Antibacterial activity of green microalgae from stressed environmental conditions

by

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ABSTRACT

Bacterial resistance to antibiotics is a growing problem in worldwide health care. The discovery of new antibiotics has been less successful in recent years which have so far not proven to be as successful or safe as natural products. Photosynthetic fresh (non-saline) water green microalgae have, however, been recently shown to be a promising source of compounds with antibacterial activity. Extracts from microalgae from a variety of environments have inhibited growth of both gram-negative and gram-positive bacteria. By investigating untapped microalgal species that thrive in stressed environments, most likely through the production of protective secondary metabolites, there is a greater likelihood of discovering strains capable of producing bioactive compounds with antibacterial activity. In this study, microalgal species obtained from environments with low pH, high metal concentrations or municipal wastewater were tested against a variety of bacterial species. The results confirm that microalgae from stressed environments are a promising source of compounds exhibiting antibacterial activity.

Keywords: Antibacterial activity, stress, green microalgae, extracts

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CHAPTER 1: INTRODUCTION

1.1 ANTIBIOTIC RESISTANCE

Prior to the discovery of antibiotics, vaccination, and overall improvement in cleanliness and living conditions, infectious diseases were the main cause of mortality across the globe (Boutayeb 2006; Fernandes 2006). Improvements in public health are highlighted by the changes in mortality rates due to common infectious diseases. The top causes of mortality in 1900 were pneumonia, tuberculosis and gastrointestinal infections, whereas by 2010 the top causes had become heart disease and cancer (Jones *et al*. 2012). Bacterial infections have become readily treatable due to the discovery of antibiotics, which are considered one of the most effective medicines discovered thus far. Most active compounds were discovered in the 1940s to 1960s, or the so called "Golden Era" of antibiotics (Fernandes 2006).

However, antibiotic resistance is a growing problem in modern healthcare, with the number of deaths from drug-resistant bacterial infections rising each year (CCA 2018; CDC 2019). Currently it is estimated that approximately 700 000 people die each year from antibiotic-resistant infections, but this is expected to rise to 10 million by 2050, making infectious diseases the number one cause of death worldwide (Caniça *et al*. 2019; Ragheb *et al*. 2019).

The available antibiotics would continue to be effective against bacterial infections if it were not for the development of antibiotic resistance. Mechanisms of antibiotic resistance in bacterial cells can be intrinsic or acquired (Sagar *et al*. 2019a). Intrinsic mechanisms are encoded in chromosomes and do not require prolonged or repeated exposure to the antibiotic in order to become activated (Hollenbeck & Rice 2012; Sagar *et al*. 2019a). These mechanisms likely occur

as a natural response to an environmental threat, which also provides resistance to antibiotics. An example of intrinsic resistance is found with β-lactam antibiotics, which have low activity against some enterococci due to low affinity to the penicillin binding proteins within these cells (Hollenbeck & Rice 2012; Sagar *et al*. 2019a).

Acquired resistance is not innate to the cell and occurs due to sporadic mutations, mobile and horizonal gene transfer, or recombination (Hollenbeck & Rice 2012; Sagar *et al*. 2019b). The mutations may result in reduced affinity for binding of the drug to the target within the cell, increased expulsion of the drug, metabolic changes that reduce the effect of the drug or a decrease in uptake of the drug (Munita & Arias 2016). Horizontal gene transfer is considered the major mechanism for proliferation of antibiotic resistance, and occurs when genes are transferred between bacteria through non-hereditary pathways (Hollenbeck & Rice 2012; Munita & Arias 2016). For example, the use of transposons and plasmids can transfer resistant genes between bacteria, therefore causing other bacteria in the community to develop resistance to the antibiotic (Hollenbeck & Rice 2012; Munita & Arias 2016).

The discovery of novel antibacterial compounds has been greatly reduced in the 21st century due to challenges in finding novel effective and non-toxic molecules (Fernandes 2006; Fernandes & Martens 2017). Historically, soil microbes, including both fungi and bacteria, provided the majority of the classes of antibiotics used today, and, therefore, have been continuously screened, but without much recent success (Fernandes 2015). The immediate need for new antibacterial compounds may be supplied by ecologically diverse and little investigated natural sources (Butler & Buss 2006; Peláez 2006; Demain 2009; Moloney 2016; Balaban & Liu 2019).

1.2 GREEN MICROALGAE – AN OVERVIEW

Microalgae is a broad term used to describe the group of microscopic unicellular organisms commonly found in water, which includes many different phyla, such as *Heterokontophyta* (golden and brown algae)*, Glaucophyta, Euglenophyta, Cryptophyta, Haptophyta, Rhodophyta, Dinophyta, Chlorophyta, Cyanophyta* and *Prochlorophyta* (Parsaeimehr & Chen 2013). The *Chlorophyta* phylum is known as green algae for their visual green colour that is produced mainly due to photopigment chlorophyll *a* that acts as the main source of primary metabolism within the cells (Masojídek *et al*. 2013). Green microalgae are unicellular, hardy, eukaryotic microorganisms that are capable of surviving in a wide range of environments (Skjånes *et al*. 2013; Bashir *et al*. 2018). The first strain of microalgae cultured in the laboratory was *Chlorella* in 1919, and since then it has served as a model organism to not only to study green microalgae, but also for higher plants (Liu & Hu 2013; Skjånes *et al*. 2013).

The use of photosynthetic green microalgae is not new to industry, as for the last few decades it has been used in both human and animal food production, as well as for intense biofuel research (Skjånes et al. 2013; Shannon & Abu-Ghannam 2016; Seyed Hosseini et al. 2018; Laamanen & Scott 2020; Desjardins et al. 2020). The ability for algae to survive in a variety of conditions makes these organisms attractive for commercial growth, even under extreme environments, such as high and low temperatures (Lyon & Mock 2014), light intensities (Gim *et al.* 2014), pH (Milledge 2011), salinities (Milledge 2011), or nutrient depravation (Skjånes *et al.* 2013).

1.3 SECONDARY METABOLITES FROM GREEN MICROALGAE

Secondary metabolites are compounds produced for purposes other than primary metabolism, and are, therefore, not involved in growth or reproduction. They are usually associated with functions such as defence against predators, protection from harsh sunlight, competition with other microorganisms, and communication (Uma *et al*. 2011; Markou & Nerantzis 2013; Alassali *et al*. 2016). Unlike primary metabolism, secondary metabolism is not a well understood process, as it can be strain specific, or results only under specific conditions (Shimizu 1996). As it is believed that secondary metabolites are synthesized in order to promote survival during specific conditions the algal cells are exposed to, this suggests that an increase in production will occur under stressful environmental conditions (Leflaive & Ten-Hage 2007; Markou & Nerantzis 2013; Guihéneuf *et al*. 2016).

Secondary metabolites produced by green microalgae that have been discovered include fatty acids, carotenoids, glycolipids, phenolics, indole alkaloids, terpenes and other small bioactive compounds (Ördög *et al*. 2004; Alassali *et al*. 2016). These compounds can act as bioactive molecules, and have been found to have some antibacterial activity (Ghasemi *et al.* 2007; Senhorinho *et al.* 2018), as well as anticancer (Jayappriyan *et al*. 2013; Senhorinho *et al*. 2019), antiviral (Santoyo *et al*. 2012), antifungal (Ghasemi *et al*. 2007; Senhorinho *et al*. 2018) and antioxidant (Hemalatha *et al*. 2013; Markou & Nerantzis 2013).

Fatty acids are chains of hydrocarbon with a carboxylic acid functional group and were originally shown to have antibacterial activity in 1880 by Dr. Robert Koch, but have been seldom researched after the discovery of current antibiotics (Yoon *et al*. 2018). Fatty acids are able to destabilize via incorporation into the cell membranes of the target bacteria due to their

amphipathic nature, thus increasing membrane permeability and eventually causing cell lysis (Jung & Lee 2016; Le & Desbois 2017; Yoon *et al*. 2018). It is also believed that fatty acids can interfere with the electron transport chain through the binding of electron carriers and reducing overall oxygen intake of the cell, thereby decreasing energy production which can lead to cell death (Desbois & Smith 2010; Yoon *et al*. 2018).

Phenolic compounds contain a hydroxylated aromatic ring, and are typically found in extracts of true plants and fruit (Rempe *et al*. 2017). Phenolics have also been associated with changes in membrane permeability, but there have also been non-membrane targets associated with antibacterial activity, including genomic DNA, DNA gyrase, FabZ, protein kinases, FtsZ, which can indicate the ability for use in susceptible species of bacteria (Wu *et al*. 2016; Rempe *et al*. 2017; Wang *et al*. 2017). The alkaloid molecules have inspired some of the currently used antibacterial drugs, including quinolones, and provided scaffolds for linezolid or trimethoprim, however naturally occurring compounds can continue to provide a source for novel antibiotics (Cushnie *et al*. 2014). As alkaloids are a broad group of compounds, the activity varies based on the molecule of interest, however some mechanisms of action that have been proposed include the inhibition of nucleic acid synthesis by enzyme inhibition, inhibition of Z-ring formation therefore reducing cell division, and reduction of oxygen intake by bacteria (Cushnie *et al*. 2014). There has also been a study identifying pyruvate kinase as the target for alkaloids in *Staphylococcus aureus*, which causes a disruption of carbohydrate metabolism (Zoraghi *et al*. 2011).

