.25 mg/L nor 400 mg/L extract induced haemolysis in the Swiss albino mice, nor were there signs of systemic or acute toxicity at 2000 mg/kg. However, there was a reduction in the number of erythrocytes, leukocytes and platelets in the blood of the Swiss albino mice exposed to an acute dose of 2000 mg/kg. While these results were still within the normal range, Araújo *et al.,* (2013) cautioned that increasing extract concentrations used for water treatment should be done with care. The general consensus in the literature is that crude *M. oleifera* is an effective treatment for bacterially contaminated water when used at the concentrations currently recommended. However, the use is still confined to regions where the alternative is consuming untreated water, and long-term toxicity studies would be beneficial.

A disadvantage of using crude *M. oleifera* in water treatment comes from the degradation of the seed powder. As the seed powder degrades there is an increase in the concentration of nutrients in the treated water. This increase in the nutrient concentration has been shown to support the regrowth of surviving bacteria (Ndabigengesere & Narasiah 1998a, 1998b; Ali *et al.,* 2011). In addition to the increase in bacteria, this breakdown also releases unpleasant odours and renders the water non-potable (Ndabigengesere & Narasiah 1998a). When the crude extract is used as a co-treatment with chlorine there is the potential for forming disinfection by-products (Bhuptawat *et al.,* 2007). These by-products occur through the reaction of the chlorine with organic material during the disinfection process and include trihalomethanes, which are known to be carcinogenic (AWWA 2011). Another limitation with crude treatment comes from variation in the amount of useful compounds present in each seed. Factors like soil, air and water chemistry, the season and the amount of sunlight a plant receives can all affect the composition of the seed it

produces (Tsukamoto *et al.,* 1995; Shih *et al.,* 2011). Differences in a coagulant assay using a purified aqueous seed extract, where the *M. oleifera* seeds were sourced from separate continents, has been observed (Nordmark *et al*., 2016).

The increase in organic matter from crude *M. oleifera* extract can be overcome through the isolation and purification of the antimicrobial and coagulant compounds (Beltran-Heredia *et al.,* 2012). *M. oleifera* seeds contain two major types of compound known to cause bacterial inactivation, an isothiocyanate and a proteinaceous group. Both of these compounds are water soluble and the amount present in the crude extract is dependent on the extraction process. The variation in the extract concentration leads to a significant range in the reported efficacy of the seed extract, even when similar experimental parameters are followed. The variation in efficacy, increased organic content and bacterial regrowth associated with the crude treatment mean that this option is not currently suitable for large-scale water treatment.

To overcome the issues of seed degradation and active compound variation, the compounds within the seeds have been isolated, identified, and tested against a variety of bacteria. Currently there is no comprehensive review of these compounds, nor is there a comparison of the methods used to isolate them. Several review papers are available where the use of crude *M. oleifera* seeds for potable water treatment is discussed. Kansal and Kumari (2014) have published an extensive review of the efficacy of primary and secondary processing of *M. oleifera* seeds for a range of parameters including coagulation bacterial removal. Anwar *et al.,* (2007) discuss the traditional uses of the *M. oleifera* tree. Yin (2010) discusses crude *M. oleifera* seeds for water treatment as part of broader reviews into plant-based coagulants and Wang *et al.,* (2016) review the antimicrobial activity of the whole tree and conclude that the seeds displayed antimicrobial activity against pathogenic bacteria, fungi and parasites. The aim of this review is to compare the extraction and use of the compounds within the *M. oleifera* seed, which are known to act as antibacterial agents in water, specifically the proteins first isolated by Gassenschmidt *et al.,* (1995) and the isothiocyanates first used by Eilert *et al.,* (1981).

2.2. The active compounds of *M. oleifera* seeds

There are three approaches generally followed when preparing *M. oleifera* seeds for water treatment. The first two are also termed crude extract as they are the seed or seed extract without any modification or purification (Kansal & Kumari, 2014).

- Primary treatment is the direct addition of crushed seeds to water.
- Secondary treatment occurs when crushed seeds are soaked in a small volume of water, or other solution, for a given period of time. This solution is either added directly to the contaminated water or filtered and the filtrate added to the contaminated water. The extract solutions for this method include water, saline solutions, alcohol and organic solutions such as hexane and methanol.
- Tertiary treatment follows the same steps as secondary treatment, but the filtrate is processed further in order to isolate the targeted compounds. The most common process used to isolate the active compound is ion exchange chromatography.

The main compounds isolated from the seeds are glucosinolates, isothiocyanates, proteins, lectins and a 3 kDa negatively charged polyelectrolyte. The glucosinolates and isothiocyanates have been of interest since the isolation of ptergospermin (Rao *et al.,* 1946); later shown to be a precursor to benzyl isothiocyanate (Das *et al.,* 1954). The isolation of 4(α-$_L$-rhamnosyloxy)-benzyl isothiocyanate (Figure 3) by Kær *et al.,* (1979) was used to show that the compound was a potent antibacterial agent (Eilert *et al.,* 1981). Glucosinolates and isothiocyanates are sometimes referred to as mustard oils because of their prevalence in mustard seeds and are what give the spicy taste to broccoli, rocket, and mustard (Fahey *et al.,* 2001).

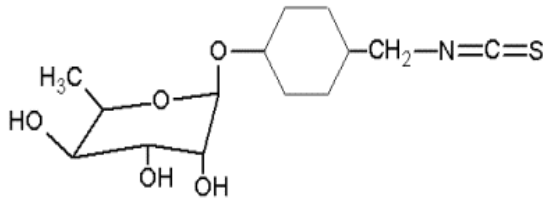

Figure 3: 4(α-$_L$-rhamnosyloxy)-benzyl isothiocyanate

The aqueous seed extract is comprised of a large mixture of proteins that share similar physical characteristics (Ghebremichael *et al.,* 2005). Only one of these has been extensively studied and is most commonly called the *M. oleifera* coagulant protein (MOCP). The MOCP is a 12 kDa dimer protein that is formed of a 6.5 kDa monomer, called $MO_{2.1}$, and a second similarly sized polypeptide (Gassenschmidt *et al.,* 1995; Shebek *et al.,* 2015). $MO_{2.1}$ and the MOCP are effective coagulants (Gassenschmidt *et al.,*1995, Ghebremichael *et al.,* 2005). Significant bacterial reduction was reported from $MO_{2.1}$ but it was originally attributed solely to removal from water via aggregation (Madsen *et al.,* 1987; Broin *et al.,* 2002). The first report of the bactericidal effect of

$MO_{2.1}$ was made by Suarez *et al*., (2003) who later confirmed that separate sections of the protein were responsible for coagulation and bacterial inactivation (Suarez *et al*., 2005). The predicted structure of MO $_{2.1}$ is presented in Figure 4 and can be viewed using the SWISS MODEL software (https://swissmodel.expasy.org/). The structure of the MOCP has not been published.

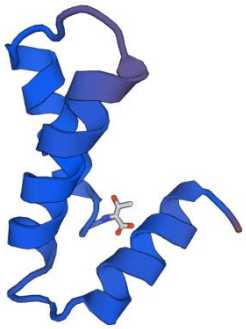

Figure 4: The predicted structure of the $MO_{2.1}$ monomer. This structure is estimated using the SWISS-MODEL Workspace (Benkert *et al*., (2011); Bertoni *et al*., (2017); Biasini *et al*., (2014); Bienert *et al*., (2017); Guex *et al*., (2009))

2.2.1 Glucosinolates and isothiocyanates

Glucosinolates are anionic, secondary plant metabolites, and are common in plants of the order Brassicales (Förster *et al.,* 2015). Dietary glucosinolates are water soluble, but are poorly absorbed with no known activity in the body (Angelino & Jeffery, 2014). The seeds and leaves of *M. oleifera* produce stable, aromatic glucosinolates that contain an L-rhamnose sugar which is unique to the *Moringaceae* (Waterman *et al.,* 2014). The major glucosinolate found in the *M. oleifera* seed is 4-(α-L-rhamnosyloxy)-benzyl glucosinolate (Eilert *et al.,* 1981; Amaglo *et al.,* 2010; Galuppo *et al.,* 2014). This glucosinolate is also called 4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate (Bennett *et al.,* 2003; Fahey *et al.,* 2003) and glucomoringin (Amaglo *et al.,* 2010; Galuppo *et al.,* 2014). However, glucomoringin has also been used to describe only the 4-(α-L-rhamnosyloxy)-benzyl component of the compound (Brunelli *et al.,* 2010). Fahey *et al.,* (2001) identified seven other glucosinolates from *M. oleifera*, and it is now known that *M. oleifera* also contains 2-propenyl glucosinolate (sinigrin) (Gueyrard *et al.,* 2010). Glucosinolates are the inert precursors of isothiocyanates (Shapiro *et al.,* 2001) and are hydrolysed in the presence of water by the enzyme myrosinase (Dufour *et al.,* 2015). The reaction is illustrated in Figure 5. This reaction occurs when plant cells are damaged, releasing the myrosinase (Herr & Büchler, 2010; Angelino & Jeffery, 2014). The enzyme is also produced by certain

intestinal microflora, and so the conversion is reported to occur during digestion (Jadin, 1900, Fahey *et al*., 2012). No antibacterial or coagulant ability is attributed to the glucosinolates found in *M. oleifera*.

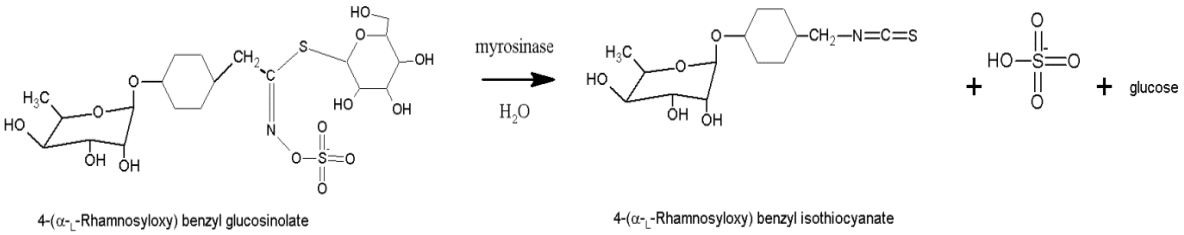

Figure 5: The hydrolysis of 4-(α-L-rhamnosyloxy)-benzyl glucosinolates to 4-(α-L-rhamnosyloxy) benzyl isothiocyanate by the enzyme myrosinase.

2.2.1.1 4-(α-L-rhamnosyloxy) benzyl isothiocyanate

4-(α-L-rhamnosyloxy) benzyl isothiocyanate is produced in the leaves and seeds of *M. oleifera* and has a molecular mass of 353 Da (Cheenpracha *et al.,* 2010). It has also been called 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate (Oluduro *et al.,* 2010) and moringin (Gueyrard *et al.,* 2010). Isolation of isothiocyanates is achieved by:

- Solvent extraction; and,
- Isolation via anion exchange chromatography.

The primary solvents used in the extraction process include water (Eilert *et al.,* 1981; Mehta *et al.,* 2011; Waterman *et al.,* 2014), methanol (Cheenpracha *et al.,* 2010; Oluduro *et al.,* 2010; Rim Jeon *et al.,* 2014; Waterman *et al.,* 2014), dichloromethane (Padla *et al.,* 2012), and ether (Badgett 1964). Chromatography techniques used to isolate or identify 4-(α-L-rhamnosyloxy) benzyl isothiocyanate are silica gel chromatography (Padla *et al.,* 2012), pressure liquid chromatography (Rim Jeon *et al.,* 2014) and high-performance liquid chromatography HPLC (Eilert *et al.,* 1981; Cheenpracha *et al.,* 2010; Oluduro *et al.,* 2010; Waterman *et al.,* 2014). A second method of isolating the 4-(α -L-rhamnosyloxy) benzyl isothiocyanate is to extract and purify the glucosinolate then react it in the presence of myrosinase following the method of Barillari *et al.,* (2005).

Eilert, *et al.,* (1981) calculated that a 0.2 g/L crude *M. oleifera* solution produced from shelled, defatted seeds would optimally contain 15 – 30 µmol/L 4-(α -L-rhamnosyloxy) benzyl isothiocyanate. Unfortunately, this concentration cannot be assumed when using crude treatment as the amount of 4-(α -L-rhamnosyloxy) benzyl isothiocyanate present is

dependent on the amount of the enzyme myrosinase, and the concentration of 4-(α -$_L$-rhamnosyloxy) benzyl glucosinolate.

Studies examining the effect of 4-(α -$_L$-rhamnosyloxy) benzyl isothiocyanate on a range of gram-positive and gram-negative bacteria are listed in Table 1. This list includes the gram-positive bacteria *Cacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, the gram-negative bacteria *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pheumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Serratia marcescens*, and *Shigella dysenteriae*, and the acid-fast bacteria *Mycobacterium phlei*.

Table 1: Bacterial susceptibility to 4-(α-L-rhamnosyloxy) benzyl isothiocyanate

Bacteria	Strain	Initial concentration (CFU/mL)	Method	Inhibitory concentration (mg/L)	Incubation time (hours)	Measure of Efficacy	Reference
Gram-positive bacteria							
B. cereus	NA	2.4×10^7	Pour plate[a]	0.005	3	Complete inactivation	(Oluduro *et al.*, 2010)
B. subtilis	NA	NA	Broth dilution	17.5	48	MBC	(Eilert *et al.*, 1981)
	ATCC 6633	1×10^8	Broth dilution and colony counts	250	18 -24	MIC	(Padla *et al.*, 2012)
				500	18 -24	MBC	
S. aureus	ATCC 6538	1×10^8	Broth dilution and colony counts	62.5	18 -24	MIC	(Padla *et al.*, 2012)
		1×10^8		125	18 -24	MBC	
		1×10^6		50	48-72	MIC	(Rim Jeon *et al.*, 2014)
		1×10^6		1000	18 -24	MBC	
S. epidermidis	ATCC 12228	1×10^8	Broth dilution and colony counts	125	18 -24	MIC	(Padla *et al.*, 2012)
		1×10^8		250	18 -24	MBC	
Gram-negative bacteria							
E. coli	ATCC 8739	1×10^8	Disk diffusion[b]	10,000	18 -24	No inactivation	(Padla *et al.*, 2012)
	NA	1.5×10^7	Pour plate[a]	0.005	5	Complete inactivation [c]	(Oluduro *et al.*, 2010)
E. aerogenes	ATCC 13048	1×10^8	Disk diffusion[b]	10,000	18 -24	No inactivation	(Padla *et al.*, 2012)
*K. pneumoniae**	ATCC 13883	1×10^8	Disk diffusion[b]	10,000	18 -24	No inactivation	
P. aeruginosa	NA	2.3×10^7	Pour plate[a]	0.005	5	Complete inactivation [c]	(Oluduro *et al.*, 2010)

Bacteria	Strain	Initial concentration (CFU/mL)	Method	Inhibitory concentration (mg/L)	Incubation time (hours)	Measure of Efficacy	Reference
P. aeruginosa	ATCC 9027	1×10^6	Broth dilution and colony counts	5000	48-72	MIC	(Rim Jeon *et al.*, 2014)
		1×10^6		10,000	18 -24	MBC	
		1×10^8	Disk diffusion[b]	10,000	18 -24	No inactivation	(Padla *et al.*, 2012)
S. typhi	NA	1×10^7	Pour plate[a]	0.005	2	Complete inactivation	(Oluduro *et al.*, 2010)
S. marcescens	NA	NA	Broth dilution	25	48	50% inactivation	(Eilert *et al.*, 1981)
S. dysenteriae	NA	2.4×10^7	Pour plate[a]	0.005	5	Complete inactivation [c]	(Oluduro *et al.*, 2010)

[a] Pour plate: 4-(α-L-rhamnosyloxy) benzyl isothiocyanate was isolated and mixed with distilled water and the organism. The solution was mixed for 1 – 6 hours. The inactivation time was determined by measuring the concentration of surviving organisms every hour.

[b] Disk diffusion: Paper disks impregnated with known concentration of 4-(α-L-rhamnosyloxy) benzyl isothiocyanate and placed on agar inoculated with organism (Jorgensen 2007).

[c] No growth observed at 5 or 6 hours.

*Pathogenic strain

The results presented in Table 1 are indicative of the antibacterial properties of 4-(α -L-rhamnosyloxy) benzyl isothiocyanate against a broad range of bacteria but since the methods followed to test the efficacy are not standardised against each other, and influence the outcome, a direct comparison is difficult. Padla *et al.*, (2012) tested seven species of bacteria using the disk diffusion method. Of these seven species, the three gram-positive bacterial strains showed inactivation and were further tested to determine the MIC and MBC as shown in Table 1. The four gram-negative strains *P. aeruginosa*, *E. coli*, *E. aerogenes* and *K. pneumoniae* were not inhibited using disk diffusion, even at the highest concentration of 4-(α -L-rhamnosyloxy) benzyl isothiocyanate (Padla *et al.*, 2012). However, in other studies 4-(α -L-rhamnosyloxy) benzyl isothiocyanate was found to be effective against both *P. aeruginosa* and *E. coli* (Oluduro *et al.*, 2010; Rim Jeon *et al.*, 2014). To improve the comparison the initial concentration of bacteria, concentration of 4-(α -L-rhamnosyloxy) benzyl isothiocyanate, incubation time and the method used to assess

efficacy are presented if available. The minimum inhibitory concentration (MIC) is the lowest concentration of antibacterial compound that prevents visible bacterial growth while the minimum bactericidal concentration (MBC) is the lowest concentration that results in a 99.9% decrease in culturable cells.

The mechanism of action for 4-(α -L-rhamnosyloxy) benzyl isothiocyanate against bacteria has not been elucidated. However, the effect of isothiocyanates on specific bacterial functions and the hypothetical mechanism of action of some other common isothiocyanates have been proposed (Dufour *et al*., 2015). In their review Dufour, *et al*., (2015) argue that isothiocyanates may accumulate within bacteria as conjugates of glutathione, bacillithiol and thioredoxin-dithiocarbamate. Once inside the cell, a number of responses have been hypothesized: the isothiocyanate binds to the thiol or amine groups of enzymes causing disruption of enzymatic activities including respiration, metabolism and gene transcription; the isothiocyanate conjugates with the thiol groups of proteins, promoting protein misfolding, protein aggregation and the activation of heat-shock response; the isothiocyanate conjugates with amino acids, leading to depletion and a stringent response; finally, the conjugation of isothiocyanates with thioredoxin and other small thiols may affect cell redox homeostasis (Dufour *et al*., 2015).

4-(α -L-rhamnosyloxy) benzyl isothiocyanate has been observed to inhibit the human pathogenic fungi *Candida pseudotropicalis, Fusarium oxysporum* f. *lycopersici,* (Eilert *et al.,* 1981), *Trichophyton rubrum* and *Epidermophyton floccusum* (Padla *et al.,* 2012). Benzyl isothiocyanate, extractable from the *M. oleifera* root, is another antimicrobial agent and has been observed to inhibit *B. subtilis, M. phlei* and *S. marcescens* (Das *et al.,* 1954; Eilert *et al.,* 1981). The compounds 4-[(2'-O-acetyl- α -L-rhamnosyloxy) benzyl] isothiocyanate, 4-[(3'-O-acetyl-α-L-rhamnosyloxy) benzyl] isothiocyanate and 4-(4'-O-acetyl- α -L-rhamnosyloxy) benzyl isothiocyanate have all been shown to be anti-inflammatory agents (Cheenpracha *et al.,* 2010; Waterman *et al.,* 2014). The MIC of another compound, 4-(4'-O-acetyl-α-L-rhamnosyloxy)-benzyl isothiocyanate, was reported to be 62.5 mg/L and 250 mg/L against *S. epidermidis* and *B. subtilis* (Padla *et al.,* 2012). The 4-(4'-O-acetyl-α-L-rhamnosyloxy)-benzyl isothiocyanate was prepared in the same way as 4-(α -L-rhamnosyloxy) benzyl isothiocyanate and when compared, required a lower dose to obtain the same result.

The catalysed reaction of glucosinolates initially results in an unstable intermediate. This intermediate rearranges to form the isothiocyanate group but can also result in formation of nitriles, thiocyanates, epithionitriles and oxazolidonethiones (Fahey *et al.,* 2003). Other antimicrobial compounds isolated from *M. oleifera* seeds are methyl N-4-(α -L-rhamnopyranosyloxy) benzyl carbamate. A 0.005 mg/L concentration has inhibited *E. coli* and *S. typhi* after one hour, *B. cereus* after three hours, and *P. aeruginosa, S. dysenteriae* after four hours (Oluduro *et al.,* 2010). A methanolic fraction containing 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxy) at a concentration of 0.005 mg/L completely inhibited *E. coli* after three hours, *P. aeruginosa* and *S. dysenteriae* after four hours, and *S. typhi* and *B. cereus* after three hours (Oluduro *et al.,* 2010). The compounds S-ethyl-N-[4- (α-L-rhamnosyloxy) benzyl)] thiocarbamate and 2-acetoxy (4- [2',3',4' – tri-O-acetyl –α- L- rhamnosyloxy] benzyl acetonitrile) have been isolated from a ethyl acetate fraction of *M. oleifera* seeds first extracted in distilled water but were not tested against any organisms (Mehta *et al.,* 2011).

Rim Jeon, *et al*. (2014) have reported that 4-(α -L-rhamnosyloxy) benzyl isothiocyanate can be encapsulated within porous zinc oxide, itself an antibacterial agent, in a 10% w/v suspension. This functionalised zinc oxide can then be used as reservoir, allowing for the slow release of 4-(α -L-rhamnosyloxy) benzyl isothiocyanate. The applications of this research are reported to be of interest for cosmetic purposes (Rim Jeon *et al.,* 2014).

2.2.1.2 Potential toxicity of isothiocyanates

A significant body of research exists regarding the consumption of isothiocyanates, especially those found in the *Brassicaceae* family, indicating consumption is beneficial for overall human health (Angelino & Jeffery 2014). The benefits of isothiocyanates, including 4(α-L-rhamnosyloxy) benzyl isothiocyanate, comprise inactivation of the expression of nitric oxide synthase (iNOS) and nitric oxide production. Nitric oxide is an inflammatory mediator that is overproduced during chronic inflammation associated with diseases such as type 2 diabetes mellitus and multiple sclerosis (Cheenpracha *et al.,* 2010; Galuppo *et al.,* 2014; Waterman *et al.,* 2014), and 4(α-L-rhamnosyloxy) benzyl isothiocyanate has also shown significant antitumor activity with low toxicity (Brunelli *et al.,* 2010).

The toxic effect of the crude seed extract is attributed to the glycosides; namely 4(α-L-rhamnosyloxy) benzyl isothiocyanate (Grabow *et al.,* 1985; Araújo *et al.,* 2013; Al-Anizi

et al., 2014) and isothiocyanates are known to break down into goitrogenic agents, which damage the thyroid gland (Al-Anizi *et al.,* 2014). Stohs & Hartman (2015) reviewed the safety of the *M. oleifera* leaf isothiocyanates and reported a high degree of safety across human studies using powdered leaf preparations, and for animal studies using aqueous, alcohol and hydroalcohol extraction techniques. They concluded that the research looks promising but caution that there are significant differences in the methods used to test the leaf extracts and so direct comparison is not currently feasible. The consensus is that 4(α-$_L$-rhamnosyloxy) benzyl isothiocyanate is a beneficial water treatment option where the risks of its use are outweighed by the efficacy of the treatment. If 4(α-$_L$-rhamnosyloxy) benzyl isothiocyanate were to be used as a primary water treatment method, then long term toxicity studies should be completed in order to verify this consensus.

2.2.2 Proteins

M. oleifera seeds contain 22.0 % ±3 % total proteins (Shebek *et al.,* 2015). Cationic proteins make up 97 ± 0.3 wt. % of the water-soluble proteins after fatty acid removal (Nordmark *et al* 2018). This fraction contains a large range of proteins that share similar physical characteristics (Ghebremichael *et al*., 2005). The defatted aqueous extract contains at least eight distinct cationic protein sub-populations with non-reduced molecular weights ranging from 11 kDa to 48 kDa, and reduced molecular weights ranging from 7 to 30 kDa (Nordmark *et al* 2018). Of these fractions, 94.9% are strongly cationic, are dominantly alpha helical in shape, and have a radius of 1.2 nm to 1.5 nm (Nordmark *et al* 2018).

Only one of these proteins has been extensively studied, and is commonly called the *M. oleifera* cationic protein (MOCP). This name is used to describe both the non-reduced 12-14kDa dimeric protein as well as the reduced monomer. In this review MOCP will be used exclusively to refer to the 12- 14 kDa protein while the monomer will be called $MO_{2.1}$, as this was the name it was originally given when first isolated by (Gassenschmidt *et al.,* 1995). $MO_{2.1}$, is often referred to as a polypeptide and the recombinant or synthetic $MO_{2.1}$ has been called Flo (Suarez *et al.,* 2003; Suarez *et al.,* 2005), the recombinant *M. oleifera* coagulant protein MOCRP (Pavankumar *et al.,* 2014b), and the water-soluble *M. oleifera* lectin (WSMoL) (Santos *et al.,* 2005).

While the monomer is usually described as being 6.5 kDa, estimating the molecular weight from the amino acid sequence gives a higher number of 6,782 Da. Sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to identify the presence of $MO_{2.1}$ and MOCP in an extract or purified solution. In SDS-PAGE the protein solution is compared to protein markers of known molar mass and is used either to estimate of the size of the protein(s) in the solution or verify the presence of a specific protein in an extract solution. The aqueous extract of *M. oleifera* contains a large variety of proteins with similar characteristics and molecular weight (Gassenschmidt *et al.*, 1995). Nordmark *et al.,* (2016) suggested that small differences in the amino acid sequence of *M. oleifera* proteins may not be distinguishable by SDS-PAGE but would affect when the proteins eluted out of a cation exchange chromatography column. This variety within the aqueous extract makes it difficult to accurately state that $MO_{2.1}$ is the only protein present, as SDS-PAGE may not be sensitive enough to distinguish between all of the coagulant proteins of similar molecular mass and in many cases there may be more than one compound present but only $MO_{2.1}$ is identified.

2.2.2.1 $MO_{2.1}$

In an effort to elucidate the components that cause flocculation, crushed seeds from *M. oleifera* were defatted and soaked in a phosphate buffer (Gassenschmidt *et al.,* 1995). The flocculating proteins were separated from the non-flocculating proteins using cation exchange chromatography with a NaCl gradient from 0 M to 2 M and the three fractions of flocculating protein produced were called MO_1-MO_3. The second of these, MO_2 was further fractionated into $MO_{2.1}$, $MO_{2.2}$ and $MO_{2.3}$ of which $MO_{2.1}$ and $MO_{2.2}$ were homogeneous polypeptides with molecular masses of 6.5 kDa and 7 kDa. $MO_{2.2}$ is not directly referenced in any other studies and there are currently no reports where $MO_{2.2}$ is examined as an antimicrobial. However, a compound with a molecular mass of 7.1 kDa has been used to coagulate haemoglobin (Katre *et al.,* 2008). This compound was isolated by reducing the disulphide bonds of a 13.6 kDa homodimer using mercaptoethanol and the N-terminal amino acid sequence, APGIMYRVQR, did not yield any significant matches to $MO_{2.1}$ (Katre *et al.,* 2008). The sizes and structures of MO_1 and MO_3 were not described (Gassenschmidt *et al.,* 1995).

$MO_{2.1}$ is reported to comprise 1.2 % ±0.2 % of the total protein in unshelled seeds (Shebek *et al.,* 2015). $MO_{2.1}$ contains eight positively charged amino acids and is highly charged in an aqueous solution with an isoelectric point (pI) of 12.6 (Broin *et al.,* 2002). The amino acid sequence mapped by Gassenschmidt, *et al.*, (1995) can be found on the

protein sequence database UniProt under the reference number P24303 (http://www.uniprot.org/). It is labelled Flocculent-active proteins $MO_{2.1}$ and $MO_{2.2}$ with the amino acid sequence QGPGRQPDFQ RCGQQLRNIS PPQRCPSLRQ AVQLTHQQQG QVGPQQVRQM YRVASNIPST. The molecular weight of this sequence is 6,782 Da .Another sequence can be found under the reference number Q93YG0 as 2.1 Protein. In this sequence the Gly-13 and Glu-23 amino acids were replaced with Cys residues (Broin *et al.,* 2002). The molecular weight of this sequence is higher than the reported $MO_{2.1}$ at 6,803 Da and as previously mentioned, may be a distinct protein labelled as $MO_{2.1}$. The European Bioinformatics Institute has $MO_{2.1}$ under catalogue number AJ345072 (Broin *et al.,* 2002). Tandem mass spectrometry (MS/MS) has resulted in a four peptide sequences of similar mass but slightly different composition, one of which is identical to the mapped sequence of $MO_{2.1}$ (Ghebremichael *et al.,* 2005).

The secondary structure of $MO_{2.1}$ is comprised of three α-helices and β-loop regions (Suarez *et al.*, 2005). Three conformations have been proposed. The first was modelled on the NMR structure of 2S-albumin, the napin B chain, and had a sequence identity of 71% with $MO_{2.1}$ (Suarez *et al.,* 2005). The second is based on the Mabinlin II protein and has a 97% similarity to $MO_{2.1}$ (Okoli *et al.,* 2012). The third has 100% similarity to the peptide sequence published by Broin, et al., (2002) and 97% similarity to the sequence of $MO_{2.1}$ reported by Gassenschmidt, et al., (1995) and was presented earlier in Figure 4 (Pavankumar *et al.,* 2014a). The reported size of $MO_{2.1}$ is 3.1 nm (Okoli *et al.,* 2012), which is larger than the 1.2 nm to 1.5 nm cationic fractions reported more recently by Nordmark *et al.,* (2016).The tertiary structure is proposed to be a dimer with another stable formation as a tetramer with a molecular mass of 26 kDa (Pavankumar *et al.,* 2014a).

The thermostability of the coagulating protein fractions was established by Ghebremichael *et al*., (2005) who found that the coagulating protein fraction could withstand heating for 5 hours at 95ºC and that heat-treating the proteins increased the coagulant efficiency. This has been observed in several other studies of other *M. oleifera* proteins (Katre *et al*., 2008; Luz *et al*., 2013) and boiling the crushed seed kernels to remove the oil before the seeds use used to treat water is recommended in a protocol for using the seeds in water treatment (Lea, 2010). Recently is has been reported that the *M. oleifera* coagulant proteins may belong to the class of intrinsically disordered proteins (Dezfooli *et al*., 2016). Intrinsically disordered proteins are characterised by a high thermal stability, a high percentage of polar

amino acids, and a notable depletion of hydrophobic amino acids, leading to a high solubility in aqueous solutions. Dezfooli *et al.*, (2016) argued that as $MO_{2.1}$ contains 11.7% arginine residues, 11.7% proline residues and has minimal hydrophobic residues it could be categorised as an intrinsically disordered protein. They supported their argument by treating $MO_{2.1}$ to sterilising conditions (121 ºC, 1.2 bar, 15 minutes) and reported that it retained 70% of the coagulation activity of the crude extract. The general method used to extract $MO_{2.1}$ is:

- Defatting;
- Solvent extraction; and,
- Isolation via cation exchange chromatography.

The solutions used to remove the fat from the seed powder were trichlorofluoromethane (Gassenschmidt *et al.,* 1995), ethanol (Ghebremichael *et al.,* 2005; Ghebremichael *et al.,* 2006; Sanchez-Martin *et al.,* 2010) or petroleum ether (Kwaambwa *et al.,* 2010; Nordmark *et al.,* 2016). Defatting is not reported to cause any difference in the coagulation activity of the extract (Ghebremichael *et al.,* 2005; Nordmark *et al.,* 2016). *M. oleifera* seed oil is a valuable commodity so is beneficial that the oil is not necessary for coagulation (Nordmark *et al.,* 2016).

Most studies used distilled water to extract the $MO_{2.1}$ from the crushed seeds (Ghebremichael *et al.,* 2005; Santos *et al.,* 2005; Ghebremichael *et al.,* 2006; Kwaambwa *et al.,* 2010; Shebek *et al.,* 2015). The other solutions used were sodium chloride (Suarez *et al.,* 2003; Ghebremichael *et al.,* 2005), ammonium acetate (Ghebremichael *et al.,* 2006; Sanchez-Martin *et al.,* 2010) and a sodium phosphate buffer (Gassenschmidt *et al.,* 1995; Nordmark *et al.,* 2016). The solution is then generally filtered at this stage to remove the undissolved particles.

After extraction, the $MO_{2.1}$ was isolated by running the extract solution through a cation exchange chromatography column. The solutions used to equilibrate the chromatography columns were ammonium acetate (Ghebremichael *et al.,* 2005; Ghebremichael *et al.,* 2006; Sanchez-Martin *et al.,* 2010), phosphate buffer (Gassenschmidt *et al.,* 1995; Nordmark *et al.,* 2016) or water (Kwaambwa *et al.,* 2010; Shebek *et al.,* 2015). The columns were then eluted using 0.6 M sodium chloride (Gassenschmidt *et al.,* 1995; Ghebremichael *et al.,* 2006; Sanchez-Martin *et al.,* 2010; Shebek *et al.,* 2015), 1 M sodium

chloride (Kwaambwa *et al.,* 2010), a sodium chloride gradient of 0% to 65% phosphate buffer Nordmark *et al.*, 2016), or ammonium acetate (Ghebremichael *et al.,* 2005).

The solution may be further purified, with loss of yield, by running it through a second cation exchange column (Gassenschmidt *et al.,* 1995). In one instance the polypeptide was precipitated after the extraction step using ammonium and re-suspended in water, purified by dialysis and then run through the cationic exchange column (Kwaambwa *et al.,* 2010). An ammonium acetate buffer can be used in the place of water negating the need to change buffers during the purification process and simplifying this method of extraction (Ghebremichael *et al.,* 2006).

Recombinant production of $MO_{2.1}$, both exactly and with slight changes to the amino acid sequence, has been previously described (Broin *et al.,* 2002; Suarez *et al.,* 2003; Suarez *et al.,* 2005; Pavankumar *et al.,* 2014b). The $MO_{2.1}$ was then isolated using cation exchange chromatography, similar to the method used in the extraction from seeds, and protein tags were used to improve this process (Broin *et al.,* 2002; Suarez *et al.,* 2003). Synthetic production of $MO_{2.1}$ has been achieved using an Applied Biosystems synthesizer 433A and an Abimed AMS 422 multiple peptide synthesizer. Purification was achieved using gel filtration on a Sephadex column and reverse phase HPLC (Suarez *et al.,* 2005).

The $MO_{2.1}$ can be precipitated from an aqueous extract solution using 40% ammonium sulphate, commonly called the salting out method. The ammonium sulphate is them removed by dialysis (Dezfooli *et al*., 2016). Salting out is used in the isolation of the MOCP and is discussed in more detail later in this review. After dialysis, the other proteins were removed by heating the solution at 40 ºC, 60 ºC, 80 ºC, 100 ºC and 121ºC (Dezfooli *et al*., 2016).

An emerging field of research involves the development of $MO_{2.1}$ functional surfaces. These surfaces include silica, super-paramagnetic iron oxide nanoparticles, magnetic iron oxide nanoparticles, rice husk ash and granular activated carbon.$MO_{2.1}$ is cationic and can be adsorbed onto the surface of silica where the polypeptide has been observed to form a layer greater than 60Å (Kwaambwa *et al.,* 2010). This method has been developed, and simplified, by Jerri, *et al.,* (2012) who reported that the polypeptide adsorbed on the surface of washed quartz sand. This functional-sand (f-sand) electrostatically adsorbs anionic colloids and inhibits *E. coli*. To prepare f-sand, a crude aqueous extract is mixed with clean quartz sand and the excess biological material is rinsed off. The $MO_{2.1}$ remains

attached to the sand unless eluted with a 0.6 M NaCl solution (Jerri *et al.,* 2012). The adsorption of defatted total proteins (cationic and non-cationic) to the surface of ionic strength of water can be varied from 2.3 mM to 9.2mM interfere with protein binding, with hard and soft water displaying the same adsorption isotherms (Nordmark *et al.,* 2018). Sand prepared in this manner has been shown to remove some species of microalgae from water (Li & Pan 2013). A similar idea was examined by Santos, *et al*., (2011) using a 30 kDa protein: however, this protein did not bind to sand, clay or cellulose.

$MO_{2.1}$ has been successfully attached to the surface of magnetic iron oxide nanoparticles and super-paramagnetic iron oxide nanoparticles that had been coated with SiO_2, coated with trisodium citrate or where uncoated (Okoli *et al*., 2011a, 2011b, 2012).Both types of iron oxide nanoparticle preparations retained the coagulant capabilities of the unattached $MO_{2.1}$ to varying degrees (Okoli *et al*., 2011a, 2012). The iron oxide nanoparticles could then be removed from the solution using magnets (Pavankumar *et al.*, 2014b). The amount of $MO_{2.1}$ isolated in this manner ranges from 1 mg/L to 42 mg/L (Pavankumar *et al.,* 2014b). So far this method of $MO_{2.1}$ isolation has not been examined against bacteria.

The method developed by Jerri *et al.,* (2012) has been modified to use rice husk ash and granular activated carbon as well as sand (Barajas & Pagsuyoin, 2015, 2017). For the parameters used in this study, the functional activated carbon and rice husk ash samples were able to remove more *E. coli* than the sand (Barajas & Pagsuyoin, 2015) Further research by Barajas *et al.,* (2016) determined that the maximum amount of polypeptide attached to the rice husk ash was 32 mg/g. Interestingly, they also found that the amount of bacteria removed using the functional rice husk ash was less than that using untreated rice husk ash; however, the untreated rice-husk ash did not inhibit the *E. coli* and so posed a risk of recontamination.

2.2.2.2 $MO_{2.1}$ as an antimicrobial agent

$MO_{2.1}$ is an active antimicrobial agent. The amino acid sequence responsible for the antimicrobial effect is RCGQQLRNIS PPQRCPSLRQ beginning at the 11th amino acid residue, and does not exhibit any coagulant activity (Suarez *et al.,* 2005). Removal of the PSLRQ residues further along the chain caused a loss of efficacy, indicating that the entire sequence is required for the antimicrobial effect (Suarez *et al.,* 2005). The proline amino acid residues at positions 21 and 22, bracketed by the positive side chains containing

arginine amino acid residues, form a kink in the peptide and are responsible for the antimicrobial effect of $MO_{2.1}$. This activity was proposed to be due to the positively charged arginines attracting the negatively charged bacterial membrane and the proline residues penetrating the membrane, disrupting cell activity and resulting in cell lysis (Suarez *et al.,* 2005). The interaction of $MO_{2.1}$ with the bacterial membrane is reported to cause fusion between the inner and outer membranes of *E. coli* cells (Shebek *et al.,* 2015). In order to elucidate whether membrane fusion is the key inhibitory mechanism, lipid vesicles were prepared from commercial *E. coli* lipid extracts and treated with $MO_{2.1}$. The lipid vesicles in the presence of $MO_{2.1}$ aggregated while the control vesicles did not aggregate. Using molecular dynamic simulations, it has been proposed that the $MO_{2.1}$ facilitates binding by bringing the membranes into contact with each other where they form a narrow stalk. This stalk widens until the two vesicles fuse with the stalk formation being the rate limiting step (Shebek *et al.,* 2015). The report of Shebek *et al*., (2015) supports the work of Broin *et al.,* (2002), who observed the aggregation of *Paenibacillus polymyxa* CF43 and *Pseudomonas brassicacearum* NFM421 in the presence of $MO_{2.1}$ and also explains why the antimicrobial activity of $MO_{2.1}$ is so often reported as a factor of the coagulation. The attachment of $MO_{2.1}$ to *E. coli* was made apparent using f-sand where live-dead staining showed that the organisms attached to the sand were dead and that once attached, would not separate nor transfer between f-sand particles (Jerri *et al.,* 2012).

Studies examining the effect of $MO_{2.1}$ on a range of gram-positive and gram-negative bacteria are listed in Table 2. The list includes the gram-positive bacteria *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mitis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*, and the gram-negative bacteria *Escherichia coli*, *Legionella pneumophila*, *Proteus mirabalis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Salmonella typhimurium*. As with Table 1, the results presented in Table 2 are indicative of the antibacterial properties of $MO_{2.1}$ against a broad range of bacteria. In addition, the optical density (OD) is was considered synonymous with the concentration of the organism and is estimated using a spectrophotometer, at 600 nm (Suarez *et al.,* 2003; Pavankumar *et al.,* 2014b) or 620 nm (Suarez *et al.,* 2005). An optical density of 0.1 is approximately 1 - 2 x 10^8 CFU/mL of bacteria (Wikler 2006).

Table 2: Bacterial susceptibility to $MO_{2.1}$

Bacteria	Strain	Initial concentration (CFU/mL)	Method	Inhibitory concentration (mg/L)	Incubation time (hours)	Inactivation method	Reference
Gram-positive bacteria							
B. subtilis	NA	1 - 2 × 10^8	Overnight culture diluted and mixed with $MO_{2.1}$	10 50	4	Low growth rate [c]	(Pavankumar *et al.*, 2014b)
B. thuringiensis	Bt7 Bt75	4 × 10^8	Overnight culture diluted and mixed with $MO_{2.1}$	NA	2	4 log reduction	(Ghebremichael et al., 2005)
E. faecalis	Clinical isolate	7 - 8 × 10^8	Exponential phase organism re-suspended in buffer, incubated with $MO_{2.1}$	1950 [a]	2	Low growth rate [c]	(Suarez *et al.*, 2005)
		5 ×10^5	Broth dilution and colony counts	10,000	24	MIC	(Suarez *et al.*, 2003)
S. aureus	NA	1 - 2 × 10^8	Overnight culture diluted and mixed with $MO_{2.1}$	10 50	5	Low growth rate [c]	(Pavankumar *et al.*, 2014b)
	P8 methicillin resistant	5 ×10^5 5 ×10^5	Broth dilution and colony counts	2000 - 5000 5000 - 10,000	24 24	MIC MBC	(Suarez *et al.*, 2003)
	P8, methicillin resistant	7 - 8 × 10^8	Exponential phase organism re-suspended in buffer, incubated with $MO_{2.1}$	1950 [a]	2	Low growth rate [c]	(Suarez *et al.*, 2005)
S. mitis	NA	5 ×10^5	Broth dilution and colony counts	10,000 10,000	24 24	MIC MBC	(Suarez *et al.*, 2003)
S. pneumoniae	NA			1000 2500	24 24	MIC MBC	
S. pyogenes	ATCC 700294*			2000 - 5000 2000 - 5000	24 24	MIC MBC	
S. pyogenes	ATCC 700294*	7 - 8 × 10^8	Exponential phase organism re-suspended in buffer, incubated with $MO_{2.1}$	1950 [a]	2	Low growth rate [c]	(Suarez *et al.*, 2005)

Bacteria	Strain	Initial concentration (CFU/mL)	Method	Inhibitory concentration (mg/L)	Incubation time (hours)	Inactivation method	Reference
Gram-negative bacteria							
E. coli	ATCC 25922	5×10^5	Broth dilution and colony counts	100	24	IC_{50}	(Suarez *et al.*, 2003)
		5×10^5		10,000	24	MIC	
	D31	4×10^8	Overnight culture diluted and mixed with $MO_{2.1}$	NA	2	1.1 log reduction	(Ghebremichael et al., 2005)
	K12					4 log reduction	
L. pneumophila	serotype 1	5×10^5	Broth dilution and colony counts	800	48	MIC	(Suarez *et al.*, 2003)
		5×10^5		2000 - 3000	48	MBC	
P. mirabalis [b]	NA	$1 - 2 \times 10^8$	Overnight culture diluted and mixed with $MO_{2.1}$	10	5	Low growth rate [c]	(Pavankumar *et al.*, 2014b)
				50			
P. aeruginosa	NA	$1 - 2 \times 10^8$	Overnight culture diluted and mixed with $MO_{2.1}$	10	5	Low growth rate [c]	(Pavankumar *et al.*, 2014b)
				50			
	PAO1 / ATCC 15692*	$7 - 8 \times 10^8$	Exponential phase organism re-suspended in buffer, incubated with $MO_{2.1}$	1950[1]	2	Low growth rate [c]	(Suarez *et al.*, 2005)
	K12	4×10^8	Overnight culture diluted and mixed with $MO_{2.1}$	NA	2	4 log reduction	(Ghebremichael et al., 2005)
S. enterica	Typhimurium	$7 - 8 \times 10^8$	Exponential phase organism re-suspended in buffer, incubated with $MO_{2.1}$	1950[1]	2	Low growth rate [c]	(Suarez *et al.*, 2005)
S. typhimurium	NA	$1 - 2 \times 10^8$	Overnight cultures diluted and mixed with $MO_{2.1}$	10	5	Low growth rate [c]	(Pavankumar *et al.*, 2014b)

[a] Synthetically produced polypeptide, converted using a molecular mass of 6500 g/mol

[b] Referred to as *Serratia mirabalis*

[c] Result reported as the organism growth rate in a control solution compared to the organism growth rate in the presence of $MO_{2.1}$

*Pathogenic strain

There is substantial variation in the concentrations of $MO_{2.1}$ reported to reduce or inhibit even one species of bacteria. This variation is evident in Table 2 as illustrated by *E. coli* where the half maximal inhibitory concentration (IC_{50}) is 100 mg/L, but the minimum inhibitory concentration is 10,000 mg/L (Suarez *et al.*, 2003). Further to this, the ability to cause bacterial aggregation using $MO_{2.1}$ can differ between bacterial species. This was observed using *E. coli* (D31) and *E. coli* (K12) where the latter did not noticeably aggregate (Ghebremichael *et al.*, 2005). In this study the $MO_{2.1}$ caused a 4 $\log_{10}$ reduction of the *E. coli* (K12) but only a 1.1 log reduction of the *E. coli* (D31) (Ghebremichael *et al.*, 2005). As noted with the isothiocyanates, the difference in the methods used to measure the effect of the $MO_{2.1}$ on bacteria may be misleading. Larger, standardised studies to determine the actual inhibitory concentrations would provide clarity on this issue Bacteria that have shown inactivation for both $MO_{2.1}$ and 4-(α-L-rhamnosyloxy) benzyl isothiocyanate are *B. subtilis*, *S. aureus, E. coli*, and *P. aeruginosa*. The specific quantities of each compound in the seed extract comparable to each other compound have not been determined.

The haemolytic effect of $MO_{2.1}$ on human red blood cells has been reported to occur at 300 µM. This is two magnitudes higher than the bactericidal concentration (Suarez *et al.*, 2005). No haemolytic effect on mouse erythrocytes was observed by Araújo *et al.*, (2013). The protein fraction that most likely contained the $MO_{2.1}$ and the MOCP exhibited no mutagenic activity against the TA97, TA98, TA100 and TA102 *Salmonella* strains (Rolim *et al.*, 2011). The effect of $MO_{2.1}$ on the human microbiome, or the beneficial bacteria which reside there, and the potential implications for people relying on water containing the protein is something which could be explored. The coagulant proteins are very robust with the $MO_{2.1}$ shown to maintain its structure when exposed to sterilising conditions (121 ^{o}C, 1.2 bar, 15 minutes). Likewise, a *M. oleifera* seed lectin with 81% similarity in its amino acid sequence to $MO_{2.1}$ was shown to maintain its secondary structure across a pH range of 2 to 12 so it is possible that these type of proteins may be able to pass into the intestines and influence the bacteria that reside there.

2.2.2.3 The coagulant protein

The MOCP is isolated under non-reducing conditions. Structural analysis indicated that the secondary structure is dominantly α-helical and is stable between pH 4 and 10 in an ionic solution of 0.5 M NaCl (Kwaambwa & Maikokera 2008). As with $MO_{2.1}$, there is a

general method followed to isolate the MOCP. In all cases, the general method is based on the method developed by Ndabigengesere *et al.,* (1995). The steps are:

- Defatting;
- Solvent extraction;
- Protein precipitation (salting out);
- Dialysis; and,
- Isolation via cation exchange chromatography.

Petroleum ether was the reagent used in all cases to remove the seed oil (Ndabigengesere & Narasiah 1998a; Kwaambwa & Maikokera 2007, 2008; Hellsing *et al.,* 2013; Kwaambwa & Nermark 2013). The protein was then extracted using distilled water (Ndabigengesere *et al.,* 1995; Ndabigengesere & Narasiah 1998a; Okuda *et al.,* 1999; Kwaambwa & Maikokera 2007, 2008; Hellsing *et al.,* 2013; Kwaambwa & Nermark 2013), and a salt solutions of NaCl (Okuda *et al.,* 1999), KNO_3, KCL and $NaNO_3$ (Okuda *et al.,* 1999). The protein was not soluble in petroleum ether, acetone, chloroform or hexane (Ndabigengesere *et al.,* 1995).

Ammonium sulphate was used to reduce the solubility of the protein so that it precipitated out of the extraction solution. The protein was then re-suspended in water, and the salt was removed by dialysis (Ndabigengesere *et al.,* 1995; Ndabigengesere & Narasiah 1998a; Kwaambwa & Maikokera 2007, 2008; Hellsing *et al.,* 2013; Kwaambwa & Nermark 2013). Isolation was achieved by loading the protein solution into a cation exchange column. The solutions used to equilibrate the column are water (Ndabigengesere *et al.,* 1995; Ndabigengesere & Narasiah 1998a; Kwaambwa & Maikokera 2007, 2008; Hellsing *et al.,* 2013; Kwaambwa & Nermark 2013), Tris-HCL and phosphate buffers (Ndabigengesere *et al.,* 1995). The solutions used to elute the protein are sodium chloride (Ndabigengesere *et al.,* 1995; Ndabigengesere & Narasiah 1998a; Kwaambwa & Maikokera 2007, 2008; Hellsing *et al.,* 2013; Kwaambwa & Nermark 2013) and sodium chloride in Tris-HCL buffer or phosphate buffer (Ndabigengesere *et al.,* 1995). To date, MOCP has not been tested against any pathogenic organisms, as the research has been focused on the protein as a coagulant. As MOCP is partly comprised of the $MO_{2}.1$ polypeptide exploring its efficacy against bacteria is of interest for further study.