As secondary metabolites may only be produced by specific strains in certain environments, it would seem logical to bioprospect stressed natural environments for green microalgae that may produce compounds of interest as part of their survival mechanisms (Challouf *et al*. 2012;

Mezzari *et al*. 2017; Senhorinho *et al*. 2018). Alternately, one can also alter the environment that the cells are grown in the laboratory, in order to induce stress and thereby encourage production of increased amounts of specific secondary metabolites needed to cope with the change in environmental conditions (Markou & Nerantzis 2013; Guihéneuf *et al*. 2016).

CHAPTER 2

HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

Strains of eukaryotic green microalgae that originate from extreme environmental conditions produce secondary metabolites that can enhance survival in these environments. These secondary metabolites may also represent a new source of novel and much needed compounds with antibacterial activity.

2.2 OBJECTIVES

The objectives of this study were to conduct a literature review of the potential of extremophilic green microalgae to produce antibacterial compounds and to then investigate the antibacterial activity of green microalgae bioprospected from extreme environmental conditions, in particular areas of low pH and wastewater.

CHAPTER 3:

HOW EXTREME ENVIRONMENTAL CONDITIONS INFLUENCE PRODUCTION OF ANTIBACTERIAL COMPOUNDS BY GREEN MICROALGAE: A REVIEW

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3.1 ABSTRACT

Increased proliferation of bacterial resistance to antibiotics is a critical issue that has increased the demand for novel antibacterial compounds. Antibacterial activities have been measured in extracts from photosynthetic green microalgae, with varying levels of subsequent potential for development based on the strain of algae, strain of bacterial pathogen, and solvent used to extract the metabolites. Green microalgae bioprospected from extreme environmental conditions have had to adapt to environments that exclude most other organisms, with the production of antibacterial compounds aiding directly or indirectly in the survival, and potentially with fortuitous antibacterial properties of compounds serving other primary roles in algal cells. This review investigates antibacterial activities of green microalgae from both extreme in-situ environmental conditions and induced extreme laboratory conditions and highlights.

KEYWORDS: Antibacterial activity, bioprospecting, environmental stress, extracts, green microalgae

3.2 INTRODUCTION

The discovery and synthesis of antibiotics has been one of the most successful developments in the field of human health care, with prevention and cure of various infections made available at low cost and easily accessible to most patients (Fernandes 2006). However, with the success of antibiotics has also come the wide-spread proliferation of bacterial resistance to some of the most commonly prescribed broad and narrow spectrum drugs (Livermore 2003). The incidence of patients with infections caused by resistant bacterial strains is rising and leading to increased untreatable infections and mortality rates (Luepke *et al.* 2017). The Centers for Disease Control and Prevention (CDC) released a report on antibiotic resistance in 2013 and an updated version in 2019, which showed an increase in the number of infections in the United States from 2.0 million to 2.8 million per year, and a rise in deaths from 23,000 to 35,000 per year between 2013-2019 (Luepke *et al*. 2017; CDC 2019). A report in 2018 estimated that 26% of bacterial infections were resistant to the first line of treatment, and that the biggest risk factor was previous treatment with antibiotics (CCA 2018). Changes in prescribing habits and regimens have attempted to prevent further antibiotic resistance to some drugs, but novel antibacterial agents are urgently needed as resistance to antibiotics is increasing (Livermore 2003; Fernandes & Martens 2017).

Since Fleming's seminal discovery of penicillin, different classes of antibiotics have been developed, with nine of the twelve currently marketed originating from natural sources (Coates *et al*. 2011; Parsaeimehr & Chen 2013). The most common natural sources have been fungi and filamentous bacteria isolated from common environments like soils, leading for example, to the discovery of cephalosporins and vancomycin respectively (Peláez 2006). However, research on antibiotics from bacteria and fungi now usually results in rediscovery of known compounds,

indicating a need to look at metabolites produced by microorganisms that have not been previously extensively investigated, and from environments that might select for unique metabolites (Pidot *et al*. 2014; Giddings & Newman 2015a).

One such group are non-marine photosynthetic unicellular microalgae that can be found in diverse habitats, ranging from glaciers to even hot springs, despite that thermophilic lifestyles are rare in eukaryotes (Thamilvanan *et al*. 2016). The term microalgae currently describes ten phyla (Parsaeimehr & Chen 2013) : *Heterokontophyta* (golden and brown algae)*, Glaucophyta, Euglenophyta* (euglenoids)*, Cryptophyta* (cryptophyte algae)*, Haptophyta, Rhodophyta* (red algae)*, Dinophyta* (dinoflagellates)*, Chlorophyta* (green algae)*, Cyanophyta* (cyanobacteria) and *Prochlorophyta.* The species of microalgae are classified based on colour largely due to the photopigments they possess for the purpose of photosynthesis. For example, *Cyanophyta* contain large amounts of phycocyanin which appears as a blue green colour, and *Rhodophyta* contain large amounts of phycoerythrin which appears as a red colour (Kannaujiya & Sinha 2016). The variation in microalgal metabolism based on habitat and the competitive nature of each environment has allowed for a wide variety of secondary metabolites to be produced (Cakmak *et al*. 2014). Currently eukaryotic green microalgae are being investigated for other commercially valuable compounds such as biofuels, nutraceuticals and pigments (Priyadarshani & Rath 2012; Panis & Carreon 2016; Randhir *et al*. 2020). This highlights their potential as sources of a wide variety of compounds, including potential antibacterial agents (Challouf *et al*. 2012; Cakmak *et al*. 2014; Senhorinho *et al*. 2015; Ruiz *et al*. 2016; Patel *et al*. 2019). As a consequence, there have been a growing number of studies focused on investigating antibacterial activity from green microalgae isolated from extreme environments with the hope to find unique antibacterial

compounds (Challouf *et al*. 2012; Giddings & Newman 2015a; Navarro *et al*. 2017; Senhorinho *et al*. 2018; Patel *et al*. 2019; Santhakumaran *et al*. 2020).

Green photosynthetic microalgae are eukaryotic organisms that rely on chlorophyll a and b found in chloroplasts to produce energy (Lee 2012). The appearance and structure of *Chlorophyta* is very variable, as are the environments in which they live. These include marine, terrestrial, and freshwater environments, and in extreme conditions such as hypersaline or within snow (Kvíderová *et al*. 2005; Andersen 2013; Giddings & Newman 2015a; Lauritano *et al*. 2016). Green microalgae are able to survive in extreme environments in which other eukaryotic planktonic algae cannot (Gimmler 2001a). Their cell processes require these microorganisms to maintain a constant intracellular environment by adapting to external changes, an adaptation that can cause an increase in energy consumption leading to a decrease in the photosynthetic metabolism (Gerloff-Elias *et al*. 2005). This decrease can result in an accumulation of intermediate compounds within the cells, which are acted upon by subsequent pathways to form secondary metabolites (Malik 1980).

Secondary metabolites are a diverse group of molecules that are not necessary for basic cell functioning and are known to exhibit other important biological properties including antibacterial activities (Lustigman 1988; Das & Pradhan 2010; Sasso *et al*. 2012; Jyotirmayee *et al*. 2014). In general, secondary metabolites seem to aid cells in their interactions with their environment, including signaling other organisms and protecting themselves against predators and competitors (Lustigman 1988; Leflaive & Ten-Hage 2007; Challouf *et al*. 2012; Senhorinho *et al*. 2018; Dantas *et al*. 2019; Patel *et al*. 2019).

Stress can, therefore, play an important role on microalgal production of antibacterial compounds. For example, strains of *Dunaliella salina* collected from waters contaminated with human sewage and industrial wastes produced more compounds displaying antibiotic activity than strains from low pollution areas (Lustigman 1988). Therefore, secondary metabolites have been a valuable source in the development of new pharmaceuticals, such as antibiotic, anti-inflammatory and anti-cancer drugs (Namdeo 2007). Compounds originated from microalgae exhibiting antibacterial activity include fatty acids, glycolipids, phenolics, terpenes, β-diketone and indole alkaloids (Ördög *et al*. 2004). However, most antibacterial activity is usually attributed to long chain unsaturated fatty acids (Trick *et al*. 1984; Borowitzka 1995; Plaza *et al*. 2012). The interest in microalgae as a source of antibiotics seems to have originated with the work of Pratt *et al.* (1944). They investigated *Chlorella,* a genus of freshwater green microalgae capable of producing chlorellin, an antibacterial compound able to inhibit the growth of both Gram-positive and Gram-negative bacteria.

As microalgae under stress- inducing conditions need to adapt in order to survive, they may produce unique compounds as a result of metabolic changes (Gerloff-Elias *et al*. 2005). Therefore, studies on the abilities of green microalgae sampled/enriched from extreme environments to exhibit antibacterial activity against human pathogens are highlighted in this review. This includes reviewing the range of extreme environments that harbour microalgae producing antibacterial compounds, the spectrum of purported antibacterial compounds, the specific environmental conditions that may impact compound formation, and how efficacious the compounds are.