2.2.2.4 Lectins

Another class of proteins that can be isolated from the *M. oleifera* seeds are the lectins. Lectins are proteins which recognise and bind to monosaccharides and oligosaccharides, including carbohydrates on the surface of blood erythrocytes. The binding of lectins to these carbohydrates causes aggregation of the erythrocytes (Luz *et al.,* 2013). Several coagulating lectins have been isolated (Katre *et al* 2008; Santos *et al* 2005; Santos *et al* 2009). Though a lectin isolated from *M. oleifera* has been found to act as an anticoagulant (Luz *et al.,* 2013) The lectins have been shown to be structurally similar to the other *M. oleifera* proteins (Luz *et al.,* 2013), and effective at reducing the number of mosquito larvae (Coelho *et al.,* 2009

2.2.2.5 Other isolated proteins

Santos *et al.,* (2009) reported the presence of a 30 kDa, thermo-stable, basic protein in the water extract that denatured under reducing conditions and appeared as a 26.5 kDa band on SDS-PAGE (Santos *et al.,* 2009). This protein has also been shown to cause coagulation and is comparable with alum (Santos *et al.,* 2009). A further study showed that the 30 kDa protein did not cause coagulation in the presence of calcium and magnesium ions (Santos *et al.,* 2011). The lectins have been shown to be structurally similar to the other *M. oleifera* proteins (Luz *et al.,* 2013), and effective at reducing the number of mosquito larvae (Coelho *et al.,* 2009

Other proteins ranging in size from 20 kDa to 66 kDa have been identified in *M. oleifera* seed extracts. This protein has since been reported to be a stable oligomeric tetramer comprised of the $MO_{2.1}$ and $MO_{2.2}$ polypeptides (Shebek *et al.,* 2015). A 66 kDa protein with coagulant activity was observed in an extract solution that also contained $MO_{2.1}$. This protein bound to an anion exchange column and was eluted using 0.3 M NaCl. Two proteins sized 20 kDa and 40 kDa were observed in the same solution (Agrawal *et al.,* 2007). None of these proteins have been tested for antimicrobial activity; however, testing these compounds may be of interest in future studies since at least one of them contains the amino acid sequence responsible for the antimicrobial activity of $MO_{2.1}$.

2.2.3 The 3 kDa polyelectrolyte

The final *M. oleifera* seed compound that is reported to cause coagulation is a 3 kDa polyelectrolyte. This polyelectrolyte is included here as it is often referred to in reports discussing the use of the crude extract as a coagulant. However, there are only two reports

that describe the isolation and use of this polyelectrolyte (Okuda *et al*., 2001a; Okuda *et al*., 2001b).

The process of isolation was achieved in four steps:

- Solvent extraction;
- Dialysis with a molecular mass cutoff of 12 kDa to 14 kDa;
- Defatting; and,
- Anion exchange chromatography.

The electrolyte was extracted from crushed seeds with a 1 M NaCl solution used in the extraction process followed by dialysis with a molecular mass cut-off of 12 kDa to 14 kDa was used to remove the salt. An anion exchanger was used to purify this compound and explains why the authors did not isolate any of the other proteins or polypeptides. Cation exchange chromatography was used in all cases where the $MO_{2.1}$ or MOCP was isolated. When the same process was followed using a DEAE sephadex column none of the polypeptide or protein bound to the matrix (Ndabigengesere *et al.,* 1995; Katre *et al.,* 2008). As the isolation of this compound is achieved using anionic exchange chromatography it would not appear in the literature discussing the coagulant protein fractions but may be found in comparisons with crude extracts.

The proposed coagulation mechanism was that the negatively charged compounds bind to divalent cations in solution to form an insoluble, matrix and remove the suspended solids by sweep coagulation (Okuda *et al.,* 2001a). When the polyelectrolyte was added to non-turbid tap water (quality not specified) it precipitated; however, this did not occur when distilled water was used (Okuda *et al.,* 2001a). The precipitation was proposed to occur because of an association between the electrolyte and the $CaCl_2$ in the water. A 0.2 mM concentration of Ca^{2+} was required for a 0.3 mg/L concentration of the polyelectrolyte to cause coagulation (Okuda *et al.,* 2001a). Despite the frequent referencing in the literature, the two studies have not been repeated and it would be of interest to test the polyelectrolyte for efficacy against any bacteria and to ascertain its long-term stability.

This polyelectrolyte is approximately 34 times more efficient than the salt water crude extract and was shown to reduce kaolin concentrations of 5 mg/L, 10 mg/L, 25 mg/L and 50 mg/L to below 0.5 mg/L (Okuda *et al.,* 2001b). The zeta potential of the

polyelectrolyte in a pH 9 solution was around -25 mV (Okuda *et al.,* 2001a). The polyelectrolyte was ineffective when the solution pH was below 7 (Okuda *et al.,* 2001b).

2.4. Conclusions

This review discussed the active compounds that can be isolated from the seed of *M. oleifera*. While it is beneficial to use the crude seed extract if there are no other options available, the disadvantages, such as the increase in biological material, currently prevent any large-scale use of the seed. Simple, affordable procedures still need to be developed for the isolation of these active compounds which are available in regions where traditional water treatment methods are scarce. The majority of methods currently used to purify the compounds are cost-restrictive and complex, especially for the areas where they are most needed, and the use of the purified compounds for point-of-use water treatment is not currently feasible. Further development of novel methods of isolating the active compound could provide a cost effective, functional method for sustainable water treatment. Functionalised super-paramagnetic iron oxide nanoparticles, silica and sand can be easily removed from the treated water after they have been used. Studies into whether communities in low-socioeconomic regions could practically gain access and use for these methods would be valuable as the dissemination of research to these groups could have a significant impact on their standard of living. The functional sand is currently the most accessible method of isolating the *M. oleifera* bactericidal proteins as the sand does not require specialist equipment to prepare and could greatly benefit regions where accessible, small-scale and low cost water treatment is required. Development of effective ways to regenerate the functional sand will remove the reliance on the *M. oleifera* seeds and is an area for more work. Large-scale studies to determine the effectiveness of these treatment systems using environmental and contaminated water sources would be beneficial to elucidate the role of *M. oleifera* seeds against currently available technologies.

2.5 Summary

This literature review has outlined the major antibacterial compounds that can be extracted from *M. oleifera* seeds. The reports of the versatility of $MO_{2.1}$ were considered promising as an emerging area of research. Removal of water-borne bacteria using $MO_{2.1}$ functionalised sand and subsequent removal of the inhibited bacteria was chosen as an area to research further. The following chapters outline the experiments undertaken to achieve this goal.

2.6 Acknowledgements

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Chapter 3. Materials and Methods

3.1 Introduction

This chapter outlines the general analytical methods used for the experimentation required to achieve the aims of this research. Specific experimental designs and procedures are presented in the relevant chapters. All laboratory work was performed at the University of Adelaide with the majority undertaken in the School of Chemical Engineering.

3.2 Materials and Reagents

All reagents were analytical grade unless otherwise specified. Solutions were prepared using Milli-Q water (Millipore Pty Ltd).

3.2.1 *M. oleifera* seeds

M. oleifera seeds were obtained from AustraHort Pty Ltd, Australia. These were manually de-husked using a mortar and pestle. The seed kernels were ground using a spice grinder (Sunbeam, EM0405 MultiGrinder™) for 30 seconds to 1 minute immediately before use. Large particles (above approx. 0.5mm) were removed by sifting the seed powder through a mesh tea strainer.

3.2.2 Sand and Silicon dioxide

Unimin™ Quartz sand was provided by the School of Chemical Engineering, The University of Adelaide. It was thoroughly rinsed with distilled water and dried before use. Silicon dioxide (SiO_2, CAS 14808-60-7) was purchased from Sigma Aldrich and used as received. Specifications for both are detailed in **Table 3**.

Table 3: Specifications for sand and SiO_2.

Sand type	**Silica sand**	**SiO_2**
Particle size	75 μm to 300 μm	0.5 μm to 10 μm
	Approx. 80% between 116 μm to 172 μm	Approx. 80% between 1 μm to 5 μm
Purity	>99%	99%

3.2.3 Cultivation of test bacteria

Nutrient agar was prepared by dissolving either 15 g agar (Oxoid, LP0011B) with 30 g nutrient broth (Oxoid, CM0001B) or by dissolving 15 g nutrient agar (Oxoid, CM0003) into one litre of RO water. The agar solution was autoclaved at 121 °C for 30 minutes and poured onto sterile, plastic petri dishes before use.

Nutrient broth was prepared in 1L Schott bottles by dissolving 25 g/L Nutrient broth (Oxoid, CM0001B) into one litre of RO water. The solution was shaken till all the powder dissolved. The broth was distributed into 50 mL Schott bottles and autoclaved at 121 °C for 30 minutes.

A non-pathogenic strain of *Escherichia coli* (ATCC® 25922™) was sourced from the School of Animal and Veterinary Sciences, The University of Adelaide. *Micrococcus luteus* was sourced from the School of Molecular and Biomedical Sciences, The University of Adelaide. Bacteria colonies were maintained by selecting colonies with a sterile loop and spreading onto agar. The plates were incubated overnight at 37°C, and then stored at 4 °C in the refrigerator.

3.2.4 Surfactant stock solutions

Dodecyl glucoside (CAS 11061-47-9) is a non-ionic surfactant with the formula $C_{18}H_{36}O_6$ and a molecular mass of 348.5 g/mol. It was donated by the Australian suppliers FPI Oceania and Ingredients Plus and received as a 49.74% w/w concentrate solution. The concentrated dodecyl glucoside was diluted to a 1 M stock solution, and the absorbance at 223 nm was used to check the concentration of subsequent dilutions. Sodium dodecyl sulphate (as sodium lauryl sulphate, The British Drug House, CAS 151-21-3) is an anionic surfactant with the formula $NaC_{12}H_{25}SO_4$ and a molecular mass of 288.4 g/mol. The surfactant was provided by the School of Chemical Engineering and was stored as a white powder. A 1 M stock solution was prepared by dissolving the powder in Milli-Q water.

3.3 Analytical Methods

For the studies involving bacteria, all glassware, materials, solutions and tubing were sterilised by autoclaving (121 °C for 30 minutes) in facilities provided by either the School of Chemical Engineering or Medical School South, University of Adelaide.

3.3.1 *M. oleifera* protein extraction and concentration determination

Crude *M. oleifera* extract solutions were prepared by mixing the finely ground seed particles with either Milli-Q water or a 0.05 M, 0.1 M or 0.15 M NaCl solution. The extract solutions were mixed on shaker plates (Ratek, OM7 Digital Orbital Mixer) at 50 RPM for 1 hour unless specified otherwise. The solutions were allowed to settle for 5 minutes, strained through a tea strainer (approx. 0.5 mm) then separated from the solids

using vacuum filtration through 0.8µm filter paper (Zetapor ® Cuno Inc.). The filtrate was used immediately, and fresh solutions were prepared as required.

The concentration of total soluble protein in each sample was tested using a UV-visible spectrophotometer (Shimadzu, UV-1601). Calibration of the protein concentration was performed by preparing five serial dilutions of a Bradford reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) mixed with a filtered *M. oleifera* extract solution. Once the concentration had been calculated the absorbance of each crude or purified solution was measured across a UV range of 190 nm to 800 nm, and the UV peak was observed at 270 nm to 280 nm. Variations in the sample turbidity were initially an issue, so the absorbance at 400 nm was used as a baseline against the absorbance at 280 nm. In later experiments this variation was removed by filtering the samples through 0.45µm filter papers (Millipore) to remove background turbidity. The total protein in each solution was determined following the method of Aitken and Learmonth (1996) using the Beer-Lambert law (Eq. 1). Quartz cuvettes with a path length of 1 cm were used for all experiments.

$$A=\varepsilon Cl \qquad \textbf{Eq. 1}$$

Where A is the absorbance at 280 nm, ε is the extinction coefficient (1520) as determined from the amino acid sequence available on the protein sequence website UniProt (http://www.uniprot.org/), C is the concentration (mol/L) and l is the cuvette pathlength (cm).

3.3.2 Isolation of $MO_{2.1}$

The 280 nm peak in the crude *M. oleifera extract* solutions is comprised of many water-soluble proteins and polypeptides (Ndabigengesere *et al.*, 1995). To isolate the $MO_{2.1}$ polypeptide, cation exchange chromatography was performed following the method of Ghebremichael *et al.*, (2005). A glass chromatography column (46 cm, 1 mm ID) was loaded with CM Sepharose Fast Flow (Sigma-Aldrich) and equilibrated with a 0.1 M NaCl solution. The filtered crude extract was loaded into the column and washed with 0.1 M NaCl. The NaCl concentration was increased stepwise from 0.1 M to 0.6 M then 1 M. The $MO_{2.1}$ eluted at 0.6 M NaCl. An example of the elution profile obtained during this research is presented in Figure 6. The first and second peaks shown in Figure 6 contain weakly cationic proteins while the third peak contains the cationic proteins and includes $MO_{2.1}$. The elution profile of the third peak was comparable to the elution profiles

reported by Ghebremichael *et al.,* (2005, 2006) who demonstrated that single step cation exchange purification for $MO_{2.1}$ was feasible using a NaCl step gradient as the other proteins eluted between 0.1 M and 0.3 M NaCl.

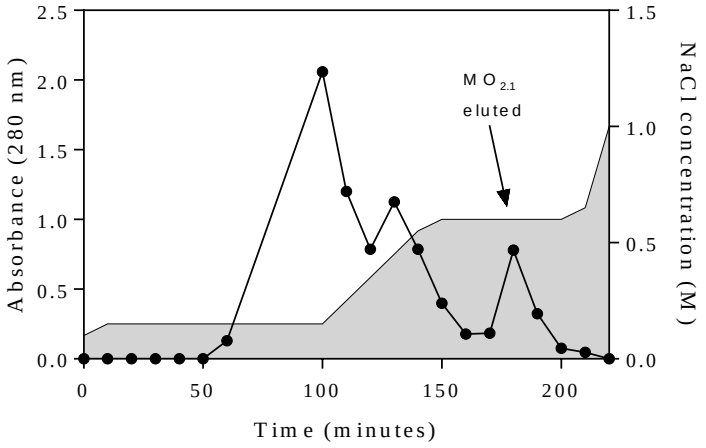

Figure 6: CM Sepharose Fast Flow cation exchange chromatography

3.3.3 Preparation of *f*-sand

The Moringa functional sand (*f*-sand) as studied by Jerri *et al.*, (2012) had scope for further development. In their study, they concluded that while the *f*-sand had potential for water treatment, it was a single use technology. The reported limitation led to the decision to develop a method that would render the *f*-sand reusable, as described in the principle aims of the thesis in Chapter 1. Firstly, the preparation of the *f*-sand was optimised for the greatest amount of bound $MO_{2.1}$ per unit mass of sand. The method of Jerri *et al.,* (2012) involved mixing 1.2 g of washed quartz sand with 9 mL of a 0.025 g/mL crude solution for 1 hour at room temperature. Jerri *et al.,* (2012) reported that the treated quartz had acquired the same antibacterial activity when higher seed concentrations were used and suggested that surface protein saturation occurred around 0.9 seeds per 1.2 g sand. The amount of $MO_{2.1}$ that would attach to the surface of sand was tested in a series of experiments. Three experimental parameters were chosen for investigation and are listed below. The first two values were based on those in the published $MO_{2.1}$ extraction literature as they had all been reported to affect the amount of $MO_{2.1}$ extracted (Jerri et al., 2011; Ghebremichael *et al.,* 2005). The third was investigated as a time saving measure to reduce the time spent preparing the *f*-sand. The total time to prepare *f*-sand from the crude extract was 3 hours while preparing the *f*-sand from the chromatography-isolated protein took over 6 hours.

The three experimental parameters selected include:

- Seed powder concentration
    - 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL;
- NaCl concentration
    - 0 M, 0.05 M, 0.1 M, 0.15 M; and,
- Time of sand addition
    - With the *M. oleifera* seeds or to the filtrate.

The amount of $MO_{2.1}$ on the sand surface was determined by eluting the $MO_{2.1}$ and measuring the absorbance. The $MO_{2.1}$ was separated from the f-sand by mixing 1 g with 5 mL of a 0.6 M NaCl solution for 1 hour on a shaker plate. The mass of $MO_{2.1}$ per gram of f-sand was determined using Equation 2, a modified version of the Beer-Lambert law (Eq. 2). The mass of $MO_{2.1}$ was determined using a molecular mass of 6781.6 g/mol, based upon the reported amino acid sequence of $MO_{2.1}$ (http://www.uniprot.org/).

$$C_{MO_{2.1}} = \frac{1000\left(\left(\frac{A_{280}}{\varepsilon}\right)V_{ex}\right)6781.6}{m_{sand}} \qquad \textbf{Eq. 2}$$

Where $C_{MO_{2.1}}$ is the concentration of $MO_{2.1}$in mg/g sand, A_{280} is the absorbance at 280 nm, V_{ex} is the volume of extracting solution (L) and m_{sand} is the mass of sand (g).

3.3.4 Reusability studies

Currently f-sand is a single use technology and once inhibited, the *E. coli* remained attached to the $MO_{2.1}$ and prevented any further inactivation from occurring (Jerri *et al.*, 2012). Development of a method to separate the inhibited bacteria without reducing the efficacy of the f-sand would allow for more versatility in the use of f-sand as it would reduce both the preparation time and quantity of *M. oleifera* seeds. The amount of $MO_{2.1}$ remaining on the f-sand was determined using Eq. 2.

The effect of surfactants on the inhibited bacteria was considered as many surfactants are widely used in domestic cleaning products. Surfactants are amphiphilic compounds that are commonly added to cleaning products to solubilise fats (Glover *et al.*, 1999). This mechanism can be utilised on a cellular level to destabilise bacterial lipid membranes and is used to extract membrane proteins and other compounds of interest (le Maire *et al.*, 2000). The surfactants sodium dodecyl sulphate and dodecyl glucoside were selected as

they are both commonly used in a large range of detergents, shampoos and cleaning products.

The effect of heat on the inhibited bacteria was considered as it would be more widely accessible than chemical methods. $MO_{2.1}$ is thermoresistant and able to withstand being heated to 95°C for 5 hours without loss of activity (Ghebremichael *et al.*, 2005). The coagulant activity of isolated $MO_{2.1}$ at 121ºC is reported to be approximately 70% of the crude extract (Deezfooli *et al*., 2016). Further information on the experiments conducted to determine the effect of temperature on f-sand can be found in Chapter 5.

3.3.5 Preparation of synthetic water

In this thesis the term 'synthetic' is used to describe the water that had been deliberately contaminated with bacteria. The amount of NaCl in this water was experimentally determined to be the amount at which the bacteria could survive but would not remove the protein from the sand or SiO_2 surface.

For the experiments in Chapters 4 to 7 the following procedure was followed to prepare the synthetic water. Colonies of bacteria were selected using a sterile loop from an agar plate and incubated at 37ºC in nutrient broth (Oxoid) overnight. Approximately 10 mL of bacterial broth was centrifuged at 3000 x g for 5 minutes (Allegra™ X-12, Beckman Coulter). The resultant bacterial pellet was then resuspended in sterile 0.85% NaCl. The bacterial pellet was centrifuged again and resuspended in sterile 0.85% NaCl to an absorbance of 0.1 ± 0.005 at 600 nm on a UV-visible spectrophotometer (Shimadzu UV-Vis 1601). This absorbance is equivalent to a bacterial concentration of 1-2 $x10^8$ (Wikler, 2006). The solutions were diluted to the required bacterial concentrations, which were quantified using UV-Vis. Cultivatable counts were performed to validate the number of bacteria in solution.

For the work reported in Chapter 7, artificially contaminated water was prepared as follows. A 0.1% NaCl solution was prepared in 1 L and 5 L glass bottles (Schott) or in 20 L plastic vessels (Nalgene) and sterilised by autoclaving (121 °C for 30 minutes). *E. coli* was introduced by pipetting a volume from a 0.1 optical density (OD) solution as described in section 3.2.3. A homogeneous bacterial concentration was maintained through the use of a magnetic stirrer (Industrial Equipment & Control Ptd Ltd.). 200µL cultivatable counts were performed to test the actual concentration immediately after preparation and after the synthetic water had been used. An example of the variation in

bacterial concentration over 3.5 hours in 5 L synthetic water is presented Figure 7. The line of best fit was fitted by calculating linear regression of the *E. coli* concentration and indicates loss of bacterial viability over the 3.5 hours. Because of the reduction in bacteria larger volumes of source water were not used for the experiments in Chapter 7.

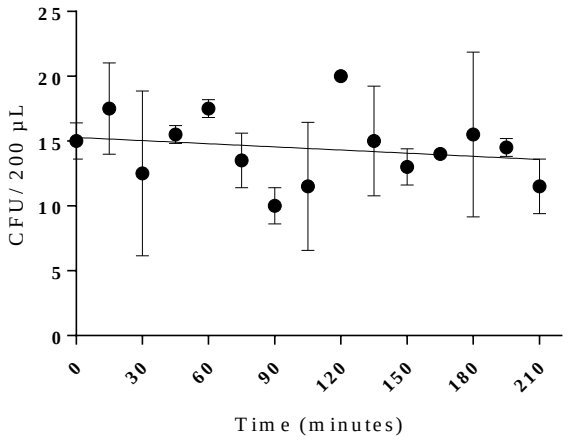

Figure 7: Concentration of *E. coli* in 5 L synthetic water.

3.2.6 Live cell imaging

Microscopy was used to qualitatively observe the attachment and separation of bacteria to the f-SiO_2 and f-sand. Staining was performed using propidium iodide (1mg/mL, Sigma Aldrich) at a 1:500 dilution of 100mM Tris/ acetic acid/ EDTA buffer (Biorad). Samples were incubated for 15 minutes at room temperature in the propidium iodide then rinsed twice in sterile Milli-Q water before imaging. The Nikon Ti E Live Cell Microscope at Adelaide Microscopy (https://www.adelaide.edu.au/microscopy/) was used for all imaging. The images were used in Chapters 4 to 6.

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Chapter 4. Laboratory scale study

Statement of Authorship

Title of Paper	Laboratory scale study
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Frances Elizabeth Williams
Contribution to the Paper	Designed and performed experiments, interpreted the data and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised development of work. Helped to interpret data and to evaluate and edit the manuscript.
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Feasibility study of using *Moringa oleifera* functional sand for water treatment

Abstract

Moringa oleifera seeds are an effective primary method of water treatment. Their efficacy as a flocculent and antibacterial agent is already well-established in the literature. The crushed seeds are traditionally used in small-scale water treatment in India, Sub-Saharan Africa and Asia, where they are added directly to turbid water and act as an effective flocculent to visibly clarify the water. This effect is caused by coagulant proteins from the *M. oleifera* seed, including the $MO_{2.1}$ polypeptide, that adsorb onto the surface of sand. This functional sand (f-sand) can then be used to inactivate *Escherichia coli*. In this study the efficacy of f-sand in *E. coli* removal was examined in lab-scale sand columns with the aim of determining the effect column configuration and length had on column efficiency. The f-sand was most efficient when a fluidised configuration was used though the highest bacterial reduction achieved was log 1 indicating that there is a place for f-sand in water treatment, but the best method is still to be determined

4.1 Introduction

Lack of access to potable water remains a major issue for a significant part of the global population with at least 10% of the inhabitants in 17 Sub-Saharan countries completely reliant on untreated surface waters (WHO, 2017). Practical and accessible methods of bacterial inactivation that use plant materials can improve health outcomes by filling the gap that currently exists in the accessibility of water treatment methods. The *M. oleifera* tree is native to the lower Himalayas and has been naturalised in Africa, South America, India and across Asia (Jahn, 1988). The seeds of *Moringa oleifera* are a low cost, low technology water treatment option that is well established in the literature (Kansal & Kumari 2014). The seeds contain several bactericidal compounds and one of these can also be used to initiate coagulation of colloidal solutions. The seeds are already used as a primary method of water treatment in many countries (Kansal & Kumari 2014).

One of these compounds is the water-soluble, cationic protein that can be isolated via adsorption onto the particle surface of sand to produce functional sand (f-sand) (Jerri *et al.* 2012). The applications of this f-sand include the inactivation of bacteria and cyanobacteria, and a reduction of turbidity of a suspended kaolin solution (Jerri *et al.*

2012; Li & Pan 2013). *Escherichia coli* is used as an indicator of recent faecal contamination. When water containing suspended *E. coli* is mixed with f-sand, the *E. coli* are attracted by the positive surface charge of the *M. oleifera* protein (Broin *et al.* 2002). The bacteria are then immobilised and inhibited via the attachment of three hydrophilic arginine residues of the $MO_{2.1}$ amino acid sequence (Suarez *et al.* 2005). These surround two hydrophobic proline residues that are forced into the lipid membrane causing lysis and cell death. Once inhibited the bacteria remains immobilised on the f-sand surface and does not separate when rinsed with water (Jerri *et al.* 2012). A major block to the large-scale use of *M. oleifera* seeds in water treatment is the biological matter that is released into the water along with the useful compounds. The breakdown of this biological matter leads to the recontamination of the water and making storage of treated water unfeasible (Ndabigengesere & Narasiah, 1998). The isolation of the *M. oleifera* cationic proteins onto sand limits the amount of biological matter released into the water during treatment (Jerri *et al*, 2011). The World Health Organization recommends a minimum water consumption rate of 8 to 15 L per person, per day for personal use, of which 2.5 to 3 L is for drinking (WHO 2013). While theoretically scalable, no studies have been done to test the efficacy of f-sand for the amounts of water that humans actually require. The aim of this research was to produce quantifiable results that could be used to determine whether f-sand can be an appropriate water treatment technology at scale.

Sand filters can be effectively used as a primary method of water treatment or as a co-treatment to remove suspended solids from water before the water is disinfected. The f-sand filter, while similar in appearance to other types of sand filters, differs as the sand is a matrix to support the *M. oleifera* proteins instead of acting as a physical barrier. This matrix configuration has also been achieved using granular activated carbon and rice husk ash (Barajas & Pagsuyoin 2017). The preparation of f-sand can be separate to the use of the f-sand and so removes the requirement to store water after it has been treated water for periods longer than immediate use, an advantage in areas where collecting and treating water is a significant daily task and re-contamination of treated water is a risk. While well outside of the scope of this study, large scale production of f-sand, including commercial production, could be a significant factor in the uptake of this low-technology method of water treatment.

The aim of this study was to assess to use of f-sand as a practical method of bacterial inactivation in the context of domestic water treatment. To achieve this aim f-sand was

immobilised in columns and exposed to water artificially contaminated with 100 CFU/mL *E. coli*. The *ƒ*-sand columns were tested individually, in series and configured to be gravity fed (flowing down) and fluidised (flowing up). All *ƒ*-sand configurations were compared to untreated sand using the same configuration and water supply. These works were performed on a laboratory scale of capacity 8L, which is the minimum necessary drinking water requirement for 3 people for one day. The results of this study could be used to guide the development of a larger trial.

4.2 Materials and Methods

Glass columns (3 cm ID, 16.5 cm height) containing a sintered filter (size 1) and reduced ends (0.7 cm) were purchased from Asis Scientific (Australia), These were flushed with acetone, rinsed with sterile Milli-Q water and exposed to UV light for 10 minutes before each use. Peristaltic tubing (ID 20 mm) was used to convey the source water from the holding vessel to the columns and from the columns to the collecting vessels. The holding vessels were glass 5L Schott bottles and the collecting vessels were glass 1 L Schott bottles. A twin head peristaltic pump (Masterflex Easy-load) was used to pump the source water through the columns. All flow rates were maintained at 11 mL/min.

4.2.1 Functional sand preparation

Quartz sand (Unimin™, approx. 80% between 116 μm to 172 μm) was thoroughly rinsed with deionised water, dried, and then heated at 1000°C for one hour to remove organic impurities that cause bacterial regrowth. The sand was cooled to room temperature and the *ƒ*-sand was prepared as described in Chapter 3. The isolation of the *M. oleifera* cationic proteins was achieved following a similar method to Ghebremichael *et al.* (2005) using cation exchange chromatography. To achieve this the crude solution was run through a cation exchange chromatography column loaded with CM Sepharose Fast Flow (Sigma-Aldrich) equilibrated with 0.1 M NaCl and eluted using an NaCl step-gradient up to 1 M. Elution was performed at 0.6 M NaCl and the extract solution was diluted to 0.1 M before the addition of the sand. The *ƒ*-sand prepared using only the crude extract was rinsed with a 0.1 M dodecyl glucoside solution to remove unwanted organic material from the seeds. Both preparations of *ƒ*-sand were thoroughly washed with deionised water then dried overnight at 55°C.

4.2.2 Preparation of contaminated water (synthetic water)

Water containing a known concentration of *E. coli* was prepared to test the efficiency of the f-sand columns. The water contained *E. coli* with enough salt to keep the *E, coli* alive but not enough salt to elute the protein from the sand. Fresh sterile saline (0.1%) was prepared for each set of experiments and the concentration of bacteria was monitored via colony counting as described above.

Escherichia coli (ATCC® 25922™) was sourced from the School of Animal and Veterinary Sciences, The University of Adelaide and maintained on Bacteriological agar (Oxoid). Colonies of *E. coli* were selected from an agar plate grown overnight at 37ºC and emulsified into 50 mL of sterile nutrient broth and grown overnight. 10mL of this was centrifuged for 10 minutes at 3000 x g, re-suspended in a sterile 0.85% NaCl solution, centrifuged again and finally resuspended in a 0.85% NaCl solution to an absorbance of 0.1+/- 0.005; equivalent to a bacterial suspension containing 1 $x10^8$ - 2 $x10^8$ CFU/mL of *E. coli* (Wikler 2006). The amount of *E. coli* in each water sample was confirmed by spreading samples onto agar plates, incubating overnight at 37ºC and counting the number of colonies that grew. Each concentration was sampled in triplicate. . The source water was prepared by pipetting 5µm aliquots of the 1 $x10^8$ - 2 $x10^8$ CFU/mL *E. coli* solution into 5L of sterile 0.1% NaCl solution to prepare water containing 100 cfu/mL.

4.2.3 Column setup

Lab-scale glass columns (30mm ID and 90 mm bed depth) with a sintered filter at the base of the column were constructed and operated in parallel (Figure 8). Before each run all tubing and sampling glassware was autoclaved and the glass columns were rinsed with acetone, allowed to dry, rinsed with sterile water then exposed to UV-light for 10 minutes. The columns were filled with either 100g of f-sand, $MO_{2.1}$-sand (purified protein used to make the f-sand) or untreated sand as a control. The two types of f-sand were compared to determine whether the purification step was necessary. For both column types, the water was pumped through the peristaltic pump to regulate the flowrate. For the fluidised-bed columns, source water was pumped through the base of the sand column causing the f-sand to fluidise. The system was closed and had a flowrate of 11 mL/min. For the gravity-fed columns the water was introduced into the top of the column with a constant flow of water that kept the sand bed submerged. The system was partially open at the top of the column and the water was introduced to the column at a flow rate of 12.5 mL/min. Each

litre was collected in a sterilised 1 L Schott bottle and was gently swirled to homogenise the bacterial concentration before sampling. Colony counts were performed on each sample in triplicate, the method followed was as described in the Methods section.

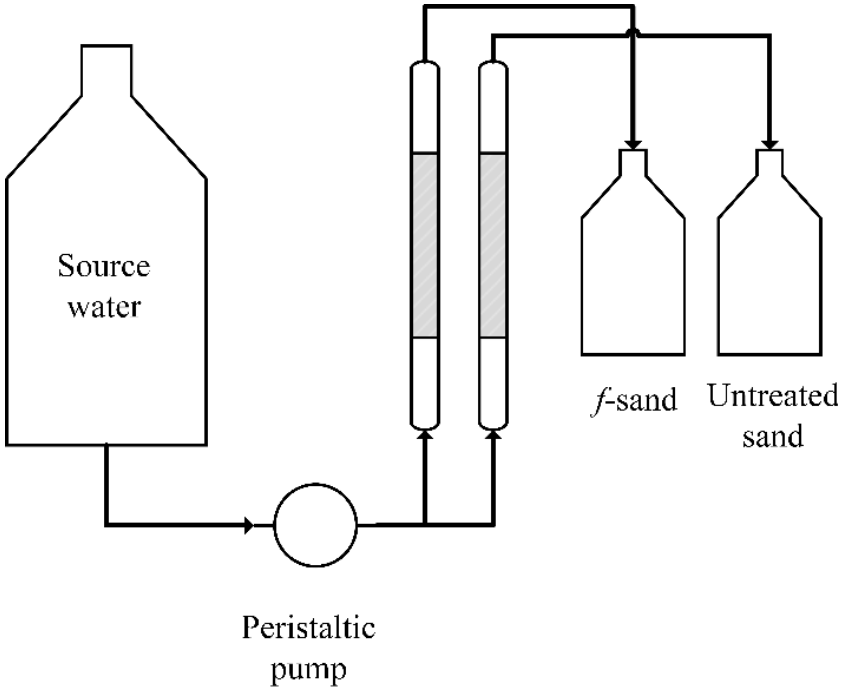

Figure 8: Fluidised bed columns

4.3 Results

4.3.1 Fluidised bed columns

Bacterial breakthrough occurred in the fluidised bed for both the *f*-sand and untreated sand exposed to 100 CFU/mL *E. coli* (Figure 9). The *f*-sand had a log reduction of 1.1 for the first litre to pass through the column with the amount of *E. coli* being removed declining for each litre until the final removal was only 0.56 log. The majority of the *E. coli* passed through the untreated sand column with the maximum amount held in the column on the third and fifth litres.

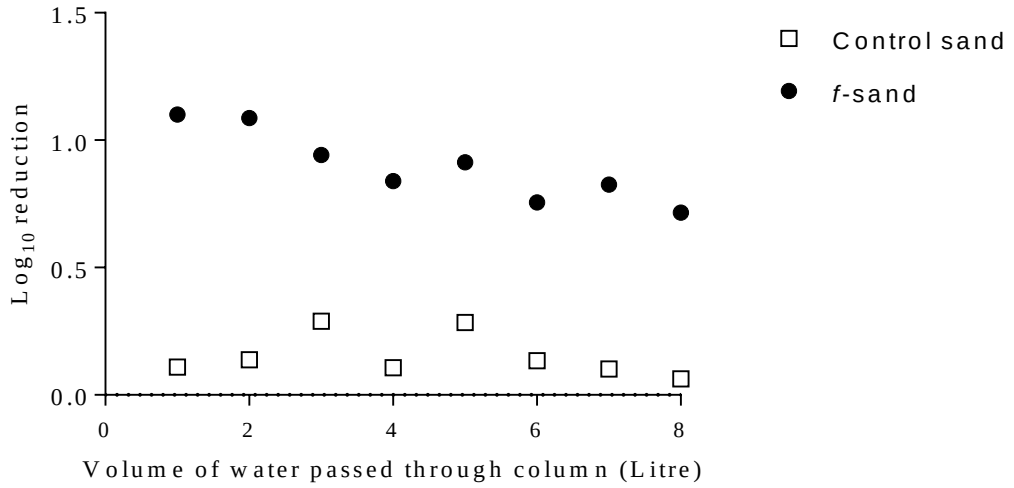

Figure 9: *f*-sand versus control sand – fluidised bed.

To determine if bacterial reduction was dependent on the concentration of the source water or the system itself, three columns were run in succession. The results from column 1 are presented in Figure 10: Column 1. Note: Data points not shown for *f*-sand columns 3 and 4. and the results from column 3 are presented in Figure 11 . Both columns are compared to the inlet concentration of column 1. Data from the second, middle column was not recorded. The outlet samples for columns 3 and 4 in Figure 10: Column 1. Note: Data points not shown for *f*-sand columns 3 and 4.did not contain any *E. coli* and so do not appear in the graph. The control sand column remained around 0 with slight increases and decreases in bacterial concentrations for the first column and below 0.5 log for column 3. The *f*-sand columns did not display consistent removal and all log reductions were below 1.

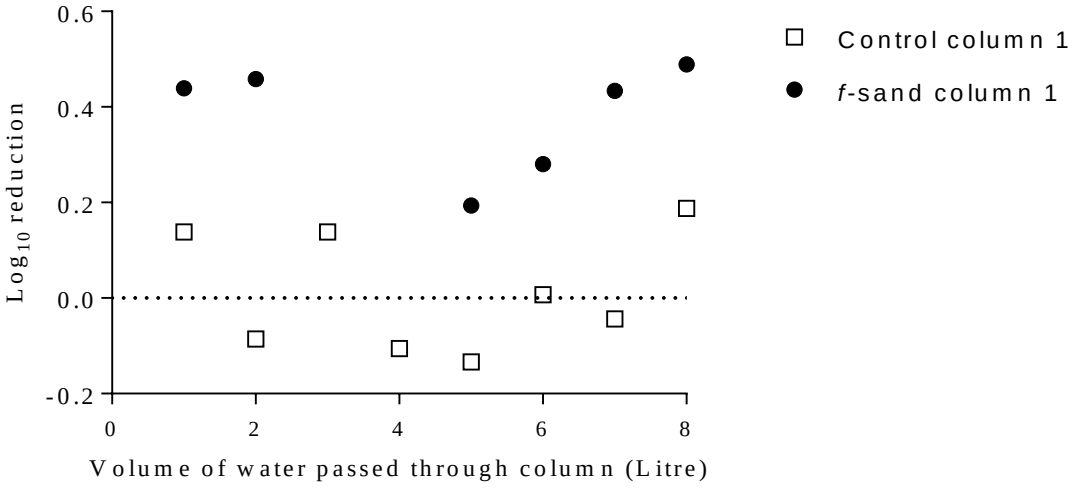

Figure 10: Column 1. Note: Data points not shown for *f*-sand columns 3 and 4.

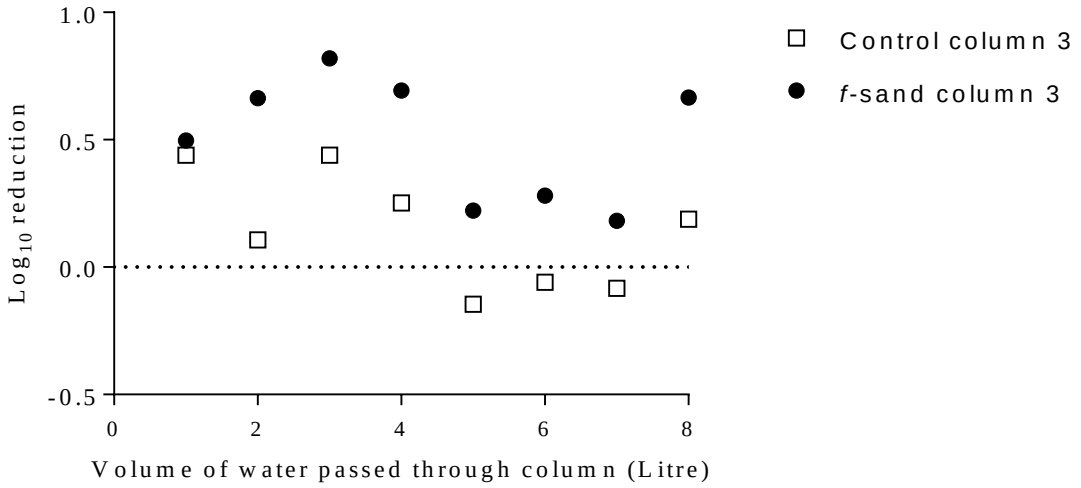

Figure 11: Column 3.

Two ways of preparing the *f*-sand were compared in order to determine whether there was any advantage to isolating the $MO_{2.1}$ prior to preparing the *f*-sand (Figure 12). Both columns followed the same removal pattern across the 8 litres which indicates fluctuating concentrations of inlet bacteria rather than one column performing better than the other. This assumption is strengthened by the result of columns 7 and 8 where the log reduction was around 0.1 then increased to 0.5 for the $MO_{2.1}$-sand and 0.34 for the *f*-sand.

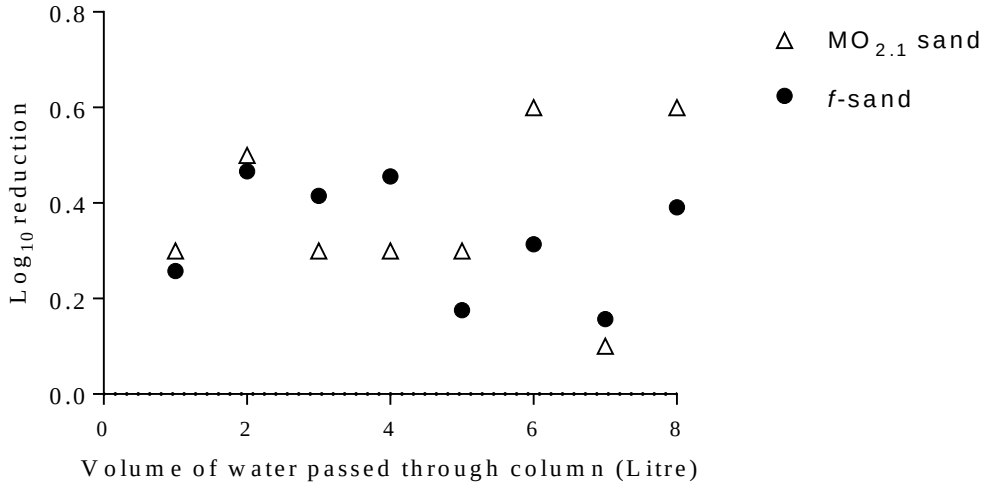

Figure 12: Comparison of $MO_{2.1}$ -sand to *f*-sand, fluidised bed.

4.3.2 Gravity fed columns

When the system was configured so that the water flows down through the packed sand the system becomes a granular-bed filter (Figure 11). They have a similar performance for the first four litres with the f-sand maintaining some efficacy until the 8th litre.

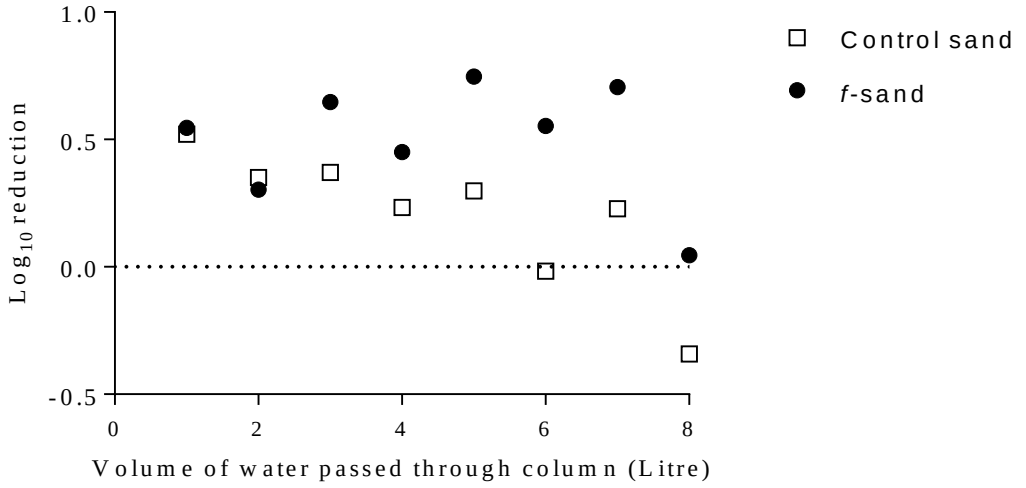

Figure 13: f-sand versus control sand – gravity fed.

4.4 Discussion

Bacterial contamination of drinking water is still a major problem worldwide. Treatment of water using *M. oleifera* seeds is an appropriate alternative that could be used where the seeds are available. The isolation of $MO_{2.1}$ and production of f-sand increases the availability of *M. oleifera* as a water-treatment option. The aim of this study was to research the efficacy of f-sand when exposed to the minimum amount of water recommended by the World Health Organization per person per day (WHO, 2013). To do this, f-sand was contained in columns and exposed to water artificially contaminated with *E. coli*. This type of biophysical filtration can be operated immediately after the f-sand is prepared and can be used with a faster flow rate than the slow sand filters. There is also no need to wait for a biofilm to develop. The f-sand has the added advantage of being reusable if washed with dodecyl glucoside and if this is inappropriate, the f-sand is simple to prepare.

The f-sand was most effective at the highest tested bacterial concentrations and when the source water was introduced through the base of the column. This configuration allows for bacterial breakthrough due to the constant movement of the sand particles in the fluidised

bed. The slow flowrate and constant mixing of the sand increases the chance of a viable *E. coli* cell contacting an unused $MO_{2.1}$ binding site. Reducing the bacterial concentration in the source water rendered the f-sand inefficient, suggesting a lower limit of bacterial inactivation and increasing the length of the bed by adding extra columns did not increase the bacteria removal. There was no significant difference between the reduction in column 1 and 3 for either the f-sand or the untreated sand. This validates the idea that there is a lower limit. Running an inlet concentration of 10 CFU/mL *E. coli* through a column did not remove any more bacteria than the control column (data not shown). When a similar system to the one used here was tested against a bacterial concentration of 7x 10^7 CFU/mL bacterial reduction of 99% was recorded for the f-sand (Williams *et al.* 2017). The volume of water used in that study was significantly less than the volumes used here and so it would be valuable to test these higher concentrations using larger volumes of water to determine if the f-sand efficacy increases with the increased bacterial load. The difference between the bacterial removals for the gravity fed columns was not significant with the bacteria passing through the column with only a small percentage of the bacteria ever coming into contact with the *M. oleifera* proteins. The constant movement of the f-sand in the fluidised columns would have increased the interactions between the *E. coli* and the unused $MO_{2.1}$ and so lead to higher removal.

Where the entrapment of the *E. coli* by two columns is of the same magnitude, the bacteria in f-sand column was inactivated compared to the bacteria in the untreated column that had only been retained. The increase in bacterial concentration in these columns is likely from the displacement of bacteria that had been held but not inactivated in the column. In a similar study using functional rice husk ash (RHA), Barajas *et al.* (2017) reported that untreated RHA removed more bacteria than f-RHA but that the f-RHA inactivated the bacteria that were removed. While the f-sand removed as much or more than the untreated sand, the same reasoning applies.

The method used to prepare the f-sand did not influence the efficacy of the column as isolating the $MO_{2.1}$ before attaching it to the sand did not increase the efficacy of the f-sand. From this it can be concluded that the method of preparing the f-sand does not affect the treatment outcome. One of the most important factors to consider before using f-sand is the inconsistency of bacterial removal. All f-sand was prepared following the same method and using the same materials. However, in Figure 9 a log reduction of 1.1 was achieved whereas when the three columns were run in succession, column 1 only removed

a maximum of 0.47 log. The first column as equivalent to the single column trial but there was significant difference between the results. The the composition of the sand, including the surface charge and silica content; the age of the f-sand; the age of the *M. oleifera* seeds; the cleanliness of the sand, and the conditions under which the f-sand is used all factors that influence the efficacy of the f-sand. The outcomes of this study justify the need to test the f-sand on a larger scale to determine the real-world application; especially for regions where water treatment infrastructure is lacking, or where current treatment methods are not cost-effective, available or applicable.

4.5 Conclusions

In this study it is demonstrated that 100 g of f-sand can be used to improve bacterially-contaminated water. However, this water still contained bacteria and is not yet comparable to other methods of treatment. The bacterial reduction is highly dependent on the bacterial concentration of the source water and that fluidisation of the f-sand allowed for *E. coli* breakthrough in all trials. The results of this study indicate that there is a place for f-sand in water treatment, and this may be as a co-treatment with chlorination, but the best method is still to be determined. Future studies should include a range of turbid and non-turbid natural surface waters, and surface waters that contain other aquatic microbes.

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Chapter 5. f-sand reuse

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis: and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Moringa oleifera functionalised sand – reuse with non-ionic surfactant dodecyl glucoside

Frances E. Williams, Andrew K. Lee, Sanaz Orandi, Sarah K. Sims and David M. Lewis

ABSTRACT

Moringa oleifera seeds are well known for their ability to cause flocculation in turbid water and facilitate bacterial inhibition. These effects are due to the cationic polypeptide $MO_{2.1}$, which affects the surface charge of suspended particles and causes lysis of bacterial cells. However, the attachment of bacteria to $MO_{2.1}$ prevents further bacterial attachment, reducing the effectiveness of the seeds. This research investigated the effect of surfactants on functionality and reuse of *Moringa* seeds to develop a sustainable water treatment technique. The seed extracts ($MO_{2.1}$) were used with a functionalised sand system, and the sands were exposed to commercially available (ionic and non-ionic) surfactants, dodecyl glucoside and sodium dodecyl sulfate. Artificially polluted water contaminated with *Escherichia coli* was used to evaluate the efficiency of the system. The non-ionic surfactant was found to be effective at separating *E. coli* from the functionalised sand without the detachment of the $MO_{2.1}$ and subsequent loss of the system efficiency. This was successfully repeated four times. The results demonstrated a sustainable, reusable technique to inhibit bacterial contamination in water.

Key words | dodecyl glucoside, *Escherichia coli*, functionalised sand, *Moringa oleifera*, sodium dodecyl sulfate, sustainable water treatment

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INTRODUCTION

Eleven percent of the world's population relies on untreated surface or ground water as a primary source of drinking water (WHO 2014). This water is often contaminated with pathogenic bacteria, and treatment for immediate use is commonly undertaken using tablets of sodium hypochlorite or calcium hypochlorite (Arnold & Colford 2007). This method is mainly used in regions which lack access to suitable water treatment infrastructure. The use of the tablets prevents recontamination, as the chlorine provides residual protection lasting from hours to days (Arnold & Colford 2007). High cost, an unpleasant taste and low availability of these chemicals have been identified as the main reasons which lead to their underuse or misuse (Islam *et al.* 2014).

The crushed seed kernels from *Moringa oleifera* are known to be an appropriate antibacterial agent for water treatment (Madsen *et al.* 1987; Jahn 1988). The seeds can be used directly, or as a crude water extract, to inhibit bacterial and viral organisms. They also cause coagulation of suspended particles in water (Ali *et al.* 2004; Kansal & Kumari 2014). However, the disadvantage of using the seeds in such a way is that the crushed seed particles quickly decompose and increase the amount of organic carbon in the water, promoting bacterial regrowth (Jerri *et al.* 2012).

The compound in the *M. oleifera* seed that causes the effects has been identified as a cationic polypeptide called $MO_{2.1}$ (Gassenschmidt *et al.* 1995; Suarez *et al.* 2005).

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This polypeptide is part of a dimer protein comprised of two polypeptide subunits linked by di-sulfide bonds (Ndabigengesere *et al.* 1995). The amino acid sequence of $MO_{2.1}$ has been documented (Gassenschmidt *et al.* 1995) and the molecular weight is 6,782 g/mol (http://www.uniprot.org/, accessed 28/09/2016).