Table 1: Studies documenting antibacterial activity from bioprospected green microalgae isolated from extreme environments.

Chlorophyta **– Green Microalgae**					
Environment	**Strain**	**Active against**	**Solvents**	**Minimum Inhibitory Concentration**	**Reference**
Mining impacted waters	*Coccomyxa onubensis*	*Escherichia coli, Salmonella enterica, Proteus mirabilis, Staphylococcus aureus*	Chloroform, hexane, dichloromethane	106-305 µg/mL	(Navarro *et al*. 2017)
	Chlamydomonas sp., *Coccomyxa* sp., *Scenedesmus* sp.	*Staphylococcus aureus, Bacillus subtilis*	Methanol	16-1024 µg/mL	(Senhorinho *et al*. 2018)
Hot Spring	*Cosmarium* sp.,	*Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermis, Salmonella typhimurium, Escherichia coli*	Methanol, hexane, acetone, water	28-85 µg/mL	(Challouf *et al*. 2012)
High Salinity	*Dunaliella primolecta*	*Staphylococcus aureus (*MRSA)	Methanol	10 µg/disc (pure compound)	(Ohta *et al*. 1995)
	Dunaliella salina	*Yersinia ruckeri, Lactococcus garvieae, Vibrio anguillarum, Vibrio alginolyticus, Yersinia enterocolitica, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa, Shigellla sonnei, Bacillus subtilis,*	Hexane, dichloromethane, methanol, ethanol	630-10000 µg/mL	(Cakmak *et al*. 2014)
	Dunaliella sp.	*Bacillus subtilis, Brochothrix thermosphacta, Escherichia coli, Staphylococcus aureus,*	Ethanol, methanol, hexane, chloroform, Tris-HCl, water	N/A	(Kilic *et al*. 2018)
	Dunaliella salina	*Streptococcus mutans*	Chloroform:methanol: acetone (2:1:1)	6250 µg/mL	(Jafari *et al*. 2018)
Wastewater	*Dunaliella salina*	*Escherichia coli, Pseudomonas aeruginosa, Pseudomonas vulgaris, Staphylococcus epidermis, Micrococcus luteus*	Water, acetone, ethyl acetate, propanol, butanol	N/A	(Lustigman 1988)
	Coelastrum sp., *Scenedesmus quadricauda, Selenastrum* sp. (Mixed culture)	*Pseudomonas fluorescens, Serratia marcescens, Staphylococcus epidermidis*	Methanol, hexane, ethanol	N/A	(Corona *et al.* 2017)
	Scenedesmus spp.	*Salmonella enterica*	N/A	N/A	(Mezzari *et al*. 2017)
Terrestrial, high light	*Chlorella vulgaris, Dunaliella salina,*	*Streptococcus suis, Pseudomonas aeruginosa, Escherichia coli, Shigella sonnei,*	Methanol:acetone: diethyl ether (5:2:1)	N/A	(Al-Wathnani 2012)

3.3 GREEN MICROALGAE GROWING IN STRESSED ENVIRONMENTS

Microalgae can be found in a wide range of stressed or extreme environments, such as fresh or salt water bodies with low pH and/or municipal wastewater contamination(Giddings & Newman 2015b). Whilst investigation of secondary metabolites from green microalgae has grown since the work of Pratt *et al*. (1944), these have been less explored, and only recently become the focus of attention (Mudimu *et al*. 2014; Taş *et al*. 2015; Alwathnani & Perveen 2017; Senhorinho *et al*. 2018). The environments classified as extreme are those with non-circumneutral pH, high temperatures, high dissolved solutes potentially including metals, organic-rich wastewater (e.g. from municipal waste effluents), and arid/desert environments, which have been summarized in Table 1. The microalgae found in these environments are currently underrepresented as cultured type strains, and often must be first collected and isolated through bioprospecting, which adds an additional level of difficulty in contrast to testing existing isolates established culture collections, which are more typically from mesophilic environments. Since novel strains from extreme environments have not been widely explored, this may increase the chances of finding species with unique metabolites and capabilities (Senhorinho *et al*. 2018).

3.3.1 MINING IMPACTED ENVIRONMENTS

3.3.1.1 LOW pH

Green microalgae obtained from mining-impacted water bodies with pH lower than 3 have been shown to produce antibacterial compounds capable of inhibiting the growth of both gram-negative and gram-positive bacteria (Navarro *et al*. 2017; Senhorinho *et al*. 2018). In order for green microalgae to survive in low pH waters they have to maintain a circumneutral intracellular pH (Gimmler 2001b; Gerloff-Elias *et al*. 2005). To achieve this, high amounts of H^+ ions need to be prevented from entering the cell, which can also restrict the access of other important ions that are necessary for survival, such as K^+ (Gimmler 2001a). Although not well understood, this increased ionic stress seems to influence the production of secondary metabolites by microorganisms (Malik 1980), which may indirectly be related to antibacterial activity.

Although studies investigating antibacterial activity of green microalgae induced at low pH have not yet identified specific compounds, crude extracts from these microorganisms have shown promising activity suggesting these extreme environments as potential sources of inocula be further investigated. According to Navarro *et al*. (2017), intracellular extracts from the acidophilic (pH 2.5-4.5) green microalga, *Coccomyxa onubensis* obtained using either hexane, diethyl ether, chloroform or dichloromethane, exhibited antibacterial activity, particularly against gram-negative bacteria. The green microalgae were grown at the original pH of 2.5 throughout the experiments and the results from minimum inhibitory concentration (MIC) assays ranged from 106-305 μg/mL. It was suggested that fatty acids seemed to be involved in the activity due to increased potency from extracts obtained with non-polar solvents compared to polar solvents. The presence of fatty acids was confirmed using gas chromatography (Navarro *et al*. 2017). As

no pure compounds were tested and the active compounds are likely to be found only in small concentrations within the cell, further fractionation to determine what compounds were responsible for the activity should also decrease the MIC, assuming synergistic effects of multiple compounds are not involved (Navarro *et al*. 2017).

3.3.1.2 HIGH IRON LEVELS

Mining impacted areas often have increased levels of metals in the soil and water surrounding them, including an increased level of iron, copper and nickel (Cummings *et al*. 2000). If microalgae grow in areas with increased metal concentrations higher than what is needed for growth the cells, then they may be impacted by oxidative stress (Hu 2013). It was found that carotenogenesis, the synthesis of carotenoids, such as bioactive β-carotene, could be increased in the presence of reactive oxygen species, which as shown in Figure 1 could be generated by an increase of Fe^{3+} as this can cause the creation reactive oxygen species through the Fenton reaction (Hu 2013). The carotenoids are secondary metabolites, and since iron impacts the biochemical synthesis of these secondary metabolites, it is possible that this change in metabolism could also provide a link to the production of antibacterial activity in green microalgae from mining contaminated environments (Hu 2013).

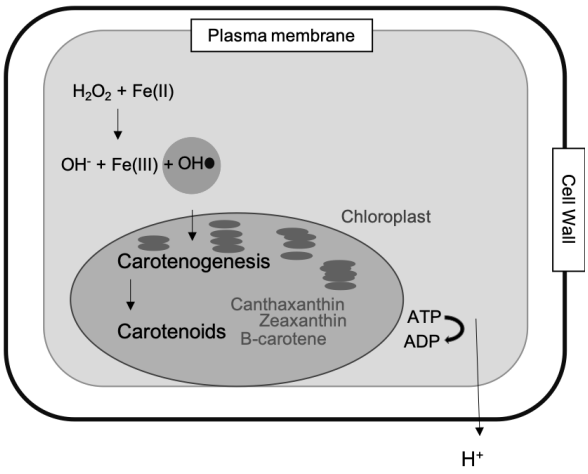

Figure 1: Mining impacted environments have high quantities of metals, including iron, as well as a commonly low pH due to acid mine drainage. To survive the cells need to be able to actively pump out excess protons, as well as degrade reactive oxygen species present due to the Fenton reaction that takes place with Fe(II), through increased carotenogenesis.

Antibacterial activity was observed from green microalgae bioprospected from water bodies (pH 2.9-8.4) near abandoned mines with high metal concentrations (Senhorinho *et al*. 2018). Levels of iron were up to 22 ppm, compared to water guidelines for protection of aquatic life that indicate the acceptable limits as less than 0.2 ppm (Hem 1972). Their results indicated that green microalgae, particularly *Chlamydomonas* spp., were active against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. The extracts obtained through the use of methanol exhibited some very low MIC values, such as 16 µg/mL against *S. aureus.*

3.3.2 EXTREME TEMPERATURES

Most commonly, high microalgal growth rates are associated with 20-30°C, but microalgae have been found in a wide range of temperatures, from Antarctic polar waters to geothermal springs reaching 70°C (Costas *et al*. 2008; Xin *et al*. 2011; Lyon & Mock 2014). The temperature for optimum growth rate and maximum production of antibacterial compounds by *Chlorella marina* was found to be 25°C compared to the other tested temperatures between 20-40°C (Elkomy *et al*. 2015). However, other studies investigating antibacterial activity from microalgae have shown that the optimum growth temperature does not necessarily equate to the highest production of antibacterial compounds, as the enhanced production of secondary metabolites can be independent of optimum level of growth (Kirrolia *et al*. 2012; Schuelter *et al*. 2019).

Geothermal springs typically have temperatures between 40°C and 70°C (Costas *et al*. 2008; Ghozzi *et al*. 2013), and the ability for microalgae to survive in this temperature range is thought due to spontaneous genetic mutation (Costas *et al*. 2008). In general, when temperature is increased green microalgae have been found to have an overall decrease in lipids, including polyunsaturated fatty acids, which have been associated with antibacterial activity (Hu 2013). It has been suggested that at temperatures above the optimal point, carotenogenesis may increase due to a temperature dependent enzymatic reaction, therefore, producing more carotenoids including ones different from those usually produced, as shown in Figure 2 (Hu 2013; Forján *et al*. 2015).

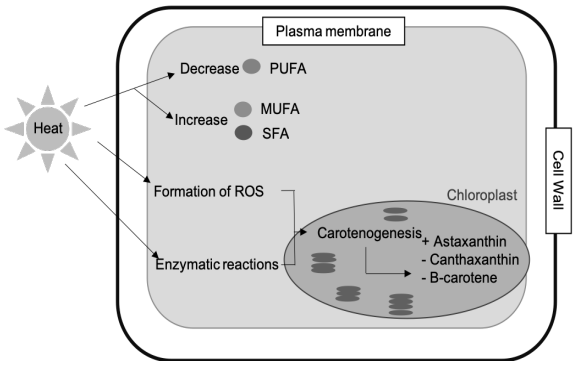

Figure 2: High temperatures create reactive oxygen species that can be mediated through carotenogenesis. There is also an overall decrease in polyunsaturated fatty acids (PUFA), as well as an increase in monounsaturated and saturated fatty acids (MUFA and SFA respectively), which changes the lipid ratio within the cell.

Cosmarium sp. collected from a hot spring (60°C) in Tunisia exhibited antibacterial activity against gram-positive and gram-negative bacteria (Challouf *et al*. 2012). Since solvents with different polarities (hexane, acetone, or water) were used to obtain the crude extracts, it is likely that different compounds exhibited antibacterial effects that resulted in the production of MIC below 100µg/mL for all susceptible bacteria (Challouf *et al*. 2012). Whereas, a previous study looking at *Cosmarium laeve* did not find significant antibacterial activity when the microalga was collected from a non-extreme river environment (Abdo *et al*. 2012). What is not known is the impact of different temperatures for the extremophilic *Cosmarium* sp. on antibacterial activity (Forján *et al*. 2015).

3.3.3 HYPERSALINE ENVIRONMENTS

Marine environments typically have a salinity around 3.5% (35000 mg/L), but there are inland lakes that have salinities reaching 35% (350000 mg/L), where some halophilic species of green microalgae such as *Dunaliella salina* have been found (Cakmak *et al*. 2014). Areas of high salinity has shown to result in increased lipid content in some strains of algae, for instance *Dunaliella* spp., and *Chlamydomonas nivalis,* whereas in others high salinity led to a reduced lipid content in species such as *B. braunii* (Hu 2013). Increases in salt concentration have been reported to increase the quantity of canthaxanthin and astaxanthin found in microalgal cells, due to the upregulation of the enzyme responsible for formation triggered by high salinity (Hu 2013). In studies investigating the effect of increasing salt concentrations (0.1-4.0M) on cell survival, the intercellular concentration of NaCl was found to be minimally impacted, indicating that pumps removing the Na^+ ions were active (Erdmann & Hagemann 2001). This would require increased photosynthesis to meet the energy demands and, therefore, impact metabolic changes that could potentially impact the production of secondary metabolites with antibacterial activity, as shown in Figure 3 (Erdmann & Hagemann 2001).

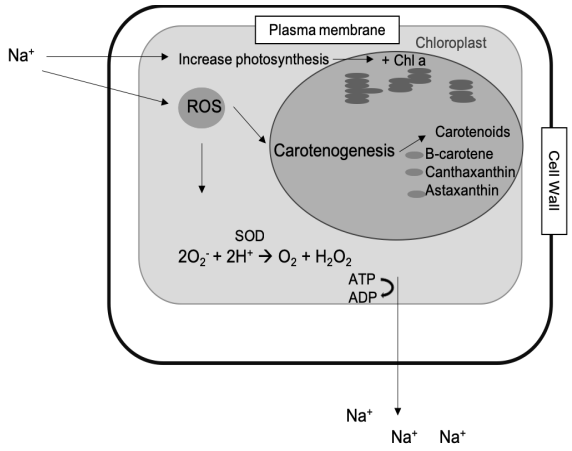

Figure 3: High salinity in the environment can cause an influx of Na^+ ions to enter the cell, which can cause a number of cellular changes within salt tolerant species. Photosynthesis has been found to increase in *Duniella* sp., as a result of the increased energy consumption to actively pump Na^+ out of the cell. The reactive oxygen species increase carotenogenesis, and other pathways such as the upregulation of superoxide dismutases in order to neutralize the free radicals.

Dunaliella is a genus of green microalgae that thrives in high saline environments, and is the only group of well-known eukaryotic photosynthetic organisms capable of surviving in 45% salinity (450000 mg/L) (Cakmak *et al*. 2014; Kilic *et al*. 2018). This genus of green algae is currently used in the production of the pigment ß-carotene for food colouring and for the antioxidant properties it possesses (Burton & Ingold 1984; Jin & Melis 2003). In high salinity, *Dunaliella* has been found to increase its production of certain secondary metabolites, such as ß-carotene, by up to 14%, which suggests an ability to produce metabolites with antibacterial activity (Jin & Melis 2003; Cakmak *et al*. 2014).

In all four studies listed in Table 1, *Dunaliella* species were cultured in a standard marine medium, except for Kilic *et al* (2018) where multiple salt concentrations were tested to determine the effect salinity would have on antibacterial production (Ohta *et al*. 1994; Cakmak *et*

al. 2014; Jafari *et al*. 2018). Increasing salinity from 10% to 20% was shown to increase antibacterial activity of the chloroform extract using a disc diffusion assay (Kilic *et al*. 2018). However, in another study where salinity was not maintained at a high concentration during growth, the extracts obtained from *Dunaliella salina* inhibited gram-positive and gram-negative bacteria only at relatively high concentrations (MIC 630-10000 μg/mL) (Cakmak *et al*. 2014; Jafari *et al*. 2018). These high values may be associated to either low concentrations of antibacterial compound(s) within the crude extract, or the culture medium, as previous reports on *Dunaliella* showed increased production of secondary metabolites in hypersaline environments (Jin & Melis 2003; Cakmak *et al*. 2014; Jafari *et al*. 2018). By looking at the antibacterial activity of unsaturated and saturated fatty acids from *Dunaliella primolecta* extracts, Ohta *et al* (1994) determined that *γ*-linolenic acid exhibited the most activity against *Staphylococcus aureus* (10 μg per disc).

3.3.4 WASTEWATER

Green microalgae have been used to mitigate contaminants from wastewater and excess nutrients that result from the disposal of human and animal waste (Zamora-Castro *et al*. 2008; Zhou *et al*. 2014). Algae that are naturally found in areas with high levels of industrial or human waste, or those that are added in order to further help with decontamination, are likely to have to cope with high levels of resource competition from other members of the microbial community . Studies have suggested that these contaminated environments could potentially encourage microalgal cells to produce antibacterial compounds (Zamora-Castro *et al*. 2008; Zhou *et al*. 2014; Mezzari *et al*. 2017).

Butanol extracts from *Dunaliella salina* bioprospected from waters that had been polluted with industrial waste and sewage demonstrated higher level of antibacterial activity compared to strains from uncontaminated waters (Lustigman 1988). The strains of bacteria that were susceptible to the extracts included *E. coli* and *Proteus vulgaris*, which are commonly found in fecal matter (Lustigman 1988). Rather than using extracts, whole cells of *Scenedesmus* spp., isolated from swine wastewater were able to eliminate *Salmonella enterica* growth within 48 hours (Mezzari *et al*. 2017). However it was unclear in this study whether the activity was due to secondary metabolites produced by the green microalgae, or the high pH 11 that was reached in the water during photosynthetic activity (Mezzari *et al*. 2017).

Polyunsaturated aldehydes from *Coelastrum* sp., *Scenedesmus quadricauda* and *Selenastrum* sp. obtained from a wastewater treatment plant inhibited the gram-negative bacterium *Serratia marcescens* in a disc diffusion assay (Corona *et al.* 2017). This study specifically targeted the extraction of polyunsaturated aldehydes, which are a product of the degradation of free polyunsaturated fatty acids after cell integrity is compromised (Ribalet *et al*. 2008). Polyunsaturated aldehydes have been shown to not only possess antibacterial activity, but also reduce the cell proliferation of human cancer cell lines, and reduce the growth of fungi (Ribalet *et al*. 2008). The cascade known to produce the polyunsaturated aldehydes is well documented in diatoms, but there are few studies specifically investigating this activity in green microalgae (Ribalet *et al*. 2007; Ribalet *et al*. 2008; Vidoudez & Pohnert 2008; Bartual *et al*. 2020).