$MO_{2.1}$ has been observed to adsorb to the surface of negatively charged solids, including silica nanoparticles, quartz sand, aluminum oxide and micro-emulsion prepared magnetic iron oxide nanoparticles (Kwaambwa *et al.* 2010; Jerri *et al.* 2012; Okoli *et al.* 2012; Hellsing *et al.* 2013). When the $MO_{2.1}$ is adsorbed to sand, this 'functionalised' sand (known as *f*-sand) displays the bactericidal and coagulant properties of the non-bonded polypeptide (Jerri *et al.* 2012). This simple method of purifying $MO_{2.1}$ has scope to be used in water treatment, as it negates the previously described disadvantages of the crude extract as well as reducing the costs associated with isolating the polypeptide by other methods. The *f*-sand can be used to attach and inhibit *Escherichia coli* in water (Jerri *et al.* 2012). In practice, the obstacle which currently prevents the use of *f*-sand to treat water is that the *E. coli* does not desorb once bonded to the *f*-sand, preventing its reuse (Jerri *et al.* 2012).

Surfactants are a class of amphiphilic compounds able to wet surfaces and solubilise fatty material (Glover *et al.* 1999). Anionic surfactants, such as sodium dodecyl sulfate, are reported to be bactericidal; affecting the permeability of cell membranes and causing cell lysis at high surfactant concentrations (Glover *et al.* 1999; Van Hamme *et al.* 2006). Non-ionic surfactants are also bactericidal and are known to solubilise the bacterial lipid membrane (le Maire *et al.* 2000). When solubilised in water, surfactants self-assemble into compounds called micelles, and the minimum surfactant concentration required for micelle formation is called the critical micelle concentration (Privé 2007).

M. oleifera seed extracts have been observed to interact with sodium dodecyl sulfate and other anionic surfactants (Kwaambwa & Maikokera 2007; Maikokera & Kwaambwa 2007; Beltran-Heredia & Sanchez-Martin 2009). The crude seed extract has been reported to remove up to 80% of sodium dodecyl sulfate via flocculation (Beltran-Heredia & Sanchez-Martin 2009). Both $MO_{2.1}$ and the dimer protein are cationic and readily adsorb to the anionic sodium dodecyl sulfate (Beltran-Heredia & Sanchez-Martin 2009; Beltran-Heredia *et al.* 2012). This has been reported to neutralize the charge (Maikokera & Kwaambwa 2007). The process begins below the critical micelle concentration of $8.2–8.3 \times 10^{-3}$ M, at the critical aggregation concentration of 1×10^{-5} M, and can be used to remove sodium dodecyl sulfate from solution (Beltran-Heredia & Sanchez-Martin 2009).

Dodecyl glucoside is a non-ionic surfactant, and no studies investigating this surfactant and the *M. oleifera* crude or purified seed extracts were identified in the literature. Okoli *et al.* (2012) report that a 0.1 wt% solution of the non-ionic surfactant Tween 20 can be used to wash coagulated clay particles off *M. oleifera* protein functionalised magnetic nanoparticles. These particles can then be reused as coagulants with some loss of efficiency. Triton X, another non-ionic surfactant, does not interact with the *M. oleifera* coagulant protein (Kwaambwa & Maikokera 2007).

This study demonstrates the use of surfactants in regenerating *f*-sand for continued *E. coli* removal. All concentrations of dodecyl glucoside tested against the *f*-sand were above the critical micelle concentration of 1.9×10^{-4} M (Neugebauer 1990). This is a novel approach for the regeneration of *f*-sand and has the potential to be applicable in water treatment for developing countries.

MATERIAL AND METHODS

This investigation was conducted in two stages. The first stage was focused on testing two surfactants, dodecyl glucoside and sodium dodecyl sulfate, to examine their effect on attachment of the $MO_{2.1}$ to the sand surface. The second stage evaluated the efficiency of these surfactants at separating *E. coli* from the $MO_{2.1}$.

Preparation of *Moringa oleifera* functionalised sand

The functional sand (*f*-sand) was prepared using *M. oleifera* seeds. The preparation was based on the method described by Jerri *et al.* (2012) with some modifications. Dry, whole seeds were purchased from AustraHort, Australia. The seeds were dehusked and the kernels pulverised to a fine powder. A 0.05 g/mL crude solution was prepared by mixing 20 g of the seed powder with 400 mL of a 0.1 M

sodium chloride solution for 1 hour at 150 rpm. The solution was sieved and filtered to remove the seed particles.

100 g of commercially available, fine grained silica sand was thoroughly rinsed with deionised water, dried then autoclaved. The sand was combined with 400 mL of the filtered crude extract solution and mixed on a shaker plate at 150 rpm for 1 hour. After the *f*-sand settled, the crude extract supernatant was removed and the *f*-sand was rinsed with deionised water. The *f*-sand was dried at 50 °C.

Mass determination of $MO_{2.1}$

The mass of $MO_{2.1}$ per gram of *f*-sand was determined by eluting the polypeptide on 1 g of *f*-sand using 5 mL of a 0.6 M NaCl solution. A baseline for the absorbance at 280 nm was set against the absorbance at 300 nm. A Shimadzu 1601 UV-visible spectrophotometer capable of scanning from 190 nm to 1,000 nm was used to detect the absorbance throughout this study. The Beer-Lambert law, $Abs = \varepsilon lc$, was applied using a path-length (l) of 1 cm and an extinction coefficient (ε) of 1,520 (Aitken & Learmonth 1996). The mass of $MO_{2.1}$ was determined in mg per gram *f*-sand using a molecular weight of 6,781.6 g/mol, based upon the reported amino acid sequence of $MO_{2.1}$ (http://www.uniprot.org/, accessed 28/09/2016).

Preparation of surfactants

Two surfactants were examined in this study: anionic sodium dodecyl sulfate (sodium lauryl sulfate, CAS 151-21-3) and non-ionic dodecyl glucoside (lauryl glucoside, CAS 11061-47-9) at concentrations ranging between 0.0005 M and 0.1 M. The concentrations of dodecyl glucoside were determined by UV-spectrophotometry. The absorbance was measured at 223 nm and was initially determined by serially diluting the supplied surfactant from a 49.74% w/w solution. A commercially available dishwashing detergent (Earth choice dishwashing concentrate, Natures Organics, Australia) was purchased from a local Australian supermarket. The detergent contained 10%–30% dodecyl glucoside as coco glucoside and <10% sodium dodecyl sulfate, and was tested at 1% and 2% v/v dilutions. The amount of dodecyl glucoside in the 1% solution was equivalent to 0.01 M dodecyl glucoside solution as confirmed by UV-visible spectroscopy.

Effect of surfactants on protein adsorption

The effect of the surfactants sodium dodecyl sulfate and dodecyl glucoside on the attachment of $MO_{2.1}$ to the sand was measured by exposing 1 g of *f*-sand to 5 mL surfactant at known concentrations. This was mixed for 1 hour and rinsed with deionised water. The samples were mixed between one and four times to determine the effect of multiple washes on the *f*-sand. The amount of $MO_{2.1}$ was measured as described above and the experiment was repeated in triplicate. The concentrations of sodium dodecyl sulfate examined were 0.01, 0.001 and 0.0005 M, and the concentrations of dodecyl glucoside were 0.1, 0.01 and 0.001 M.

Synthetic water preparation

Synthetic water with a known bacterial concentration was prepared to simulate non-potable water. *Escherichia coli* (ATCC® 25922™) was sourced from the School of Animal and Veterinary Sciences, The University of Adelaide and maintained on Bacteriological agar (Oxoid). Overnight bacterial suspensions were grown in nutrient broth (Oxoid). The suspension was centrifuged for 5 minutes at 3,000 g and the pellet was washed in a sterile 0.85% NaCl solution. This was centrifuged, and the pellet was re-suspended in sterile Milli-Q water diluted to an absorbance of 0.1 ± 0.005 at 600 nm. This was equivalent to a bacterial suspension containing 7×10^7 CFU/mL of stationary phase *E. coli* as confirmed by cultivatable counts. Fresh synthetic water was prepared for each set of experiments.

Functional sand column tests

Stage two evaluated the efficiency of the surfactants at separating *E. coli* from the $MO_{2.1}$. To do this, 50 g of dry *f*-sand was poured into a vertical glass column of 1.5 cm diameter. The *f*-sand was rinsed with Milli-Q water until the absorbance of the outgoing solution was less than 0.001 at 280 and 600 nm. The column was then subjected to a set of solutions in the following order: synthetic water, 2 × Milli-Q water, surfactant, 4 × Milli-Q water. Each set was repeated four times, so that the *f*-sand had been exposed to four discrete synthetic water treatments.

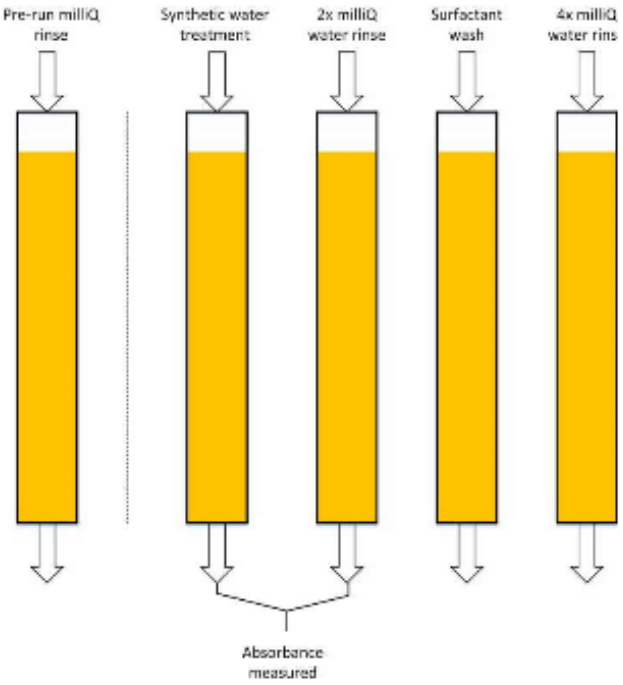

Figure 1 | The steps used to test efficacy of surfactants for the separation of *E. coli* (ATCC® 25922™) from $MO_{2.1}$ without separating $MO_{2.1}$ from the sand. The inlet solutions for the steps are: Step 0 - sterile Milli-Q water rinse; Step 1 - synthetic water; Steps 2 and 3 - Milli-Q water; Step 4 – surfactant; Steps 5–8 - Milli-Q water.

A schematic of this process is presented in Figure 1. New columns were prepared for sodium dodecyl sulfate concentrations of 0.001 and 0.0005 M and for dodecyl glucoside concentrations of 0.1, 0.01 and 0.001 M.

The solutions passing out of the columns were collected and the absorbance of the eluting solutions was measured at 600 nm. This was used to estimate the bacterial concentration of each solution and is used here as an alternate method of measuring bacterial concentrations in non-turbid solutions. To justify this method, the absorbance of bacterial solutions from 0.01 to 0.1 at 600 nm was compared with the cultivatable counts of these solutions. Colony counting was also performed on select columns. The percentage of *E. coli* inhibited by the column was determined by Equation (1),

$$\mathrm{Eq}_1 = 100\% - 100\% \left(\frac{\mathrm{Ab}_{\mathrm{outlet\ 1}} + \mathrm{Abs}_{\mathrm{outlet\ 2}} + \mathrm{Abs}_{\mathrm{outlet\ 3}}}{\mathrm{Abs}_{\mathrm{inlet\ 1}}} \right) \quad (1)$$

where Abs is the absorbance of the eluting solution at 600 nm for the solutions entering the column, inlet 1, and passing out of the column, outlets 1, 2 and 3.

Bacterial staining

Sand was heated at 600 °C in a high temperature oven for 5 hours to remove any impurities and organic matter, including dead bacteria, from the surface. 10 g of *f*-sand was prepared as above and exposed to synthetic water, 0.01 M dodecyl glucoside and synthetic water in the same manner as the columns. 2 g samples were collected after each step and were stained with a 1:500 dilution of propidium iodide in Tris buffer for 15 minutes, then rinsed twice with sterile Milli-Q water. Staining was performed on all samples, including controls of bare sand and *f*-sand, to confirm that only the dead *E. coli* was being stained. The samples were viewed on a Nikon Ti Live Cell Microscope at 40× optical zoom. A 100 ms exposure for fluorescence was used on all samples with a 195 ms exposure for the live view.

RESULTS

Mass determination of $MO_{2.1}$

The mass of $MO_{2.1}$ per gram of *f*-sand was calculated using the Beer-Lambert Law. The maximum, minimum and average masses of eluted $MO_{2.1}$ in mg per g of *f*-sand were 1.19, 0.48 and 0.79, respectively. Variation in turbidity was observed across all of the elution-type experiments. To overcome the variation between samples and batches, the absorbance at 300 nm was used as a baseline and subtracted from the absorbance of the protein peak at 280 nm.

Effect of surfactants on $MO_{2.1}$ attachment

The effect of sodium dodecyl sulfate on the attachment of $MO_{2.1}$ to the sand was immediately apparent. At 0.01 M, the highest concentration tested, there was an 80% reduction in the amount of $MO_{2.1}$ but there was no further significant loss after the first wash. At 0.001 M, there was a gradual reduction in the amount of $MO_{2.1}$, and at 0.0005 M, the amount present was relatively stable. The

results of the interaction of sodium dodecyl sulfate and *f*-sand are presented in Figure 2.

Washing the *f*-sand with dodecyl glucoside caused a slight and immediate reduction in the amount of $MO_{2.1}$. The loss was not as significant as that observed with the sodium dodecyl sulfate, and for 0.01 and 0.001 M the mass of $MO_{2.1}$ remained above 80% of the initial value. The results are presented in Figure 3.

FUNCTIONAL SAND COLUMN TEST RESULTS

Synthetic water treatment results with sodium dodecyl sulfate in an *f*-sand column

Based on the results from part one, the 0.01 M concentration of sodium dodecyl sulfate was excluded from further investigation. The treatment results of functional sand columns with sodium dodecyl sulfate, 0.001 and 0.0005 M, are presented in Figure 4 and demonstrated that the surfactant was not effective at separating the *E. coli* from the *f*-sand. In the first treatment run, when the synthetic water was passed through the *f*-sand column, between 60% and 70% of the *E. coli* was removed. Further treatment results did not show any reduction in *E. coli* and even increased the turbidity of the water.

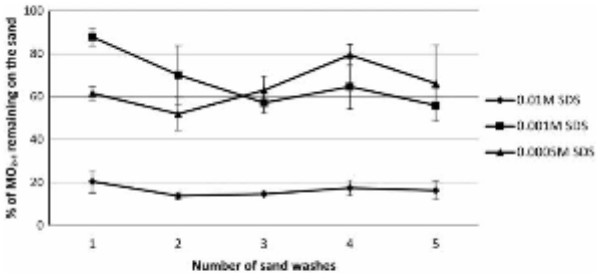

Figure 2 | Effect of sodium dodecyl sulfate (SDS) on attachment of $MO_{2.1}$ to sand used at three concentrations of 0.01, 0.001 and 0.0005 M and percentage of $MO_{2.1}$ remaining on the sand.

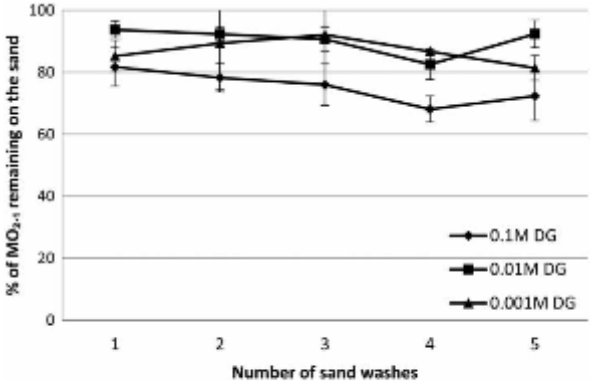

Figure 3 | Effect of dodecyl glucoside (DG) on attachment of $MO_{2.1}$ to sand used at three concentrations of 0.1, 001 and 0.001 M, and percentage of $MO_{2.1}$ remaining on the sand.

Synthetic water treatment results with dodecyl glucoside in an *f*-sand column

The dodecyl glucoside was effective at separating the *E. coli* from the *f*-sand without affecting the capacity of the *f*-sand to remove *E. coli* from water. The 0.01 M dodecyl glucoside was the most consistently effective, as shown in Figure 5. To confirm the use of UV-visible spectroscopy in bacterial estimation, colony counts were performed on the *f*-sand columns treated with 0.01 M dodecyl glucoside. For the first treatment, the percentage reduction in the *f*-sand columns determined by colony counting was 12% greater than that determined by UV-visible spectroscopy. For the second, third and fourth treatments, the difference in the values was less than 4%. When testing by UV-visible spectroscopy, the absorbance includes both viable and inhibited cells, whereas with cultivatable counts, only the living cells are recorded, indicating that UV-visible spectroscopy underestimates the

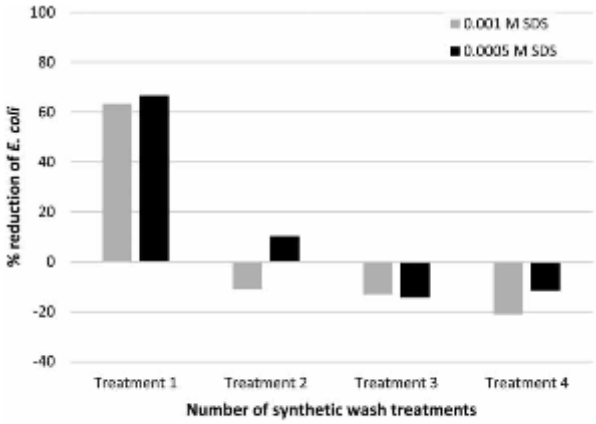

Figure 4 | Synthetic water treatments with sodium dodecyl sulfate (SDS) in an *f*-sand column.

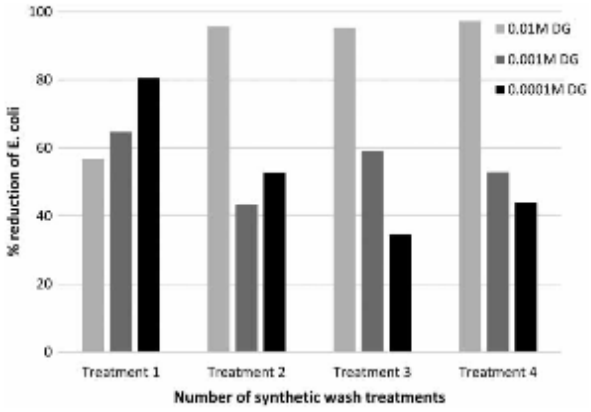

Figure 5 | Synthetic water treatments with dodecyl glucoside (DG) in an *f*-sand column.

amount of bacterial reduction. In this study, the absorbance between 0.1 and 0.001 at 600 nm was comparable to the cultivatable count taken from the solutions. However, for concentrations below 1×10^5 CFU/mL, colony counting only should be performed.

To confirm that the dodecyl glucoside was effective at separating the *E. coli* from the *f*-sand, three further columns, A1, A2 and A3, were prepared and are presented in Figure 6. For A1, the column was washed with 0.01 M dodecyl glucoside after each *E. coli* suspension, and each treatment retains the efficiency of the first run. Two synthetic water treatments were undertaken in succession for A2 and three for A3 without exposure to dodecyl glucoside between synthetic water treatments. For A2, the column was washed with dodecyl glucoside after the second treatment, and the restoration of the column is apparent on the third treatment. A fourth synthetic treatment was immediately run through the column and the efficiency was reduced. For A3, the column was run three times before it was washed with the dodecyl glucoside, and by the third treatment the *E. coli* removal dropped to below 10%. After washing with the dodecyl glucoside, the *f*-sand regained its initial efficacy.

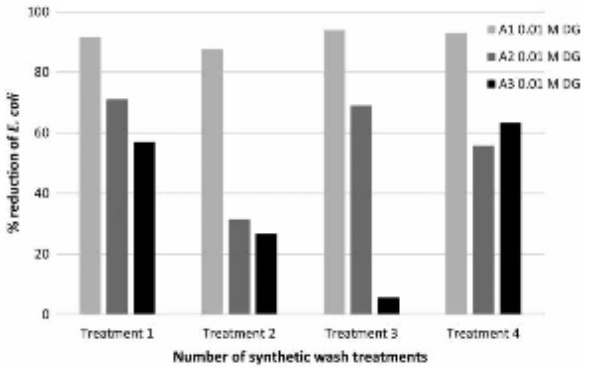

Figure 6 | 0.01 M dodecyl glucoside (DG) column tests. The order of washes is A1: *E. coli* suspension, 0.01 M dodecyl glucoside, *E. coli* susp., 0.01 M dodecyl glucoside, *E. coli* susp., 0.01 M dodecyl glucoside, *E. coli* susp., 0.01 M dodecyl glucoside. A2: *E. coli* susp., *E. coli* susp., 0.01 M dodecyl glucoside, *E. coli* susp. A3: *E. coli* susp., *E. coli* susp., *E. coli* susp., 0.01 M dodecyl glucoside, *E. coli* susp.

Commercially available dishwashing detergent

Dodecyl glucoside is a surfactant that is widely used in commercial detergents and can be readily sourced in local supermarkets. The efficacy of a detergent mixture reported to contain between 10 and 30% v/v dodecyl glucoside was tested on the *f*-sand columns. 1% and 2% solutions were prepared, which corresponded to approximately 0.25 and 0.57 M dodecyl glucoside, respectively, and were compared against the 0.01 M v/v dodecyl glucoside solution as shown in Figure 7. The 2% solution was comparable to the 0.01 M dodecyl glucoside, whereas the 1% solution exhibited a loss of efficacy for each run. This result further confirms the use of dodecyl glucoside as an effective treatment for the removal of *E. coli* from *f*-sand.

Bacterial staining

The attachment of *E. coli* to the *f*-sand was clearly visible when viewed on the live cell microscope, as presented in Figure 8. After washing with the 0.01 M dodecyl glucoside,

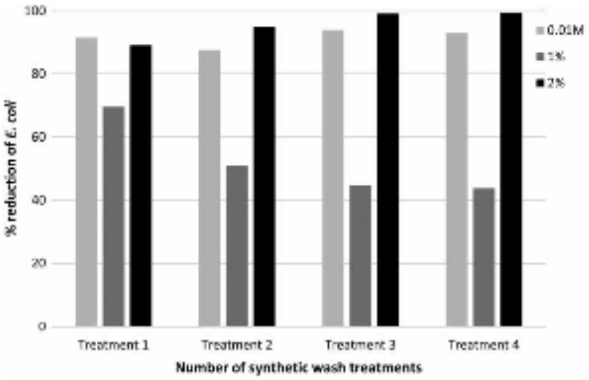

Figure 7 | Synthetic water treatments with commercially available detergent and dodecyl glucoside in an *f*-sand column.

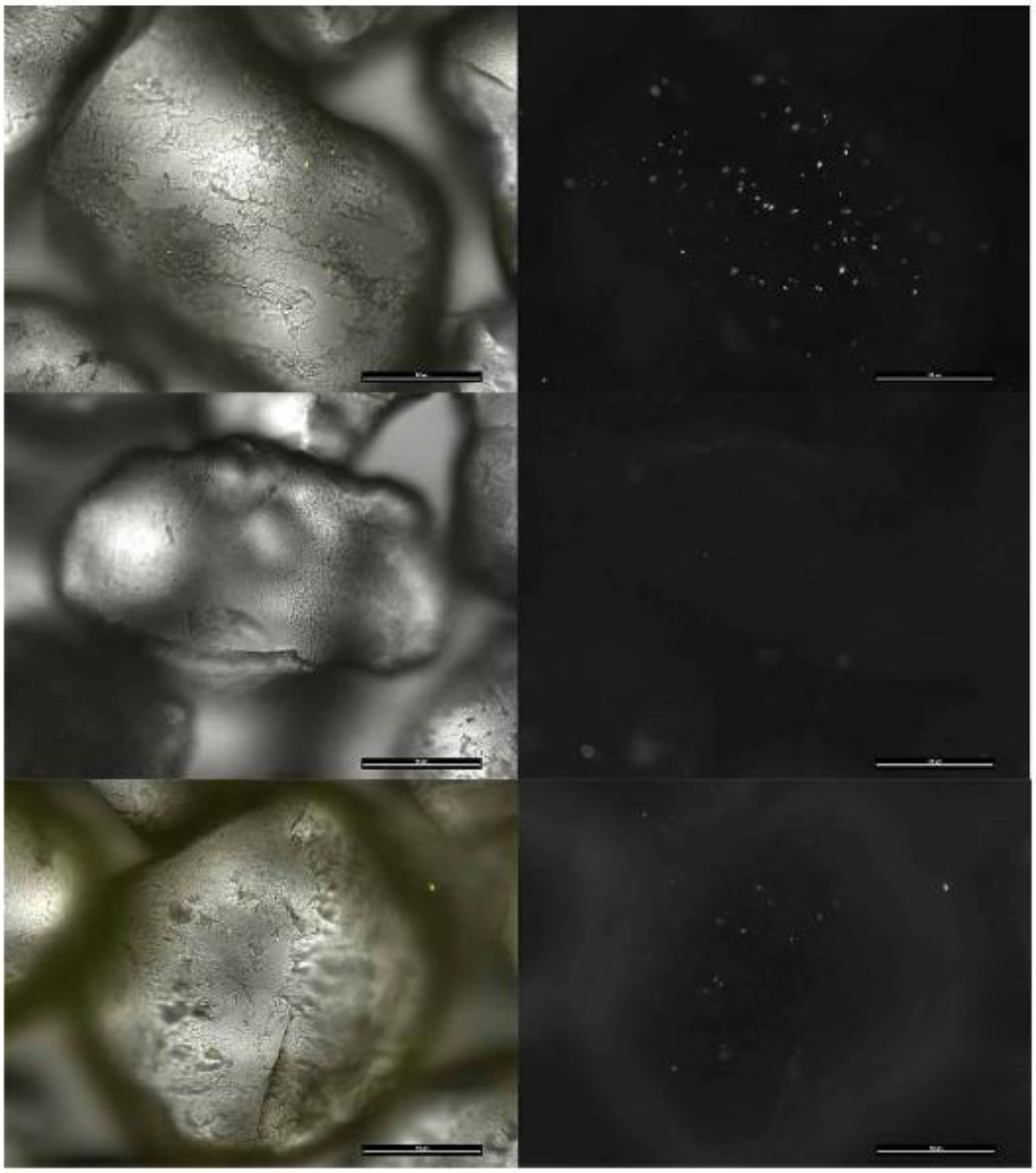

Figure 8 | Top: untreated sand, unused *f*-sand; middle: *f*-sand with *E. coli*, *f*-sand washed with 0.01 M dodecyl glucoside; bottom: washed *f*-sand with reattached *E. coli*. Scale bar 100 μm.

the *E. coli* was no longer visible on the *f*-sand. After further exposure to the bacterial solution, the *f*-sand attached and inhibited more *E. coli*. For each image, the left-hand image corresponds to the superimposed fluorescence-photo image, and the right-hand image is the fluorescence image only. The untreated sand and unused *f*-sand did not display any fluorescence.

DISCUSSION

$MO_{2.1}$ is a potent inhibitor of a range of bacterial and viral organisms (Broin *et al.* 2002). The key mechanism of action of $MO_{2.1}$ involves the interference and absorbance of the polypeptide through the hydrophobic regions of bacterial phospholipid membranes, reported in *E. coli* to

cause lysis and cell death (Suarez *et al.* 2005; Shebek *et al.* 2015). When $MO_{2.1}$ is attached to *f*-sand, the bacterial attachment prevents any further bacterial attachment, and the aim of this study was to develop a method of renewing the *f*-sand. Kwaambwa *et al.* (2010) examined the attachment of the coagulant protein to the oxide layer on an III face of a cut silicon crystal, reporting that saturation occurred at 5.5 mg/m^2. Rinsing with water or exposing the surface to a 2×10^{-3} M sodium dodecyl sulfate solution did not cause the protein to separate from the silica surface. In a similar experiment, the coagulant protein was shown to adsorb onto the surface of aluminium oxide (Kwaambwa *et al.* 2015). However, in that study, the coagulant protein could be removed by rinsing with water after exposure to concentrations of sodium dodecyl sulfate below the critical micelle concentration (Kwaambwa *et al.* 2015). Additionally, washing the surface with a 9×10^{-3} M solution of the cationic surfactant cetyltrimethylammonium bromide caused all of the protein to be removed (Kwaambwa *et al.* 2015).

In our study, the *f*-sand sample washed with a sodium dodecyl sulfate solution above the critical micelle concentration had an 80% reduction in the amount of $MO_{2.1}$ attached to the sand. The next sample was just below this and also showed a loss of $MO_{2.1}$. All of the samples were exposed to concentrations above the critical aggregation concentration. It is likely that the 0.01 M sodium dodecyl sulfate was able to overcome the electrostatic adsorption of most of the $MO_{2.1}$ polymers; however, some strongly bonded polypeptides remained. The third sample, which was below the critical micelle concentration but still above the critical aggregation concentration, did not exhibit a loss of $MO_{2.1}$. Kwaambwa *et al.* (2010) reported that the 2×10^{-3} M sodium dodecyl sulfate solution exposed to a cut silicon crystal co-adsorbed onto the silica surface with a ratio of four molecules per one *M. oleifera* protein molecule.

When the 0.001 and 0.0005 M sodium dodecyl sulfate were run through the *f*-sand column, it became apparent that the surfactant did not affect the attachment of the *E. coli*. Here, the formation of surfactant-$MO_{2.1}$ complexes and the solubilising of organic contaminants previously attached to the sand surface contribute to the increase in turbidity of the eluting solutions. The increase in turbidity of the outlet solutions was the most obvious on the first surfactant wash, where the eluting solution was visibly turbid.

Studies examining the effect of dodecyl glucoside on $MO_{2.1}$ have not been undertaken previously; however, reports of other non-ionic surfactants were encouraging. While all of the *f*-sand samples washed with dodecyl glucoside showed an immediate loss of $MO_{2.1}$, they remained stable around 80% for the subsequent washes. When the dodecyl glucoside solutions were run through the *f*-sand column, the high percentage reduction of bacteria for both 0.01 and 0.001 M dodecyl glucoside was unsurprising, as the destabilising mechanism is similar to that which occurs when phospholipid membranes are solubilised by detergents (Helenius & Simons 1975; le Maire *et al.* 2000). Bacterial membranes are made of a complex and dynamic lipid bilayer (Seddon *et al.* 2004) and gram negative bacteria possess a high density of anionic lipids (Shebek *et al.* 2015). The use of detergents to solubilise bacterial lipid membranes has been thoroughly investigated and covered by a three-stage hypothesis addressed by le Maire *et al.* (2000). In stage one, the free detergent molecules distribute into the phospholipid membrane. In stage two, the phospholipid membranes co-exist at a thermodynamic equilibrium comprised of phospholipids and detergent. Eventually, the detergent–detergent interactions destabilize the membrane structure, causing it to fragment. This leads to stage three, where, upon exposure to more detergent, the phospholipids become fully solubilised into the detergent micelles. In our study it is assumed that this three-stage phenomenon is what is causing the *E. coli* to separate from the $MO_{2.1}$. As the *E. coli* membrane was already disrupted by the $MO_{2.1}$, the hydrophobic phospholipids would readily solubilise into the dodecyl glucoside micelles and be removed from the *f*-sand column, leaving behind the $MO_{2.1}$. Since the dodecyl glucoside did not interfere with the attachment of the majority of the $MO_{2.1}$, this method allowed the reuse of the *f*-sand.

The amount of *E. coli* which the *f*-sand removed varied significantly between experimental runs. In some cases, it was 99%; however, in other runs it was as low as 57%. This was likely due to the sensitivity of the spectroscopy as a measurement method. When it was compared with colony counting, it was observed that the difference between the two methods was greater when the *f*-sand had lower efficacy, and the spectroscopy method underestimated the amount of bacteria removed when compared with direct colony

counting. It was still a useful measurement for this type of study, as it was a quick way of determining whether the system could be regenerated. For further scale-up studies, colony counting or similar methods should be employed.

CONCLUSIONS

Bacterial contamination of water is a significant problem and many people rely on untreated water for drinking purposes. $MO_{2.1}$, found within the seed of *Moringa oleifera*, can be readily adsorbed onto the surface of silica sand particles, which can then be used for the attachment and inhibition of bacteria such as *E. coli* in contaminated water. The attached bacteria prevent any further attachment and the *f*-sand is no longer effective for water treatment. The main findings from this study are as follows. 0.01 M dodecyl glucoside, a non-ionic surfactant, is effective at separating the *E. coli* from the $MO_{2.1}$, allowing the reuse of the *f*-sand. Sodium dodecyl sulfate, an anionic surfactant, causes the $MO_{2.1}$ to separate from the sand surface. A commercially available detergent containing 10–30% dodecyl glucoside was also effective at separating the *E. coli* from the sand.

The results demonstrated in this work offer a potentially sustainable, reusable process to combat bacterial contamination of water.

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Chapter 6. Heat study

The work reported in this Chapter was presented at the 2017 International Water Association conference on sustainable solutions for small water and wastewater treatment systems (S2Small) as Ref # S2SMALL-82653. The manuscript which was used for the conference is presented here with alterations.

The aim of the experiments described in Chapters 5 and 6 was to examine two independent methods of f-sand regeneration. Chapter 6 provides an extension to the work presented in the published f-sand paper shown in Chapter 5. The experiments presented in Chapter 6 were designed to test if the exposure of f-sand to elevated temperatures would:

1. Effect on the binding of the *Moringa oleifera* protein to the sand surface,
2. Cause the separation of inactivated *Escherichia coli* from f-sand so that the f-sand could be regenerated using heat.

The results of this temperature study were compared against those reported in the surfactant study. The surfactant and heat re-activation studies were performed as proof-of-concept, and any further experimentation on the application of these, and any other reactivation methods, is encouraged as future work.

Statement of Authorship

Title of Paper	Heat study
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	N/A

Principal Author

Name of Principal Author (Candidate)	Frances Elizabeth Williams
Contribution to the Paper	Designed and performed experiments, interpreted the data and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Moringa oleifera functional sand (f-sand) can be used to remove *Escherichia coli* from water by immobilization and inactivation via an antibacterial protein from the *M. oleifera* seed that is attached to the sand surface. As the proteins inactivate the bacteria, the f-sand becomes less efficient, so the purpose of this study was to determine if exposing the f-sand to elevated temperatures caused the immobilized bacteria to separate from the protein without causing the protein to separate from the sand surface. The aim of this test was to determine if heat could be used to regenerate the f-sand. To examine the effect of heat on 'used' f-sand, the f-sand was exposed to water contaminated with *E. coli* so that the removal of the inactivated *E. coli* could be measured. This 'used' f-sand was then subjected to dry or wet heat between 60°C and 100°C. The use of the 60°C water to regenerate f-sand was successfully demonstrated. Temperatures below 50°C did not demonstrate a regenerative effect. Temperatures above 70°C reduced the efficacy of the f-sand. The results were compared to the regeneration of f-sand using the non-ionic surfactant dodecyl glucoside.

6.1 Introduction

Moringa oleifera seeds are traditionally used for water treatment due to its ability to clarify turbid water (Jahn & Dirar 1979). This clarifying effect is caused by a family of water-soluble, cationic proteins which are known to be effective coagulating agents for colloidal material and other suspended particles (Pritchard *et al.,* 2009; Pritchard *et al.,* 2010a, 2010b). Generally known as *M. oleifera* cationic proteins one of the proteins that has been isolated, referred to as $MO_{2.1}$, is reported to inactivate a range of bacterial organisms by interacting with the phospholipid membrane of the bacteria. The inactivation of the *E. coli* by $MO_{2.1}$ occurs via penetration by a hydrophobic kink in the $MO_{2.1}$ amino acid sequence into the bacterial membrane. The interaction between the *E. coli* and $MO_{2.1}$ is proposed to lead to the fusion of the inner and outer bacterial membrane, causing the antimicrobial effect (Shebek *et al.,* 2015). The section of the amino acid sequence that penetrates the membrane is separate to the section which causes coagulation, as demonstrated by Suarez *et al.*, (2005).

The cationic, antibacterial *M. oleifera* proteins have been observed to adsorb electrostatically onto the negatively charged surface of silica sand particles. This functional sand (f-sand) can be used to attach and inactivate *E. coli* (Jerri *et al.,* 2012). In

practice the attachment of *E. coli* to the f-sand prevents any further bacterial inactivation and the f-sand cannot be reused. Preparation of f-sand is laborious and the development of a method to regenerate the f-sand is worthwhile.

The $MO_{2.1}$ protein is reported to be thermostable at 95°C for 5 hours without loss of activity (Ghebremichael *et al.*, 2005). The suggestion has been made that *M. oleifera* coagulant proteins belong to a class of proteins known as intrinsically disordered proteins (Dezfooli *et al.*, 2016). Intrinsically disordered proteins are characterised by a high thermal stability and high solubility in aqueous solutions. To examine this phenomenon Dezfooli *et al.*, (2016) exposed $MO_{2.1}$ to sterilising conditions (121°C, 1.2 bar, 15 minutes) and reported that the protein retained 70% coagulation activity of the crude extract. The antibacterial efficacy of $MO_{2.1}$ post-sterilisation has not yet been reported.

The principle aim of this study was to determine if the thermostability of the *M. oleifera* coagulant proteins could be leveraged to develop a second method of regenerating the f-sand The effect of temperature from 60 °C to 100 °C on the attachment of the *M. oleifera* antibacterial proteins to the sand surface was experimentally examined along with the antibacterial activity of f-sand that had been exposed to the above-mentioned temperatures. The regenerative effect of heating the f-sand was compared to the effect of the non-ionic surfactant dodecyl glucoside as investigated previously (Williams *et al.*, 2017). Dodecyl glucoside is a non-ionic surfactant. It is non-hazardous, biodegradable and rapidly degrades in aquatic environments (Gamia *et al.*, 1997) and is known by the trade names lauryl glucoside and coco glucoside. Dodecyl glucoside is derived from glucose and fatty alcohol and is used in a wide range of household products including shampoos, clothes washing detergents and dishwashing detergents. In our previous work we determined that exposing 1 g samples of f-sand to the dodecyl glucoside resulted in a 10% reduction in the amount of protein on the sand surface and that no further loss of protein occurred despite repeat washing (Williams *et al.*, 2017). Unfortunately, while the dodecyl glucoside is widely available, it is not always clearly labelled and can be mixed with a range of other surfactants including sodium dodecyl sulphate. We previously reported that a 0.01M solution of sodium dodecyl glucoside reduced the amount of protein on the f-sand by 80%. The objective of this investigation was to develop a second method of f-sand regeneration using heat.

6.2 Materials and methods

6.2.1 *f*-sand preparation

The *f*-sand was prepared following the method presented in Williams *et al*., (2017) and based on a method modified from *Jerri et al*., (2012). A 0.05 g/mL crude *M. oleifera* solution was prepared by mixing 20 g of dry, powdered *M. oleifera* seed with 400 mL of a 0.1 M sodium chloride solution for one hour. The crude solution was sieved and filtered through 0.45µm filter paper (Millipore) to remove suspended seed particles and residue bacteria which may have been on the surface of the seeds. 100 g of commercially available, fine grained silica sand was thoroughly rinsed with deionised water, dried then autoclaved (121°C, 30 minutes). The sand was combined with 400 mL of the filtered crude extract solution and mixed on a shaker plate for one hour. The *f*-sand was thoroughly rinsed with deionised water and dried overnight at 50°C.

6.2.2 Effect of heat on protein adsorption

Initial tests were performed to determine the effect of heat on the attachment of the protein to the sand. Dry 1g samples of *f*-sand were heated between 50 °C and 100 °C for 1 to 60 minutes. The dry samples were prepared by placing 1 g of *f*-sand in a sealed glass tube and submerging the lower half of the tube in heated water. After heating, the *f*-sand was immediately rinsed in 5 mL deionised water. The proteins that remained on the surface of the *f*- sand were eluted by mixing the *f*-sand with 5 mL NaCl (0.6 M) solution on a shaker plate for 1 hour to determine the amount of protein remaining on the sand after heating. The amount of protein in the NaCl solution was determined by measuring the absorbance of the filtered solution at 280 nm using UV-visible spectroscopy (Shimadzu, 1601 UV-visible spectrophotometer). The concentration of total eluted protein was determined using the Beer-Lambert law, Abs= $\varepsilon l c$, with a path length (l) of 1 cm and an extinction coefficient (ε) of 1520 (Aitken & Learmonth 1996). The total mass of protein on the *f*-sand surface was then determined as described in the Chapter 3 and Chapter 5.

6.2.3 Preparation of synthetic water

Colonies of *E. coli* (ATCC® 25922™) were selected using a sterile loop from an agar plate and incubated at 37ºC in nutrient broth (Oxoid) overnight. Approximately 10 mL of bacterial broth was centrifuged at 3000 x g for 5 minutes (Allegra™ X-12, Beckman Coulter), and the pellet was re-suspended in sterile NaCl solution (0.85%). The bacterial

pellet was centrifuged again and re-suspended in a sterile, room temperature, NaCl solution (0.85%). The absorbance was diluted to 0.1 ± 0.005 at 600 nm with the use of a UV-visible spectrophotometer (Shimadzu UV-Vis 1601). This absorbance is equivalent to a bacterial concentration of 1-2 x10^8 (Wikler, 2006). Cultivatable counts were performed to validate the number of bacteria in solution.

6.2.4 Functional sand column tests

60°C, 70°C, and 100°C tested further to determine if those temperatures could be used to regenerate the f-sand. The method described in Williams *et al*., (2017) was followed but instead of using glass columns to contain the f-sand, glass condensers were used so that the temperature could be controlled while performing the experiments. 40 g of dry f-sand was poured into a glass column condenser (1.5 cm diameter) fitted with a filter. The f-sand was rinsed with Milli-Q water until the absorbance of the outgoing solution was less than 0.001 at 280 and 600 nm. The column was then subjected to a set of 40 mL solutions in the following order: synthetic water, 2 × Milli-Q water, hot water, 2 × Milli-Q water. Each set was repeated four times, so that the f-sand had been exposed to three discrete synthetic water treatments and the amount of *E. coli* removed each time was recorded. Fresh columns were prepared for each experimental run.

Unattached *E. coli* were removed by rinsing the f-sand column twice with sterile Milli-Q water. For each temperature tested, 60°C, 70°C, and 100°C, the f-sand column with the immobilised, inactivated *E. coli* was exposed to 80 mL heated water followed by two 40 mL rinses with sterile Milli-Q water. A uniform f-sand temperature was achieved by pumping heated water around the outside of the glass condenser column while the 80 mL heated water passed through the f-sand within the centre of the glass condenser. The f-sand was rinsed twice with room temperature, sterile Milli-Q water before it was re-exposed to the *E. coli* solution (40 mL) followed by sterile Milli-Q water rinse, the hot water treatment and final Milli-Q water rinses.

The solutions passing out of the columns were collected and the absorbance was measured at 600 nm. Measuring the absorbance of the outlet solution was used to determine the bacterial concentration and was standardised against the absorbance of bacterial solutions from 0.01 to 0.1 at 600 nm and was compared with the cultivatable counts of these solutions. Colony counting was also performed on select columns to ensure accuracy of

the absorbance. The percentage of *E. coli* removed by the columns was determined using Equation 1, Chapter 5.

6.2.5 Bacterial staining

Fluorescence imaging was performed on the f-sand at initial bacteria attachment using a Nikon live cell microscope at 40× optical zoom. A 100 ms exposure for fluorescence was used with a 195 ms exposure for the live view The *E. coli* cells were stained with propidium iodide, a fluorescent stain that only attaches to membrane-compromised bacterial cells.

6.3 Results and discussion

6.3.1 Bacterial staining

The attachment of *E. coli* to the f-sand surface was confirmed by staining with propidium iodide, as shown in Figure 14. The inactivated *E. coli* is visible as fluorescent ovals across the sand surface.

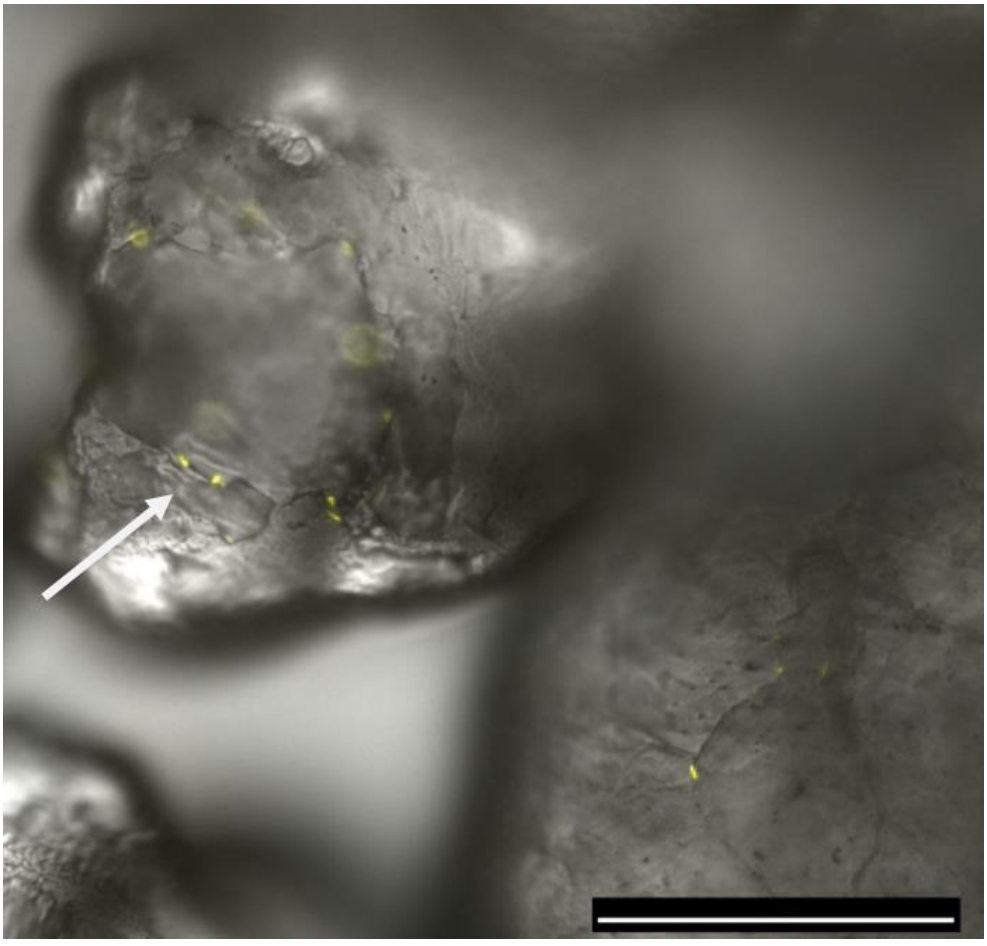

Figure 14: Fluorescence image of inactivated *E. coli* on f-sand surface. Scale bar 100µm

6.3.2 Effect of heat on protein attachment

Heating the 1g samples resulted in an immediate reduction of the amount of protein attached to the sand surface. The data presented in Figure 15 to Figure 20 shows that at 70°C and below, no further loss of protein was recorded, and at 80°C and 90°C there was consistent loss of protein over 1 hour. At 100°C there was an immediate loss of protein. The amount lost over 1 hour was more significant at 100°C than the other temperatures tested.