3.3.5 TERRESTRIAL ENVIRONMENTS

Green microalgae are commonly found in aquatic habitats, but some species are also capable of surviving in terrestrial environments, and under extreme conditions (Rindi *et al*. 2011). Green microalgae, are commonly found in soil where they contribute to nutrient cycling and uptake of certain heavy metals, such as cadmium, zinc and copper (Yoshida *et al*. 2006; Bahar *et al*. 2013). Green microalgae living near the surface of soils, on rocks or other terrestrial environments need to mitigate the effects of UV-B radiation, which are especially high in desert environments. Using mycosporine-like amino acids (MAA), a group of secondary metabolites, as a protective mechanism, both aquatic and terrestrial green microalgae have been able to endure high amounts of UV-B radiation (Xiong *et al*. 1999). The type and quantity of MAAs present in a cell can differ based on the strain of microalgae and the environment in which they are found (Xiong *et al*. 1999; Wada *et al*. 2015). Desert environments increase the production of MAAs as a response to the increased light, and, therefore, may also induce metabolic changes associated with antibacterial activity, as shown in Figure 4 (Xiong *et al*. 1999; Wada *et al*. 2015).

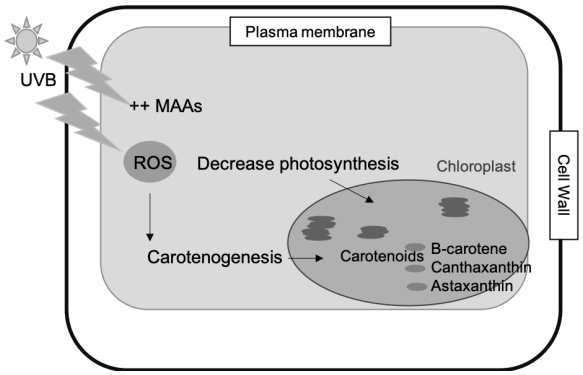

Figure 4: Terrestrial environments often have to survive under high amounts of UVB radiation, which can lead to an increase in mycosporine-like amino acids and an increase in carotenogenesis in order to absorb the radiation and mitigate any free radicals which may form. There can also be an overall decrease in photosynthesis during intense radiation.

There have not been many studies investigating green microalgae from extreme terrestrial environments. Al-Wathnani and Perveen (2017) did evaluate *Chlorella vulgaris* and *Dunaliella salina* from desert soils for antibacterial activity. The results indicated that extracts obtained with methanol:acetone:diethyl ether (5:3:1 volumes) were able to inhibit the growth of both gram-negative and gram-positive bacteria (Alwathnani & Perveen 2017). In an attempt to determine the active organic compounds mass spectrometry was performed, but without testing the active fractions through MIC tests, it cannot be definitively concluded that the most concentrated components were also the most active against the bacterial strains (Alwathnani & Perveen 2017).

3.4 MODIFIED CULTURE CONDITIONS

Studies on modifying the culture conditions of green microalgae have shown differences in antibacterial activity (Elkomy *et al*. 2015; Dineshkumar *et al*. 2017; Hamouda & Abou-El-Souod 2018). Similar to bacteria and fungi (Jain & Pundir 2011; Lo Grasso *et al*. 2016), it is suggested that by changing conditions to induce cell stress, microalgae may be stimulated to produce secondary metabolites with antibacterial activity, as well as potentially larger quantities of these secondary metabolites (Abedin & Taha 2008; Ruffell *et al*. 2016). Culture condition modifications include media composition, pH, light, and temperature.

The media typically used to grow green microalgae in the laboratory are either Bolds Basal Medium (BBM) or (Blue-Green Medium) BG-11, both of which contain generally the same nutrient compounds but at different concentrations, including N, P, K, Mg, Ca, S, Fe, Cu, Mn, and Zn. The main components that have been modified to stimulate production of antibacterial compounds are the main salts, including $MgSO_4$, $CaCl_2$, K_2HPO_4, NaCl, $NaNO_3$, and EDTA (ethylenediaminetetraacetic acid) (Ohta *et al*. 1995, Hamouda and Abou-El-Souod 2018). The required concentrations of these medium components, including the macronutrient salts, can be species or even strain specific (Grobbelaar 2013; Procházková *et al*. 2014). Each of the macronutrients plays a large role in primary metabolism, with for example, a decrease in nitrogen concentration causing a decrease in cell chlorophyll concentration, thereby altering colour and eventually the accumulation of oils (Grobbelaar 2013). By altering the primary metabolism of the green microalgae, it is possible for different secondary metabolites to be produced and hence a change in antibacterial activity.

Various concentrations of $MgSO_4$, $CaCl_2$, K_2HPO_4, NaCl, $NaNO_3$, and EDTA in microalgal medium were tested in order to determine which concentrations would stimulate higher antibacterial activity in green microalgae (Ohta *et al*. 1995). Each media component was tested individually, finding improved antibiotic production was noted when concentrations of $MgSO_4$ were increased from the 0.6 mM of standard medium to 12 mM, as well as increasing phosphate from 0.3 mM to 3 mM, and decreasing $CaCl_2$ from 30 μM to 3 μM (Ohta *et al*. 1995). Methanolic extract from green microalgae grown in the new media yielded higher anti-MRSA (methicillin resistant *Staphylococcus aureus*) activity, which suggested higher levels of antibiotic production. There was over a twofold increase of antibiotic production, but a very small increase in biomass, which indicated that the increase in antibiotic activity was not caused by higher quantities of algal cells alone (Ohta *et al*. 1995). The increase in antibiotic production allowed for purification and identification of substances responsible for the anti-MRSA activity, which were unsaturated fatty acids, with linolenic acid yielding the highest activity (Ohta *et al*. 1995).

Phosphate is considered growth limiting as it often precipitates and is unavailable for uptake by microalgae, but by increasing the concentration past the usable threshold it can induce stress via toxicity and, therefore, alter metabolism (Grobbelaar 2013). When sulphate is limited in the media, microalgal cells can reduce the rate of both photosynthesis and protein synthesis, and also affect the ratio of carotenoids to chlorophyll pigments within the cell (Procházková *et al*. 2014). The effect of changes in the concentration of phosphorus available to green microalgae on their antibacterial activity has been investigated (Hamouda & Abou-El-Souod, 2018). Phosphorus is pivotal to lipid accumulation and usually added to the medium in the form of K_2HPO_4. Different concentrations of phosphorus (0, 0.0035, 0.007, 0.01, and 0.014 g/L) were investigated and, with the highest growth rate of *S. obliquus* at 0.007 g/L, and the highest level of

activity of the microalgal methanolic extract against *S. aureus* at 0.01 g/L phosphorus (Hamouda & Abou-El-Souod 2018).

Untreated municipal wastewater, a nutrient rich medium, has been used instead to determine if an increase in algal biomass or antibacterial compound production is achieved (Dineshkumar *et al*., 2017). When *Chlorella vulgaris* was allowed to grow in either BBM medium or in wastewater, the biomass levels from wastewater were higher after 28 days (0.402 g/L) compared to BBM (0.268 g/L). Methanolic extracts from the cells grown in wastewater inhibited the growth of all the bacterial species tested (*K. pneumoniae, P. mirabilis, V. chlorerae, S. typhi, E. coli, S. aureus, B. subtilis, Enterococcus* sp., *C. botulini,* and *Nocardia* sp.) (Dineshkumar *et al*. 2017). However, unfortunately there was no comparative analysis on the activity of the microalgae grown in BBM versus the wastewater.

Of the culture conditions that have been explored, light has been the most investigated (Schuelter *et al*. 2019). The investigation of the effect of light on microalgal cells started when Pulich (1974) noticed that a strain of *Chlorella sorokiniana* was resistant to UV damage, even though other strains of the same species had a low survival rate (Pulich 1974). This suggested the potential for green microalgae to develop photoprotection in order to survive. Under low light conditions the amounts of chlorophyll *a*, *b* and *c*, and phycobiliproteins will increase in order to capture more light to maintain cellular functions. However, when light intensity is high the opposite is true, with light harvesting pigments being reduced and protective carotenoids being increased (Hu 2013). Under strong light intensities xanthophyll is also produced in order to dissipate the energy from chlorophyll a, as otherwise photosystem II can become inactivated, and needs to undergo repair (Fujita *et al*. 2001). Recently, the effect that changes in light has on

antibacterial agent production by green microalgae has been investigated (Elkomy *et al*. 2015; Kilic *et al*. 2018; Schuelter *et al*. 2019).