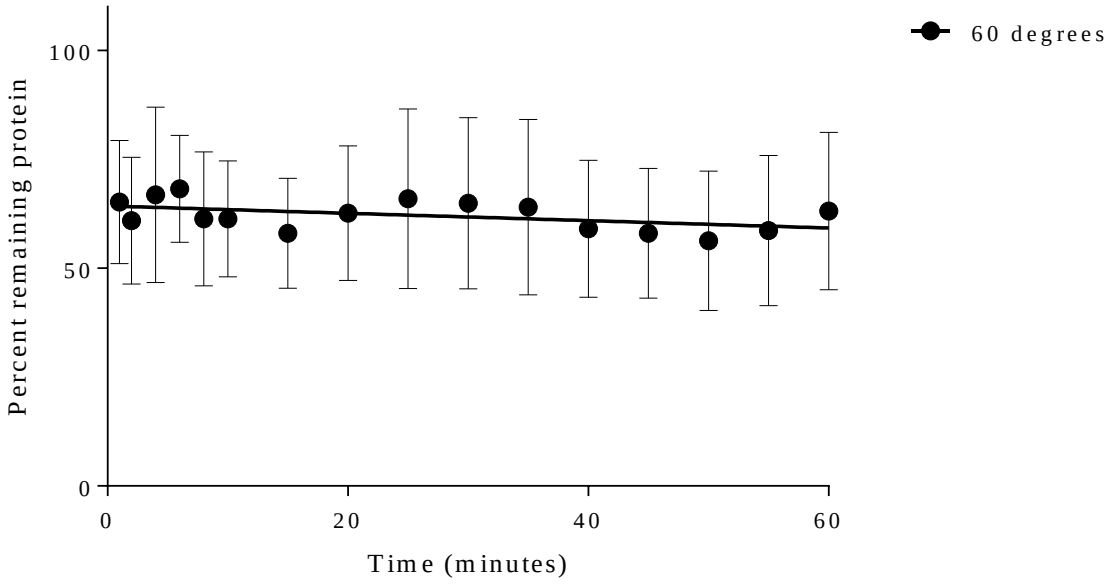

Figure 15: Effect of 60ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

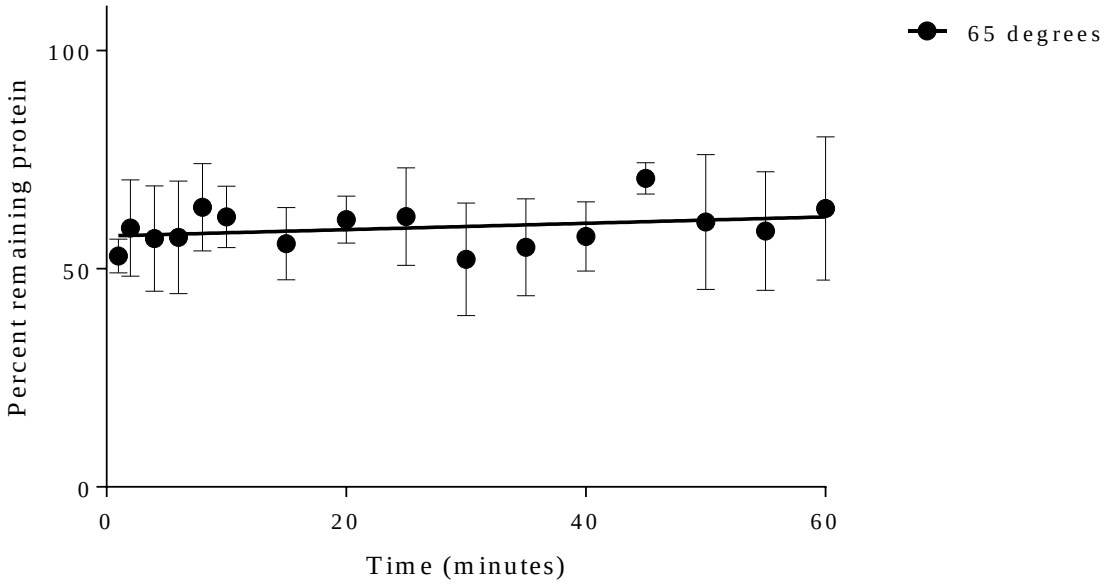

Figure 16: Effect of 65ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

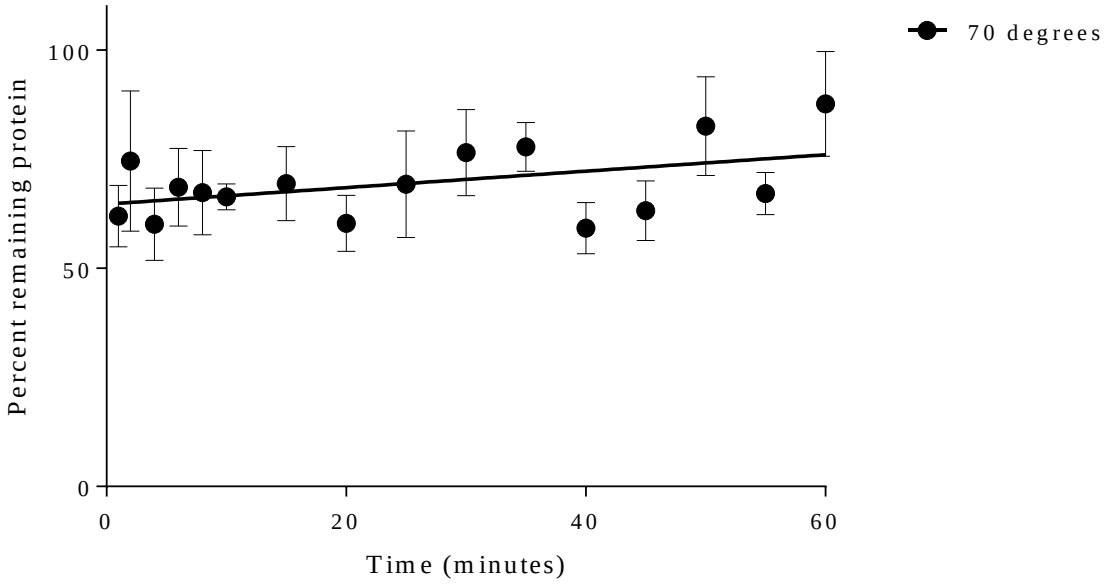

Figure 17: Effect of 70ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

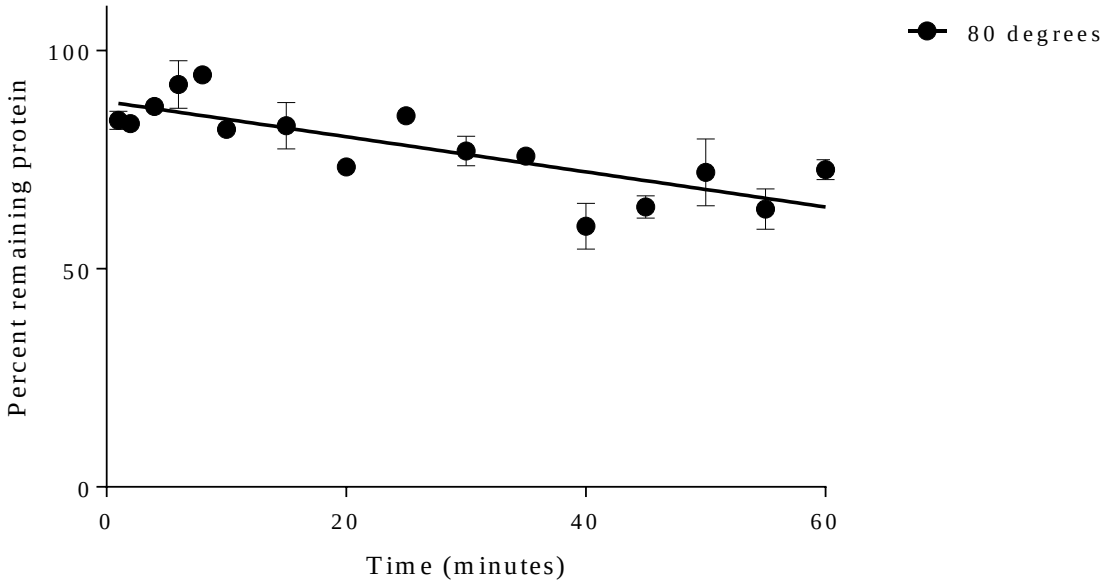

Figure 18: Effect of 80ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

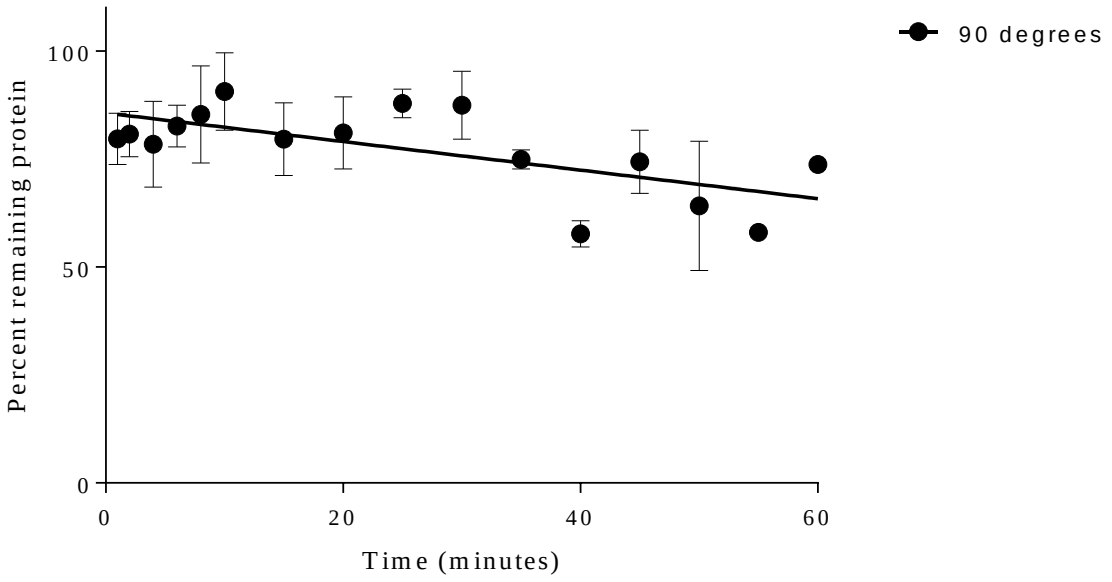

Figure 19: Effect of 90ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

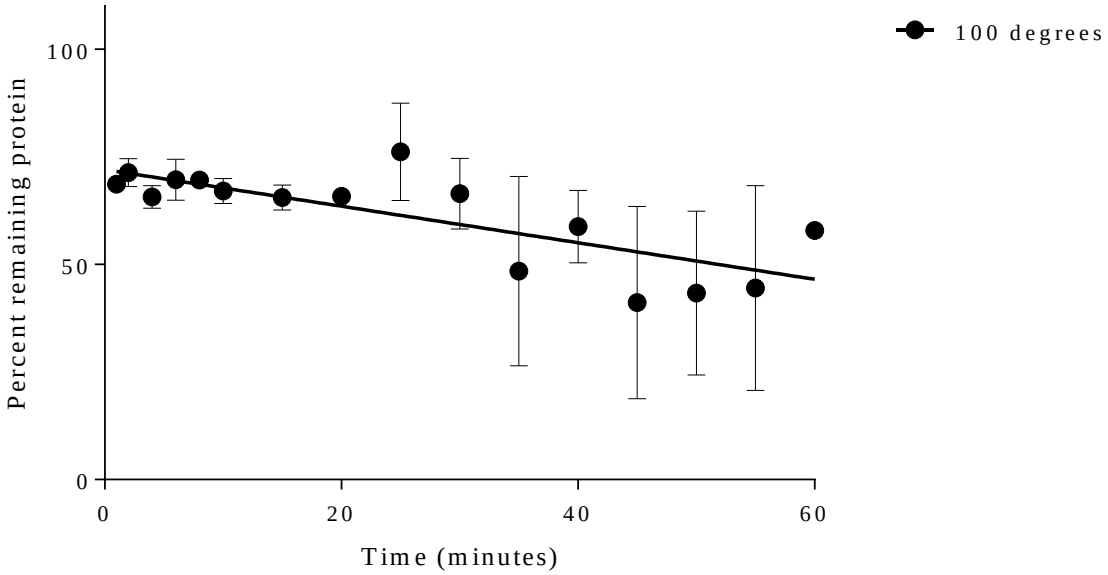

Figure 20: Effect of 100ºC heated water on the percent of *M. oleifera* proteins remaining on the sand surface.

The reduction in the amount of protein from the sand surface seen at the higher temperatures was expected to correlate to a reduction in column efficacy as the proteins are separated from the sand surface and washed out of the column. Significant variation in the amount of proteins bound to the sand surface at 100°C (displayed as the mean with the standard error of the mean in the error bars). One explanation for the significant variation in the amount of protein binding to the sand surface may be explained by the repeated separation and re-attachment of protein at 100°C.

6.3.3 Functional sand column test results

The efficacy of the *f*-sand columns after they had been exposed to water heated to 60ºC, 70ºC or 100ºC, and by extension the ability of heat to regenerate the *f*-sand, is reported in Figure 21. Water heated to 60°C was effective at regenerating the column and allowed for repeated bacterial removal across the three treatments. At 70°C and above each run lead to a loss of efficiency in the *f*-sand column with the greatest loss occurring at 100°C. Heating the column to 60°C regenerated the *f*-sand most consistently and for this study is the optimum temperature for *f*-sand regeneration.

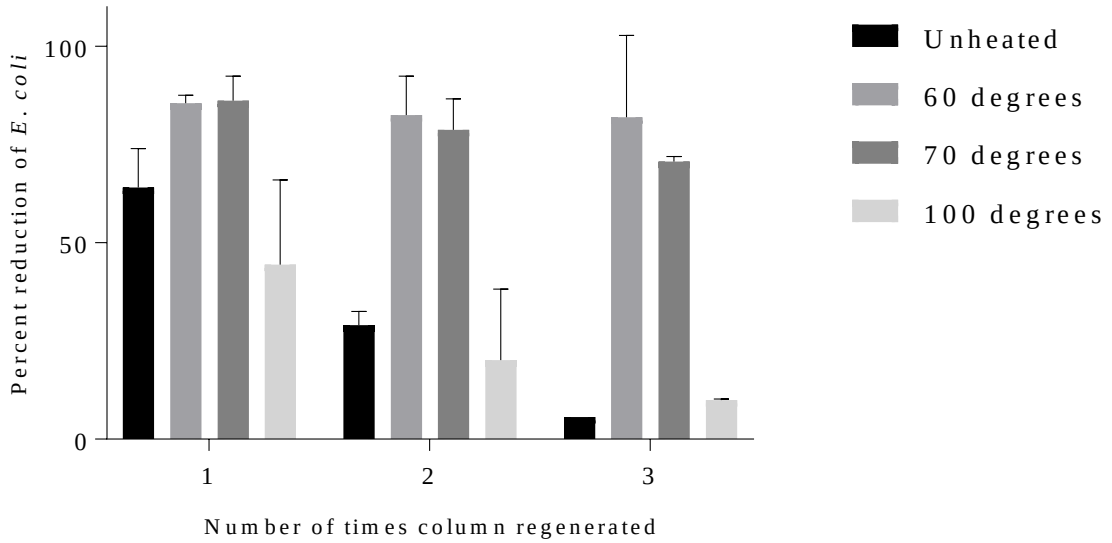

Figure 21: Heat effects on *ƒ*-sand column regeneration.

Non-ionic surfactants, including dodecyl glucoside, can be used to solubilise the lipid membrane of bacteria via integration of the surfactant into the bacterial membrane, followed by extraction of the lipids and formation of micelles (le Maire *et al.*, 2000). For dodecyl glucoside at concentrations of 0.01 M and below, this occurs without affecting the attachment of the protein to the sand surface. The regeneration of the *ƒ*-sand using heat was not as efficient as using the non-ionic surfactant dodecyl glucoside where a 99% reduction in *E. coli* was achieved. Washing the *ƒ*-sand with 0.01 M dodecyl glucoside increased the bacterial removal on the second and subsequent washes while washing with 0.001 M maintained the original efficiency of the *ƒ*-sand across the three treatments. A side by side comparison of an untreated *ƒ*-sand and the *ƒ*-sand columns treated with water heated to 60°C or 0.01 M dodecyl glucoside is presented in Figure 22.

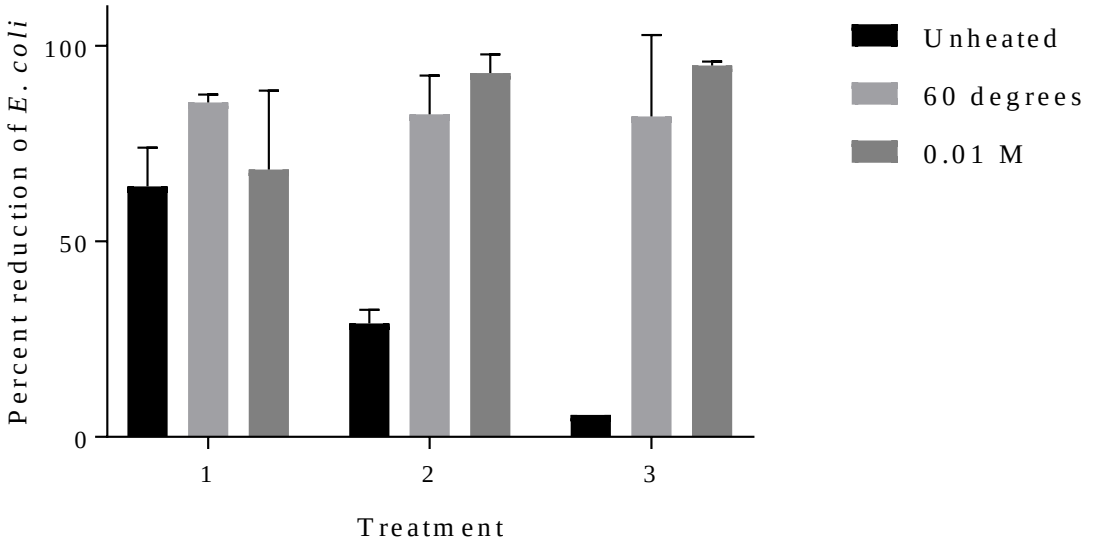

Figure 22: Comparison between untreated *f*-sand, *f*-sand treated to 60ºC heated water and 0.01 M dodecyl glucoside

6.5 Conclusions

In this study, a second method of regenerating used *f*-sand is demonstrated. Exposing the *f*-sand to water heated to 80ºC and above caused a loss of protein from the sand surface and lead to a loss of efficiency of the *f*-sand columns. A temperature of 60ºC was found to be the optimal temperature because it was not a high enough temperature to cause the protein to separate from the sand surface but was sufficient to cause a regenerative effect of the *f*-sand. Further work is required to develop this technology into a water treatment system that could be used in non-ideal conditions. However, it is a significant step in the development of an alternative treatment method to combat bacterial contamination of water with potential to become an effective alternative for water treatment in regions where accessible, affordable water treatment is lacking.

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Chapter 7. f-SiO_2

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Principal Author

Name of Principal Author (Candidate)	Frances Elizabeth Williams		
Contribution to the Paper	Designed and performed experiments, interpreted the data and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andrew K. Lee		
Contribution to the Paper	Supervised development of work. Helped to interpret data and to evaluate and edit the manuscript.		
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Contribution to the Paper	Supervised development of work. Helped to interpret data and to evaluate and edit the manuscript.		
Signature		Date	26/4/19

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Contribution to the Paper	Supervised development of work. Helped to interpret data and to evaluate and edit the manuscript.		
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Antibacterial action of functional silicon dioxide: an investigation of the attachment and separation of bacteria

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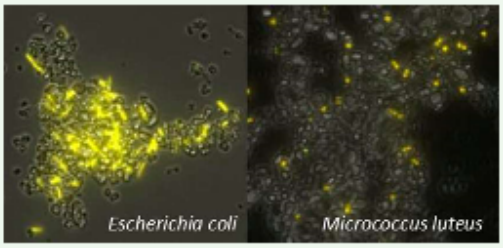
ABSTRACT

Bactericidal proteins from the *Moringa oleifera* seed are reported to be suitable alternatives to conventional methods of bacterial reduction in water. In this study the cationic bactericidal *M. oleifera* proteins were isolated by attachment onto the surface of silicon dioxide. This functionalised SiO_2(f-SiO_2) was then exposed to *Escherichia coli* and *Micrococcus luteus* to examine whether the f-SiO_2 could be used to inactivate the bacteria. The effect of the non-ionic surfactant dodecyl glucoside on the attachment of these bacteria to the f-SiO_2 was examined with the aim of developing a method of reusable bacterial inactivation. The primary result of this study was that the *E. coli* could be readily separated from the f-SiO_2, allowing the f-SiO_2 to be used for further bacterial inactivation. The regeneration of the f-SiO_2 was demonstrated using fluorescence microscopy on bacterial cells stained with propidium iodide, and zeta potential measurements. Future applications of this work include a reusable method of removing bacteria from contaminated water.



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Introduction

The use of *Moringa oleifera* seeds in water treatment is a well-established practice that is believed to have originated in rural Sudan [1]. The seeds are currently used in developing regions because they are comparatively cheap and widely available where other water disinfection practices, such as chlorination, are not obtainable or are too expensive to be used effectively [2]. Currently, a restrictionto the wider use of *M. oleifera* seeds for potable water treatment is that the treated water cannot be stored for more than one day as the decaying seed particles release nutrients and dissolved organic carbon (DOC) into the water [3]. This increase in nutrients promotes the growth of entericbacteria that either survived the treatment or are introduced latervia recontamination.

Two main types of bactericidal compound, the isothiocyanates and the coagulant proteins, have been successfully isolated from the *M. oleifera* seeds. The coagulant proteins are comprised of at least eight subpopulations that share similar physical characteristics [4–6]. Of these fractions, 94.9% are strongly cationic, dominantly alpha helical in shape, and have a radius of 1.2 to 1.5 nm [4,5]. This fraction contains the *M. oleifera* coagulant protein (MOCP), a 12 kDa dimer protein that is partly comprised of a 6.5 kDa monomer called $MO_{2.1}$ [7,8]. $MO_{2.1}$ is recognised for its bactericidal properties against a range of pathogenic bacteria [9].

The use *M. oleifera*to develop functional bactericidal surfaces is an emerging field of research that combines the benefits of the *M. oleifera* proteins with the unique optical, electronic and physiochemical properties of the material that is being functionalised [10–12]. This method of isolation overcomes the increase in DOC by effectively separating the useful proteins from the seed extract and allows for greater control over where the protein is used. The attachment of the *M. oleifera*

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proteins to materials such as sand, metal oxide and magnetic nanoparticles has the additional benefit of being easily separated from the water post-treatment [3,13]. Isolating $MO_{2.1}$ onto finely ground SiO_2 allows for visual observations of bacterial inactivation and the suspension of functional SiO_2 (f-SiO_2) in solution. When attached to SiO_2 the $MO_{2.1}$ forms a dense layer of 60 to 70 Å [10]. Quartz sand functionalised with the cationic *M. oleifera* proteins has been shown to effectively remove anionic colloids, *Escherichia coli*, and harmful marine and fresh water microalgae cells from water [3,14].

Bacteria have a net negative surface charge and are attracted to the cationic *M. oleifera* proteins, including $MO_{2.1}$, where they are then inactivated via attachment of the $MO_{2.1}$ to their lipid membrane. The attachment of *E. coli* has been elucidated for $MO_{2.1}$ and is initiated by several hydrophilic sections of the amino acid sequence that surround two hydrophobic prolines [15]. The hydrophilic, positively charged arginines interact with the negatively charged bacterial membrane, forcing the prolines into the membrane. This has been proposed to lead to fusion of the inner and outer lipid membrane [16]. Furthermore, the bactericidal and coagulant actions of $MO_{2.1}$ are attributed to separate sections of the amino acid sequence and can be isolated from each other [15]. This interaction has so far only been studied with *E. coli* and the interaction between gram-positive bacteria and $MO_{2.1}$ may follow a different mechanism of inactivation. The effect of $MO_{2.1}$ on *M. luteus* has not been examined in the literature.

The lipid membrane of *M. luteus* is strongly hydrophobic [16] especially when compared to *E. coli* [17]. The overall surface charge of gram-positive bacteria is more negative than that of gram-negative bacteria. This difference isdue to the presence of anionic polymers called teichoic acids in the thick peptidoglycan layer outside of the phospholipid membrane [18]. Surface charge neutralisation has been shown to be an indicator of the minimum inhibitory concentration of several antimicrobial peptides [19,20]. Measuring the concentration required for charge neutralisation is applicable when determining measuring protein adsorption onto the surface of silica [4,5] and the optimal coagulant dose of the *M. oleifera* protein for colloid suspensions [3,21,22].

The isolation of proteins from the bacterial lipid bilayer using surfactants is a well-established process in areas of biochemistry and can allow recovery of the proteins without loss of activity [23]. In these systems, the lipids are solubilised by the surfactants to release the proteins of interest and can be achieved using gram-positive and gram-negative bacteria [24]. The effect of non-ionic surfactants on the lipid membrane occurs in three stages. First, surfactant molecules enter the lipid membrane. This continues until the surfactant-surfactant interactions destabilise the bacterial membrane structure and causes it to fragment. Finally these lipid fragments solubilise into the surfactant micelles and the protein of interest can be isolated from the solution [23]. In our previous work, we reversed this process using dodecyl glucoside, a non-ionic surfactant, to remove the bacteria from a layer of proteins. We used this to developa method of separating inactivated *E. coli* from sand that had been functionalised with cationic *M. oleifera* proteins [24].

The primary aims of this study were to determine whether *M. luteus* could be inactivated using f-SiO_2 and tostudy the effect of dodecyl glucoside on the attachment of *Micrococcus luteus* to f-SiO_2. The second of these was performed in order to determine if this method of separation could be applied more broadly.

Materials and methods

Polypeptide extraction and attachment

The functionalised SiO_2 (f-SiO_2) was prepared using an experimentally optimised version of the method described by Jerri et al. [3]. Whole, dry *M. oleifera* seeds (AustraHort PTY Ltd, Australia) were dehusked before use. A 50 g/L solution was prepared by mixing finely ground *M. oleifera* seed kernels with a 0.1 M NaCl solution for one hour at 150 rpm. This extract solution was filtered through a biologically inert, mixed cellulose ester membrane (0.45 µm, Millipore) to remove the non-soluble seed particles and any native bacteria. A naturally occurring microcrystalline silica(SiO_2) with 0.5–10 µm diameterwas purchased from Sigma Aldrich (CAS Number 14808-60-7) and used as provided. 10 grams of SiO_2wereadded to 40 mL of the extract solution and rolled on a shaker plate for one hourat room temperature (~22°C). The solution was centrifuged for 10 min at 3000 × g, resuspended in Milli-Q water and centrifuged again. This procedure was repeated three times to remove excess biological material and salt. The attachment of the cationic proteins to the SiO_2 was confirmed using solid spectrophotometry at 280 nm (Shimadzu UV-Vis-NIR3600Plus). The spectra of the f-SiO_2 were compared to a purified protein extract containing $MO_{2.1}$ at 280 nm (Shimadzu, UV-1601). The extract was prepared by running the crude *M. oleifera* extract solution through a cation exchange chromatography column loaded with CM Sepharose Fast Flow (Sigma-Aldrich) equilibrated with 0.1 M NaCl and eluted using an NaCl gradient up to 1 M based on the $MO_{2.1}$ isolation method of Ghebremichael et al. [6]. This solution is

known to contain other homogenous proteins and some variation in the composition of the attached proteins was expected [21].

Preparation of bacterial suspensions

Escherichia coli (ATCC® 25922™) was sourced from the School of Animal and Veterinary Sciences, The University of Adelaide. *Micrococcus luteus* was sourced from the School of Molecular and Biomedical Science at the University of Adelaide. Both bacteria were maintained on Bacteriological agar (Oxoid) and suspensions were grown overnight in nutrient broth (Oxoid). 10 mL of bacterial nutrient broth was centrifuged for 5 min at $3000 \times g$ and the pellet was resuspended in a sterile 0.85% NaCl solution. The bacterial suspension was centrifuged and the pellet was resuspended in either sterile Milli-Q water or sterile 0.85% NaCl solution and diluted to an absorbance of 0.1 ± 0.005 at 600 nm. For the 0.85% NaCl solution this is equivalent to a bacterial suspension containing $1–2 \times 10^8$ Colony Forming Units (CFU)/mL of *E. coli* [25]. The Milli-Q suspension was generally lower but always above 5×10^7 CFU/mL. Fresh bacterial suspensions were prepared immediately before each experiment. The concentration in each suspension was verified by performing cultivatable triplicate spread plate counts on Bacteriological agar (Oxoid).

Attachment and separation of bacteria

The initial bacterial attachment (step 1), bacterial separation (step 2) and attachment of fresh bacteria (step 3) were defined as follows:

Step 1: The f-SiO_2 was mixed with bacterial solution for one hour. Bacteria which had not attached to the f-SiO_2 was removed by centrifuging the sample at $3000 \times g$ for 10 min, re-suspending the f-SiO_2 in sterile Milli-Q water and centrifuging the sample again at $3000 \times g$ for 10 min. Different concentrations of f-SiO_2 were used for the different analysis performed.

Step 2: The f-SiO_2 prepared in Step 1 was suspended in 20 mL 0.01 M dodecyl glucoside (supplied as 49.74% w/w solution, CAS 11061-47-9, Ingredients Plus Australia) and continuously mixed for one hour. The dodecyl glucoside was removed by repeatedly washing the sample by centrifuging the sample at 3000 g for 10 min, re-suspending the f-SiO_2 in sterile Milli-Q water and centrifuging the sample again at $3000 \times g$ for 10 min.

Step 3: The process followed for Step 3 was identical to Step 1. 1 g of f-SiO_2 was mixed with 20 mL of bacterial solution for one hour. Bacteria which had not attached to the f-SiO_2 was removed by centrifuging the sample at $3000 \times g$ for 10 min, re-suspending the f-SiO_2 in sterile Milli-Q water and centrifuging the sample again at 3000 g for 10 min.

Bacterial staining

A 10 mg/mL f-SiO_2 suspension was prepared for steps 1 to 3 with 5 mL being subtracted after each step. All f-SiO_2 samples were centrifuged then re-suspended for 15 min in 1 mL of a 1:500 dilution of propidium iodide (Sigma Aldrich) in Tris buffer. The samples were then pelleted and washed twice in Milli-Q water before being re-suspended in 5 mL Milli-Q water. A 200 µL aliquot of each sample was pipetted into an individual well of a sample holder, allowed to dry, and viewed on a Live Cell Microscope (Nikon Ti).

Zeta potential

The bactericidal effect of the protein on *E. coli* and *M. luteus* was examined by measuring the zeta potential of the SiO_2 under different treatments. SiO_2 and f-SiO_2 suspensions were prepared in in sterile Milli-Q water at 0.25 mg/mL. The bacteria suspensions were prepared in sterile Milli-Q wateras described above at 1×10^5 CFU/mL, 1×10^6 CFU/mL, 1×10^7 CFU/mL, and 1×10^8 CFU/mL. The samples measured were $SiO_2$and$f$-SiO_2, 0.01 M dodecyl glucoside, the *E. coli* and *M. luteus* suspensions, f-SiO_2 in the bacterial suspensions (Step 1), f-SiO_2 after the interaction with the 0.01 M dodecyl glucoside (Step 2), and the f-SiO_2 used in Step 2 into fresh bacterial suspensions (Step 3). The zeta potential measurements were performed using freshly prepared samples on a Zetasizer Nano (Malvern Instruments). The zeta potential of each suspension was calculated from the electrophoretic mobility using the Henry Equation (1) and the Smoluchowski approximation $f(\kappa a)$ of 1.5.

$$U_E = \frac{2\varepsilon\zeta f(\kappa a)}{3\eta} \quad (1)$$

Where UE is the electrophoretic mobility, ε is the dielectric constant, η is the viscosity of the medium. All samples were examined using folded capillary cells (Malvern Instruments) and the average charge and standard deviation is presented in the results.

Results and discussion

The use of surface-bound bactericidal proteins, including $MO_{2.1}$, for bacterial inactivation could provide a novel

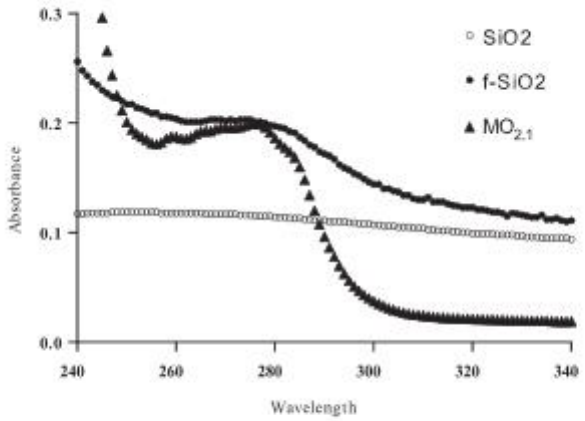

Figure 1. UV-visible spectra of $MO_{2.1}$, SiO_2 and f-SiO_2.

tool for water treatment. The separation of inactivated bacteria further develops this treatment method; however, it is still a new area of research and further analysis is required to elucidate the scope with which it can be utilised. The aim of this study was to compare the effect of dodecyl glucoside on the attachment of *E. coli* and *M. luteus* to f-SiO_2. In our previous study we reported that dodecyl glucoside could be used to separate the inactive *E. coli* from the surface of $MO_{2.1}$ functionalised sand [23]. *M. luteus* has not been previously studied in this context. The presence of the $MO_{2.1}$ on the f-SiO_2 was confirmed using UV-visible spectrophotometry (Figure 1). The $MO_{2.1}$ appears as a peak at 270 nm and is comparable to the $MO_{2.1}$ isolated from the seed extract.

Propidium iodide is a fluorescent stain that only permeates membrane-compromised bacterial cells. In this study it was used to visualise whether the inactivated bacteria remained bound to the f-SiO_2 during steps 1 to 3. The fluorescent images of the attachment, separation and further attachment of *E. coli* to the f-SiO_2 are presented in Figure 2. In the initial attachment,

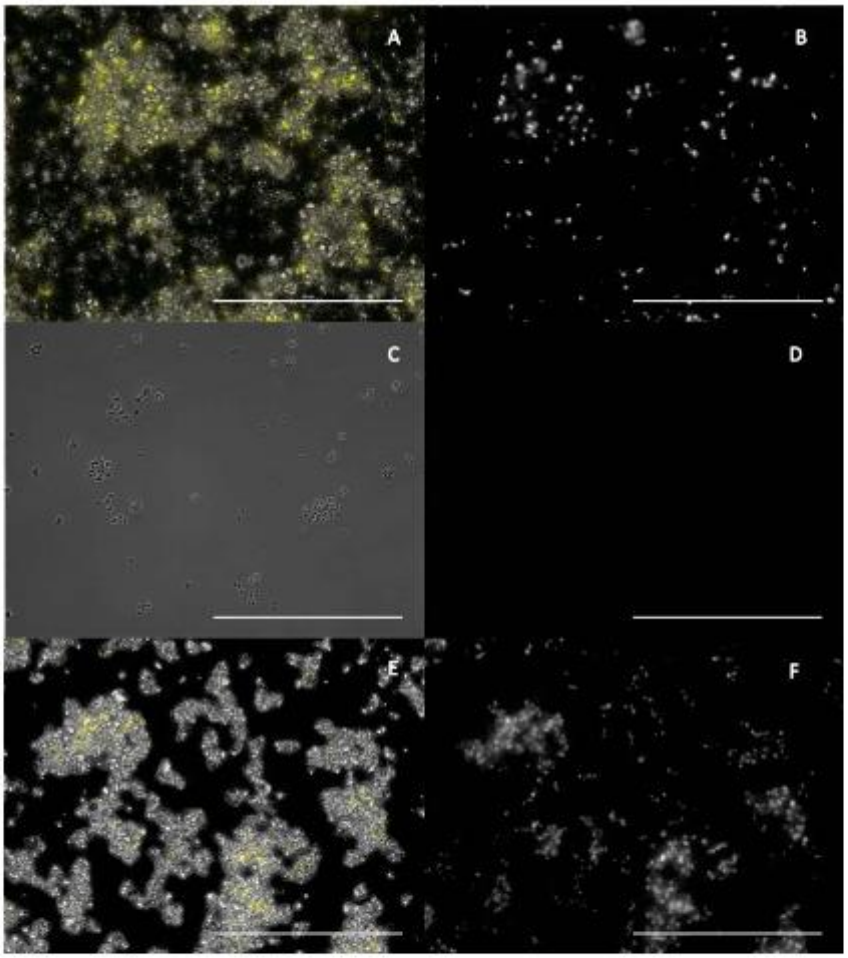

Figure 2. Fluorescence microscopy of the attachment of *E. coli* to f-SiO_2. **A**: Super-imposed fluorescence image of step 1. **B**: fluorescence image of step 1. **C**: super-imposed fluorescence image of step 2. **D**: fluorescence image of step 2. **E**: super-imposed fluorescence image of step 3. **F**: Fluorescence image of step 3. Scale bar: 100 µm.

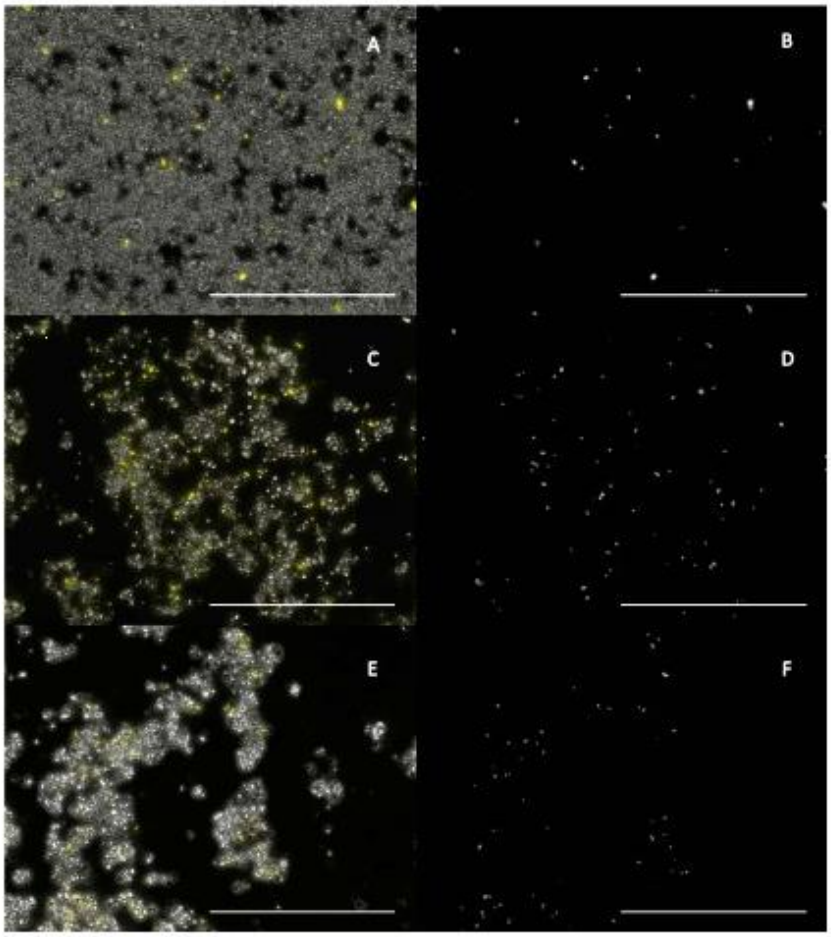

Figure 3. Fluorescence microscopy of the attachment of *M. luteus* to *f*-SiO_2. **A**: super-imposed fluorescence image of step 1. **B**: fluorescence image of step 1. **C**: super-imposed fluorescence image of step 2. **D**: fluorescence image of step 2. **E**: super-imposed fluorescence image of step 3. **F**: fluorescence image of step 3. Scale bar: 100 µm.

the *f*-SiO_2 formed flocs densely populated with *E. coli*. After washing the bound *E. coli* with the dodecyl glucoside it was observed that the bacteria had separated from the *f*-SiO_2. The exposure to fresh *E. coli* led to reattachment of the bacteria and the binding and inactivation is again apparent with larger flocs forming than in step 1.

The fluorescent images of the interaction of *M. luteus* with the *f*-SiO_2 and the dodecyl glucoside are presented in Figure 3. The *M. luteus* was inactivated by the *f*-SiO_2 but did not separate in the presence of the 0.01 M dodecyl glucoside, nor did it separate when 0.1 M doecyl glucoside was trialled. The *M. luteus* floc sizes were more consistent than the *E. coli* floc sizes.

The attachment mechanism was investigated by examining the zeta potential of the *f*-SiO_2 after exposure to the *M. luteus*, *E. coli*, and dodecyl glucoside. The *M. oleifera* coagulant protein, a 13 kDa dimer comprised partly of the $MO_{2.1}$ monomer is $+14 \pm 2$ mV [26].

In this study the zeta potential of the $MO_{2.1}$ solution isolated by cation exchange chromatography and diluted 1:10 in MilliQ water was $+3.0 \pm 0.4$ mV. SiO_2 was -24.0 ± 0.9 mV while the addition of the $MO_{2.1}$ caused ashift to $+27.0 \pm 5.6$ mV for the *f*-SiO_2. A similar shift has recently been reported by Nordmark *et al.*, [4,5]. After treatment with the 0.01 M dodecyl glucoside, the zeta potentials of the SiO_2 and *f*-SiO_2 reduce; shifting to -44.6 ± 3.5 mV and $+17.2 \pm 1.4$ mV respectively. The addition of the surfactant lowered the zeta potential which suggests that the dodecyl glucoside interacts with the SiO_2 by adsorbing to the surface as has been observed with other surfactants.

Living bacteria maintain a surface charge that is more negative than their inactivated counterparts [27]. Prior to inactivation, the *E. coli* had a zeta potential of -27.0 ± 4.9 mV and the *M. luteus* of -38.8 ± 6.9 mV. For some antimicrobial peptides, the surface charge neutralisation of bacteria has been reported to occur close to the

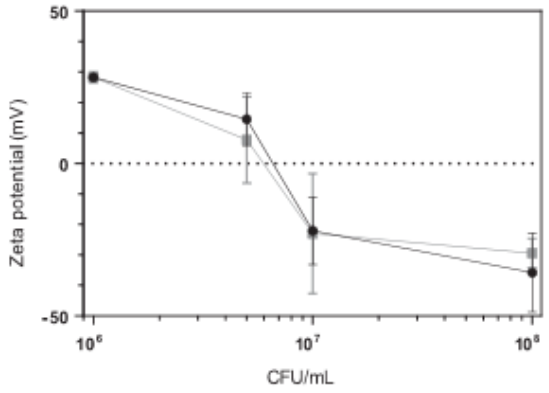

Figure 4. Effect of f-SiO_2 on zeta potential on increasing concentrations of *E. coli* and *M. luteus*. Filled circle (•): *M. luteus*, filled square (■): *E. coli*. The error bars represent the standard deviations of the measurements.

minimal inhibitory concentration (MIC) [19,20]. The charge neutralisation of *E. coli* and *M. luteus* exposed to 10 mg of f-SiO_2 occurred between 5×10^6 CFU/mL and 1×10^7 CFU/mL (Figure 4). In this study, no definite link was established between charge neutralisation and the MIC. At bacterial concentrations above 1×10^7 CFU/mL the effect of the f-SiO_2 is not apparent as the amount of $MO_{2.1}$ is limited. At concentrations of 1×10^6 CFU/mL and below, there is an abundance of $MO_{2.1}$ and the effect on the zeta potentialcan be observed.

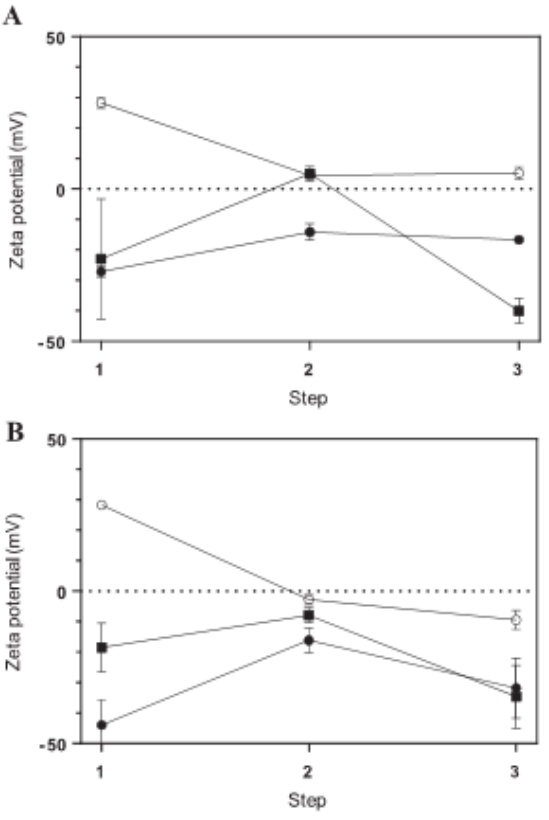

Figure 5. A: Steps 1-3 of *E. coli*. B: Steps 1-3 of *M. luteus*. Filled circle (•): 1×10^8 CFU/mL, filled square (■): 1×10^7 CFU/mL, and unfilled circle (○): 1×10^6 CFU/mL. The error bars represent the standard deviations of the measurements.

At each bacterial concentration, the effect of dodecyl glucoside on the zeta potential was investigated (Figure 5). The bacterial concentrations used in step 1 were the same as those used in step 3.

In Step 2, the two *E. coli* samples below the charge neutralisation point were both positive though neither returned to the initial f-SiO_2 zeta potential of $+27 \pm 5.6$ mV. This may be indicative of the dodecyl glucoside co-adsorbing onto the SiO_2 which was reported for the anionic surfactant sodium dodecyl sulphate [10] though not observed in our previous study [24]. The non-ionic surfactant Triton X has been found to interact with the hydrophobic region of the *M. oleifera* coagulant protein without affecting its overall charge [26]. Since the coagulant protein is comprised of $MO_{2.1}$ and another similar polypeptide, it was anticipated to behave in a similar manner.

For the *M. luteus* samples none of the suspensions had a positive zeta potential after the removal of the dodecyl glucoside in step 2, indicating the continued attachment of *M. luteus* to the f-SiO_2. The lowering of the zeta potential is due to the combined effect of the dodecyl glucoside and the bacteria. When fresh *M. luteus* was added in step 3, the entire system became more negative, likely to be due to the further reduction of available $MO_{2.1}$. The cell wall of gram-positive bacteria significantly differs to the cell wall of gram-negative bacteria. The thick peptidoglycan layer that surrounds gram-positive bacteria may interact differently to the $MO_{2.1}$ than the lipid outer membrane of gram-negative bacteria, which is predominantly a hydrophobic interaction [15]. Further research could determine if the issue of non-separation applies to all of the gram-positive bacteria or just the *M. luteus* and would clarify the mechanism of inactivation for these bacterial species.

Conclusions

This study investigated the attachment and separation of bacteria from *M. oleifera* functionalised SiO_2 using the non-ionic surfactant dodecyl glucoside. 0.01 M dodecyl glucoside was effective at removing *E. coli* from the f-SiO_2 to allow for the reuse of f-SiO_2 as a bactericidal agent. This removal was not observed with the *M. luteus* Future work will determine

whether this is specific to *M. luteus* or occurs across a range of bacterial species. Measurements of the zeta potential indicated that the surface charge reversal for both bacteria occurs at the same *f*-SiO_2 concentration and that the surfactant adsorbed onto the SiO_2 surface.

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Chapter 8. Conclusions

8.1 Summary and Discussion

The studies presented in this thesis demonstrate the potential of f-sand for the inactivation of bacteria in water. A review of the available literature confirmed that previous research had focussed on the use of the crushed *M. oleifera* seeds to directly treat contaminated water, and on the antibacterial properties of the isolated compounds. Little work had been done on the use of these compounds in a practical setting. The results highlighted the need for the development of a system that did not rely on the continued use of fresh seeds or could otherwise overcome the issues with the increased organic matter added to the water by the seeds. The growing body of research into the attachment of *M. oleifera* compounds to surfaces to create functional surfaces was also identified as an area of interest. The aims of this thesis were to investigate the use of f-sand for the removal of waterborne bacteria and to develop methods of removing inactivated bacteria from the f-sand. The principal direction of research chosen for this thesis was to advance the use and application of f-sand. The use and subsequent regeneration of f-sand was proposed to be an effective and potentially scalable, yet simple, method of isolating the *M. oleifera* antibacterial proteins including $MO_{2.1}$.

Laboratory-scale studies were used to demonstrate the capacity of f-sand to remove *E. coli* from water using different configurations. Pumping the water upwards through the column to fluidise the sand resulted in a greater removal rate of *E. coli* that was due to the constant movement of the f-sand increasing the interactions between the *E. coli* and *f*-sand

The most significant contribution of this research has been the removal of the inactivated *E. coli* from the f-sand. Preparing the f-sand is laborious and time consuming, so having the option of regenerating the f-sand reduces the cost required to treat water. The two methods described in this thesis expand on the versatility of f-sand, with the surfactant treatment increasing the bacterial reduction achievable to 99% on the second, third and fourth treatments. It also reduces the reliance on *M. oleifera* seeds as the f-sand can be used multiple times. This extends beyond water treatment and into any application to which the *M. oleifera* antibacterial proteins attached to a matrix and used to inactivate bacteria.

One limitation of f-sand treatment was the significant variation in bacterial reduction achievable on the first run of the sand column experiments. Two reasons were considered likely to cause this. The first was from organic matter attached to the sand that was used in

the preparation of the f-sand. This could be removed by heat-treating the sand, as described in Chapter 7. The second reason was due to the organic matter introduced to the sand during the f-sand preparation. This was more difficult to remove although pre-washing the f-sand with dodecyl glucoside appeared to be effective most of the time. The introduction of organic matter to the f-sand from the water passing through the column was not considered in the scope of this experimental work and was not investigated. Surface waters that contain organic matter will likely affect the f-sand performance and will need to be considered in future trials.

The use of UV-visible spectroscopy in Chapters 4 and 5 to measure bacterial reduction was based upon the method used to estimate bacterial concentration in a solution. It proved to be a fast method that did not overestimate the efficacy of the process and may be useful in other trials where cultivatable counts are not appropriate, though for this method to be appropriate, the concentration of bacterial cells must be very high.

The inactivation of *M. luteus* by *M. oleifera* cationic proteins has not been reported in the literature and the removal by f-SiO_2 adds to the list of bacteria that can be inactivated by the *M. oleifera* proteins. While *M. luteus* was inactivated, the f-SiO_2 was not regenerated when mixed with the 0.01 M dodecyl glucoside as had occurred with the *E. coli*. The reasons the *M. luteus* did not separate from the f-SiO_2 were not determined.

8.2 Conclusions and suggestions for future work

This study has shown that f-sand and f-SiO_2 is an effective method to physically remove bacteria from water and that *E. coli* can be removed by washing the f-sand and f-SiO_2 with dodecyl glucoside or heating the f-sand to 60°C.The inability of dodecyl glucoside to separate *M. luteus* and the variability in bacterial removal seen across the f-sand columns elucidate some of the challenges associated with using $MO_{2.1}$ in this manner.

It is recommended that further research is conducted to assess the viability of this process for treating environmental or polluted water that contains a large range of microorganisms, including pathogens, and would preferably include an investigation into the effect of the f-sand on a range of bacteria, waterborne viruses, parasites and algae, as this would greatly expand on the use and answer the question about whether f-sand would be feasible as a water treatment system. After this had been investigated, the f-sand could be tested at larger scales. The regeneration of f-sand using dodecyl glucoside has been successfully

demonstrated against *E. coli*. However, this method is not universally effective as demonstrated by the attachment of *M. luteus* to f-SiO_2. During the testing of a scale model, the types of organisms that can be removed from the f-sand could also be examined. This would determine the application of f-sand in the field.

Further areas of research include.

- An assessment on the f-sand to determine how many surfactant wash-cycles the f-sand could be exposed to before it loses efficacy.
- The effect of heat on the attachment of *M. luteus* and other gram-positive bacteria to f-sand has not been trialled and is worth investigating.
- The effect of water chemistry on the f-sand. It has already been demonstrated that a 0.6 M NaCl concentration causes the proteins to elute so the f-sand would not be effective in sea water but to date the effect of water hardness, pH and other chemical contaminants has not been defined.
- A separate area of research would be in determining if this method of regeneration would be effective on $MO_{2.1}$ adsorbed onto other surfaces such as granular activated carbon, rice husk ash or magnetic nanoparticles.
- An investigation into the treatment of environmental waters or wastewater where bacterial concentrations are significantly higher than 100 CFU/mL. A f-sand system could also be incorporated into a larger water treatment system as one of the primary steps in drinking water treatment or as the final stage in wastewater treatment before the water is released to the environment.