Chlorella marina under different light intensity (1000, 2000, and 3000 lux) exhibited different antibacterial activities, with cells producing more biomass under the highest light intensity (3000 lux) also being the most active at inhibiting bacterial growth (*S. aureus* and *Serratia marcescens*) (Elkomy *et al*. 2015). Extracts from *Dunaliella* sp. grown at 3600 lux yielded the highest activity against *E. coli* (a difference of 4mm in the disc diffusion assay), but when screened against *B. subtilis*, those grown at 2400 lux yielded the highest activity (a difference of 2mm in the disc diffusion assay) (Kilic *et al*. 2018). This study suggests, therefore, that the level of activity can vary between the intensity of light applied and species of bacteria screened. However, the study went onto find that in general the highest light intensity (4800 lux) yielded the overall highest level of antibacterial activity, suggesting that high light exposure is more likely to induce the production of antibacterial compounds by *Dunaliella* sp. (Kilic *et al*. 2018). Furthermore, as the chlorophyll content of the cells decreased with increasing light intensity, photoprotective compounds such as phenolics which have a role in antibacterial activity may have been produced (Kilic *et al*. 2018). The production of photoprotective compounds is necessary in order to enhance survival in the intense light conditions, as if chlorophyll *a* was able to increase light energy absorption, cellular damage would occur (Fujita *et al*. 2001). The necessary alteration in metabolism may, therefore, produce secondary metabolites which have a dual function of photoprotection and antibacterial activity (Hu 2013; Kilic *et al*. 2018).

Chlorophyll *a* absorbs light in two major areas within the visible spectrum, between 450-475 nm in the blue range and between 630-675 nm in the red range (Masojídek *et al*. 2013). In

order to optimize photosynthesis and microalgal growth, a combination of red and blue bands of light are commonly used to maximize the energy the cells are able to directly utilize through chlorophyll *a* (Baba *et al*. 2012). When white light is used to grow algae, it is exposed to all wavelengths in the visible spectrum and, however, not all of the light can be absorbed by chlorophyll *a*, and other photopigments are utilized to absorb the remaining light such are carotenoids (Masojídek *et al*. 2013). Therefore, rather than investigating light intensity, the study by Schuelter *et al* (2019) investigated the effect that a change in light source would have on antibacterial activity. By investigating the colour of light used to grow different isolates of green microalgae, it was theorized that different photoreceptors could be utilized compared to traditional blue and red bands known for aiding in photosynthesis (Schuelter *et al*. 2019). Using the green bands of light from light emitting diode (LED)- based bulbs to grow the samples yielded the highest level of antibacterial activity with inhibition halos greater than 20 mm, compared to red, blue or white LED or fluorescent light, which had inhibition halo means of 15 mm (Schuelter *et al*. 2019). Changes in green microalgae based on the colour of light have been noted to induce changes in the cell's interaction with the environment and cellular functions including reproductive process (Cepák & Přibyl 2006). The change in light could alter the photoreceptors that are responding, thus causing the formation of different pigments and a different metabolic pathway potentially resulting in the production of antibacterial compounds.

3.5 CONLCUSIONS AND FUTURE DIRECTIONS

Green microalgae from stressed environments are capable of producing compounds with antibacterial activity (Challouf *et al*. 2012; Najdenski *et al*. 2013; Senhorinho *et al*. 2018). Many studies bioprospected microalgae from extreme environments, but grew the cultures using standard media and laboratory conditions. The culture environment can be artificially manipulated in order to allow for an increased production of antibacterial agents (Ohta *et al*. 1995; Hamouda & Abou-El-Souod 2018). An interesting approach only utilized in the study by Navarro *et al* (2014), was to attempt to mimic aspects of the stressful environment while growing green microalgae in laboratory. This approach can result in a more realistic potential for antibacterial production and allow researchers to manipulate the culture conditions in order to stimulate microalgae to increase the production of active compounds.

A main challenge found in the studies investigating antibacterial activity of green microalgae is the lack of standardized protocols, which makes difficult the comparison to other studies and therefore a realistic evaluation of microalgal potential as producers of antibacterial compounds. While variances in protocols are to be expected, the determination of MIC by all studies would have increased the comparisons that could be made. This allows the concentration of the original extract to vary, but retains the ability to determine whether new compounds are in fact as active or even more active than current conventional antibiotics. Even in studies that do determine MIC, this is usually where the research is halted, with no further investigation into fractionation of the crude extract to determine the active compounds.

Further investigations should attempt to guide the medium and culture conditions that the bioprospected species of green microalgae were originally found, in order to preserve the

mechanisms that may be responsible for the production of secondary metabolites with antibacterial activity. Following the initial screening and determination of the minimum inhibitory concentration non-target toxicity studies should also be performed in order to confirm the possibility of the use of the secondary metabolites as a marketable problem in order to address the shortage of novel antibiotic pharmaceuticals.

Green microalgae have proven to be a potential new source of antibacterial agents, and the continued investigation on stressed environments may allow for new strains with such potential to be discovered.

ACKNOWLEDGEMENTS

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CHAPTER 4

ANTIBACTERIAL ACTIVITY OF GREEN MICROALGAL EXTRACTS FROM MUNICIPAL WASTEWATER AND LOW pH ENVIRONMENTS

4.1 ABSTRACT

Green microalgae have the capability to survive in a wide range of environments, including those considered extreme such as low pH and within municipal wastewaters. In order to survive in these environments green microalgae possess the capacity to synthesize protective secondary metabolites that may also exhibit antibacterial activity. In this study green microalgae from bodies of water with low pH and a municipal wastewater facility were isolated. The green microalgae genera were identified through genetic sequencing as *Scenedesmus* sp., *Chloroidium* sp., and *Chlorella* sp. Microalgal extracts from the isolated strains were then tested against eight bacterial strains, including gram-positive and gram-negative. The results showed antibacterial activity against six of the bacteria tested which were gram-positive *B. subtilis, B. cereus,* and gram-negative *S. sonnei, P. vulgaris, P. aeruginosa,* and *E. coli.* Differences in antibacterial activity were observed between the solvents used for extraction, hexane and methanol. Green microalgae from stressed environments seem, therefore, to have great potential as a source of compounds with antibacterial activity.

KEYWORDS

Green microalgae, stressed environments, extracts, antibacterial activity

4.2 INTRODUCTION

The discovery of antibiotics, beginning with penicillin in the 1920's, revolutionized the medical practice and continue to serve as a basis for modern medicine (Dcosta *et al*. 2011; Blair *et al*. 2015). Antibiotics have allowed for an increased life expectancy and decreased child mortality in nations that have access to these lifesaving drugs (Martínez 2008). However, as successful as antibiotics have been in the field of human health, the increased overuse and misuse of antibacterial drugs has led to the increase in the presence of antibiotic resistance genes in bacteria commonly found causing infections (Davies & Davies 2010). Pathogens have evolved to become multidrug-resistant (MDR), which became an urgent problem within hospital settings, where often there are very few or even no classes of antibiotics that are effective in treating the infections (Davies & Davies 2010).

Natural products have been extensively utilized in drug discovery throughout the era of modern medicine (Harvey 2008). Although the synthetic production of drugs has become highly common in drug discovery, it is estimated that up to 50% of modern medicines are either natural products themselves or have been derived from a natural product (Newman & Cragg 2007; Harvey 2008).

The worldwide need for new antibacterial drugs is increasing and it is associated to the inability of previous natural sources to produce novel molecules with antibacterial activity. This highlights the urgency to find new natural compounds from relatively unexplored sources (Clardy *et al*. 2006; Peláez 2006; Moloney 2016). To this end, studies have suggested the potential of eukaryotic green microalgae as antibiotic producers (Catarina Guedes *et al*. 2011;

Navarro *et al*. 2017; Dantas *et al*. 2019). However, based on these microorganisms' diversity, they have been little investigated for their potential as new pharmacological targets

In many cultures, microalgae have been utilized in traditional medicine for the purpose of antibacterial treatments, however in most cases the compounds produced are not known and the mechanisms by which these compounds are produced are unclear (Thamilvanan *et al*. 2016). The investigations into antibacterial activity by eukaryotic green microalgae began in 1944, with a study by Pratt and collaborators that showed *Chlorella vulgaris* and *Chlorella pyrenoidosa* produced antibacterial substances that inhibited gram-positive and gram-negative bacteria (Pratt *et al*. 1944). Although research into green microalgae has been ongoing since the 1940's, there is still a considerable gap in knowledge about the compounds involved in the activity as well as the species that have higher potential as antibiotic producers. For instance, it is estimated that only 25-30% of species have been collected and cultured, meaning that there is still much to be discovered in terms of green microalgae and their antibacterial activity (Bhattacharjee 2016). Of the studies that have been conducted on green microalgae, there has been a reliance on strains originating from culture banks rather than on bioprospecting novel strains (Abedin & Taha 2008; Uma *et al*. 2011; Thamilvanan *et al*. 2016; Dantas *et al*. 2019).

A little explored area of study for antibacterial activity from green microalgae is the potential of species originated from stressed environments. Green microalgae are capable of surviving in a wide range of environments, including, but not limited to, fresh water, marine, hot springs, soil, highly acidic environments and wastewater (Gerloff-Elias *et al*. 2005; Zamora-Castro *et al*. 2008; Lee 2012; Corona *et al*. 2017; Senhorinho *et al*. 2018). It is known that microalgae thriving in these stressed environments produce distinct secondary metabolites that allow for tolerance, thereby increasing organism survival (Malik 1980; Gerloff-Elias *et al*. 2005).

It is possible, therefore, that microalgae surviving in stressed environmental conditions will express secondary metabolites that also exhibit antibacterial activity (Malik 1980; Lustigman 1988; Ördög *et al*. 2004). In previous studies investigating both bioprospected strains and culture collection green microalgae, it has been noted that the strains originating from stressed environments have demonstrated more antibacterial activity than those from the culture collections (Senhorinho et al. 2018).

Studies have shown that different species of green microalgae obtained from wastewater are able to inhibit the growth of pathogenic bacteria (Mezzari *et al*. 2017; Corona *et al*. 2017). In order to grow and thrive in wastewater, green microalgae must be able to survive under competitive conditions that may be found in environments contaminated with high concentrations of organic or inorganic compounds (Zamora-Castro *et al*. 2008; Zhou *et al*. 2014). Similarly, green microalgae from environments affected by acid mine drainage, where stress from pH can be as low as 2.5 and high metal concentrations (Fe of 22 ppm compared to safe drinking water with concentration of 0.2 ppm (Hem 1972)) can occur, are also suggested as a promising source of antibiotics (Navarro *et al*. 2017; Senhorinho *et al*. 2018). The microalgae that survive in these acidic and potentially toxic environments may undergo changes in metabolic responses, altering the production of secondary metabolites which may lead to the production of compounds with antibacterial activity (Garbayo *et al*. 2012). It has been found that some species of green microalgae from acidophilic environments have important alteration in their genomes, such as expression of detoxification genes, in order to survive the harsh conditions (Hirooka *et al*. 2017). It is likely that genetic changes may also simultaneously promote the production of other distinct metabolites with antibacterial activity (Hirooka et al. 2017).

The aim of this study was to screen green microalgae bioprospected from acidic freshwater near abandoned and operational mine sites, as well as from tanks in a municipal wastewater treatment plant. It was hypothesized that green microalgae originated from these areas would produce unique secondary metabolites capable of inhibiting the growth of pathogenic bacteria and that may represent a new source of new antibiotics.

4.2 MATERIALS AND METHODS

4.3.1 SAMPLING

Water samples were obtained from organic contaminant removal tanks at the main Sudbury wastewater treatment plant facility, and water bodies near abandoned mine sites in Ontario that exhibited low pH (3.0-4.7). Two hundred milliliters of water samples were collected in 300 mL sterile Nalgene bottles and transported to the laboratory on ice. In order to stimulate the growth of green microalgae 20mL of Bold's basal medium was added to the water samples to increase nutrients. The bottles were then left at room temperature (21± 2°C) for two months under 12:12 hour light:dark cycle using fluorescent light (70-80 μmol photon $m^{-2}s^{-1}$).

4.3.2 ISOLATION OF GREEN MICROALGAE

When green microalgae flourished in the bottles, 20 µL was transferred and streaked onto Bold's basal medium agar plates and left under a 12:12 hour light:dark cycle using fluorescent light (70-80 µmol photon $m^{-2}s^{-1}$) for two weeks. Repeated streaking of green algal colonies was done until monocultures were obtained. The cultures were tested for bacterial and fungal contamination by streaking green microalgae colonies onto nutrient agar (BD Bacto, USA) and Sabouraud agar (BD Difco, USA), and incubating the plates at 37°C for 24 hours, and at room temperature for 7 days, respectively. Morphological identification at the generic level was performed using an inverted light microscope (Optika B290 series, Italy) as per (Shubert 2003; Bellinger & Sigee 2010)).

4.3.3 GREEN MICROALGAE IDENTIFICAITON – DNA EXTRACTION AND SEQUENCING

A procedure from Fawley and Fawley (2004) was followed to lyse the microalgal cells (Fawley & Fawley 2004). A sterile loop was used to transfer microalgal biomass from a BBM agar plate to a 2 mL conical microcentrifuge tube containing 200 µL of milliQ water. The tube was centrifuged at 16000 g for 1 minute. The supernatant was then removed, leaving the pellet in the tube which was resuspended in 200 µL of the extraction buffer containing 70 mM Tris-hydrochloride (Tris-HCl), 30 mM ethylenediaminetetraacetic acid (EDTA), and 1 M sodium chloride, at a pH of 8.6, and then vortexed until thoroughly mixed. Acid washed glass beads (Sigma G8772-10G) were added into the conical microcentrifuge tube, which was placed into a bead-beater (Biospec Mini-BeadBeater) for four 30 second cycles. Between cycles the samples

were placed on ice. The tubes were then centrifuged for 10 minutes at 4000 g and the supernatant used for DNA extraction.

In order to extract the DNA, a DNeasy Plant Pro Kit (Qiagen Montreal, Quebec, Canada) was used. The 18S region of the rDNA was amplified according to Aslam *et al.,* (2017) using 18S Forward (5'GCGGTAATTCCAGCTCCA-ATAGC-3') and 18S Reverse (5'-GACCATACTCCCCCCGGA-ACC-3') (Thermo Fisher Scientific)(Aslam *et al*. 2017). Using Phire Green Hot Start II PCR Master Mix (Thermo Scientific), the mixtures were placed into a Thermal Cycler (Bioer LifePro) with PCR conditions adopted from Mahmoud and Kalendar (2016) (Mahmoud & Kalendar 2016). The 18S amplification was initiated by DNA denaturing at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 5 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 10 seconds and the final extension at 72°C for 10 minutes before holding at 4°C.

The PCR products were confirmed using agarose gel (1.2%) electrophoresis for 2.5 hours at 70 volts. The PCR product was purified using a QUIquick PCR Purification Kit (Qiagen, Montreal, Quebec, Canada). The DNA quality was confirmed using a Synergy HT spectrophotometer (Biotek), with a Take3 microvolume adaptor and Gen5 V 2.04 software. The final product was sent to Genome Quebec in Montreal, Canada, for Sanger sequencing.

4.3.4 EXTRACT PREPARATION

Each strain of green microalgae was cultivated on three plates of BBM agar for 10 days at 21± 2°C under a 12:12 hour light:dark cycle (70-80 µmol photon $m^{-2}s^{-1}$). The total growth of microalgae from the agar plates were transferred to 1000 mL flasks with 500 mL of BBM, and left to grow for 15 days at 21± 2°C on a gyratory table orbiting at 125 rpm and under a 12:12 hour light:dark cycle (70-80 µmol photon $m^{-2}s^{-1}$). Cells were subsequently harvested by centrifuging at 2000 g for 15 minutes, and the pellet frozen at -80°C and finally freeze dried. Hexane and methanol were consecutively added to the same dry biomass at a concentration of 0.08 mL/mg biomass. The tubes were agitated for 24 hours at room temperature and the liquid removed to leave the cellular pellet. This procedure was repeated three times with hexane, followed by three more cycles using methanol as the solvent. The supernatants were combined and filtered using a 0.2µm sterile filter (Fisher Scientific, Canada). The tubes containing the supernatant were placed under vacuum until the solvent had evaporated. The extracts were then weighed and 100% DMSO (dimethyl sulfoxide) added to create a concentration of 50 mg/mL for each extract. Three technical replicates were obtained for each solvent used.

4.3.5 PRELIMINARY ANTIBACTERIAL SCREENING

The bacterial strains used in agar diffusion screening were gram-positive *Bacillus cereus* ATCC14579*, Bacillus subtilis* ATCC6051*,* and *Staphylococcus aureus* ATCC25923*;* and gram-negative *Shigella sonnei* ATCC9290*, Pseudomonas aeruginosa* ATCC10145*, Salmonella enterica* ATCC 23564*, Proteus vulgaris* ATCC 33420*,* and *Escherichia coli* ATCC11303. The bacteria were cultured using nutrient broth agar (BD Bacto, USA) and incubated for 24 hours at 37°C. After adjusting the bacterial turbidity to 0.5 McFarland scale, the strains were plated onto

Müller-Hinton agar plates (Oxoid) using sterile cotton swabs. For the screening, 2 μL of each extract was added in triplicate on top of the streaked bacteria, and incubated for 18 hours at 37°C. The negative control used was DMSO (2 μL), and positive controls were antibiotic discs containing 10 μg of ampicillin or streptomycin (Becton, Dickinson and Company, USA).

4.4 RESULTS

Four strains of green microalgae were screened against bacteria. Two of them (WW1-1 and WW1-2) were from the same tank at a municipal wastewater treatment plant in Sudbury, Ontario, Canada. The tanks these samples originated from were at pH of 4.0. Another sample (LL2A) was collected in an area close in proximity to an abandoned gold mine, again in Sudbury, Ontario, Canada from a water body of pH 4.7. The final sample (CC) was taken from Copper Cliff, Ontario, Canada, from a body of water located on an active mining operation, and with pH of 3.0.

4.4.1 DNA SEQUENCING

Sanger sequencing was used to determine the identity of each algal strain through analysis via BLAST using the NCBI GenBank. The closest BLAST match for LL2A was confirmed with 18S analysis to be *Scenedesmus* sp.. The closest BLAST match for CC was confirmed with 18S analysis to be *Chloroidium* sp.. The closest BLAST match for both WW1-1 and WW1-2, were confirmed with 18S analysis, to be *Chlorella* sp.. A morphological identification of each species was also performed and it matched the genetic identification for each genus (Figure 5).

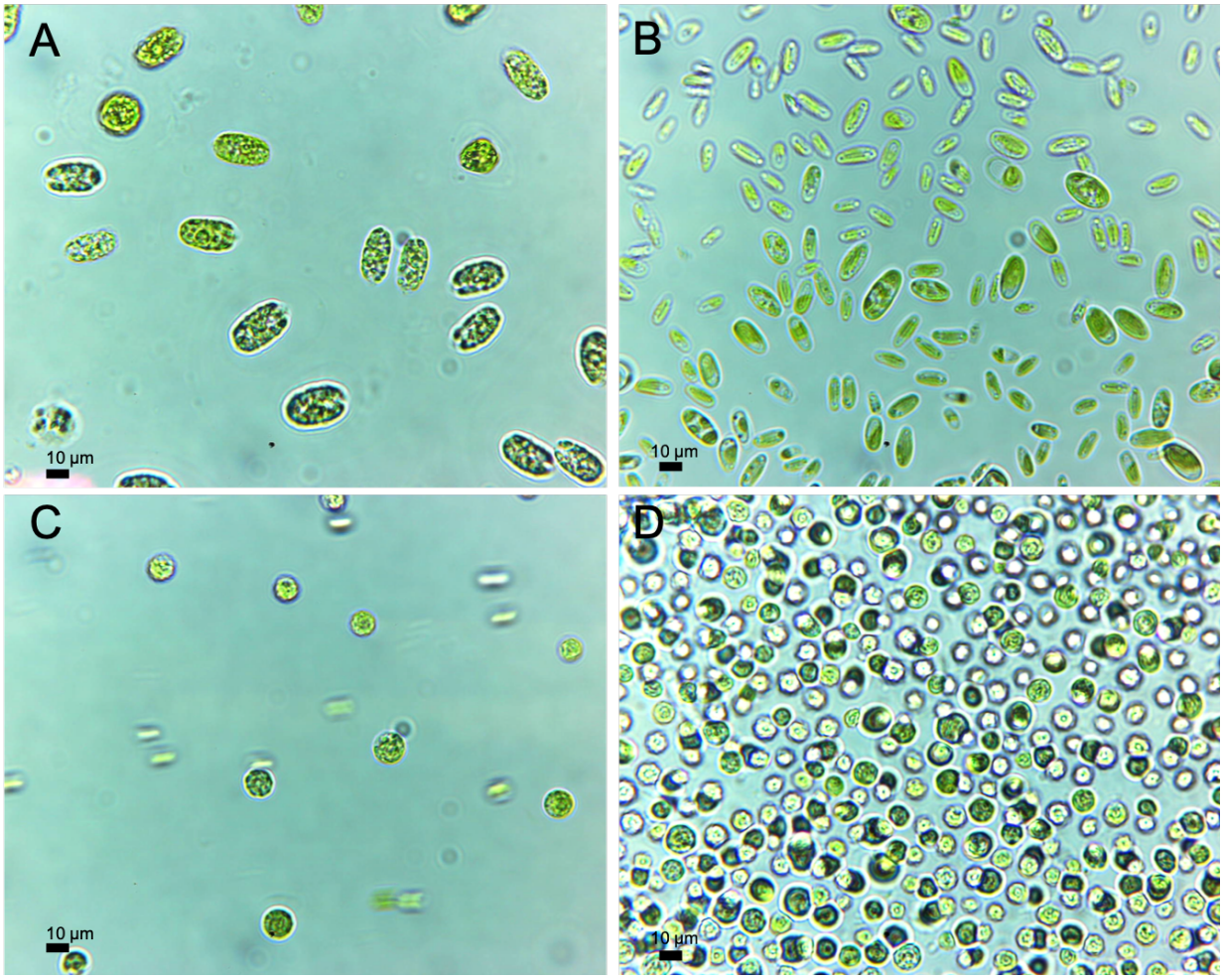

Figure 5: Micrography of each species at 1000x magnification. A: LL2A, B: CC, C: WW1-1, D: WW1-2.

4.4.2 ANTIBACTERIAL SCREENING ASSAY

The algal extracts were screened against eight strains of bacteria to determine their effectiveness. A scale was used to determine the effectiveness of the extract in preventing the growth of bacteria on the Mueller Hinton agar plates, with N/A meaning no inhibition, + little inhibition, ++ less than the total area inhibited, +++ the total area inhibited, and ++++ inhibition beyond the total area. Figure 6 displays screening results based on the inhibition scale used on this study.

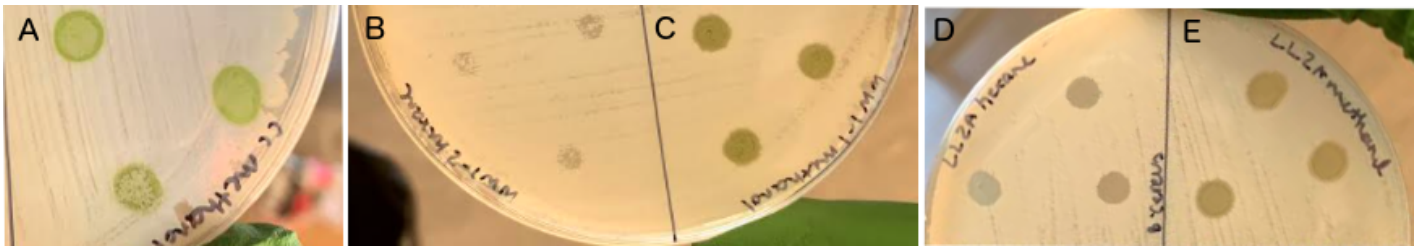

Figure 6: Scale of activity for antibacterial screening. **A:** CC methanol extract on *S. enterica* -N/A, **B:** WW1-1 hexane extract on *B. cereus* - +, **C:** WW1-1 methanol extract on *B. cereus* - ++, **D:** LL2A hexane extract on *B. cereus* - +++, **E.** LL2A methanol extract on *B. cereus* - ++++.

DMSO was used as a negative control and did not show any growth inhibition (data not shown). Streptomycin showed activity against all bacterial strains and ampicillin showed activity against all strains except for *P. aeruginosa*, which is expected due to the intrinsically resistant nature of the bacterium (Poole 2011).

All the bacterial strains showed some degree of susceptibility to the algal extracts, except *S. aureus* and *S. enterica* (Table 2). Notable extracts included the LL2A hexane extract, which displayed inhibition against six out of the eight bacterial strains, and the highest level of inhibition against *B. cereus*; and the LL2A methanolic extract, which showed activity against

five out of eight bacterial strains, with the highest level of activity against *B. cereus.* The CC hexane extract displayed inhibition against six out of eight bacterial strains, showing activity against both gram-negative *P. vulgaris, P. aeruginosa, S. sonnei,* and *E. coli* and gram-positive *B. cereus* and *B. subtilis*.

Table 2: Antibacterial screening with methanol and hexane algal extracts. No inhibition was found for *S. enterica* and *S. aureus*. All strains were screened for 18 hours with triplicate 2 µL drops on seeded Mueller Hinton agar. N/A: no inhibition, +: very little inhibition, ++: less than total area, +++: total area, ++++: beyond total area

	Gram-Negative				Gram-Positive	
	P. vulgaris	*P. aeruginosa*	*S. sonnei*	*E. coli*	*B. cereus*	*B. subtilis*
Methanol Extracts						
LL2A	++	N/A	++	+++	++++	++
CC	N/A	N/A	++	+	+	+
WW1-1	++	++	++	++	++	++
WW1-2	++	++	+	++	++	++
Hexane Extracts						
LL2A	+	+	+++	+++	+++	+++
CC	++	+	+++	++	+++	+
WW1-1	N/A	+++	+	++	+	+
WW1-2	N/A	+++	+	++	+	+
Controls						
DMSO	N/A	N/A	N/A	N/A	N/A	N/A

Ampicillin (disc)	++++	N/A	++++	++++	++++	++++
Streptomycin (disc)	++++	++++	++++	++++	++++	++++

4.5 DISCUSSION

Green microalgae are capable of adapting and surviving in a wide range of extreme environments (Giddings & Newman 2015a). Previous screenings of green microalgae from low pH environments have suggested these organisms as potential sources of antibacterial compounds (Navarro *et al*. 2017; Senhorinho *et al*. 2018), and, therefore, in this study two strains from mining associated water bodies of pH of 4.7 and 3.0 were investigated. Microalgal strains surviving in low pH need to undergo metabolic changes in order to maintain a neutral intracellular pH, such as by decreasing cell permeability to H^+ and preventing other cations such as K^+ from entering the cell (Gimmler 2001b; Gerloff-Elias *et al*. 2005).

Microalgae obtained from a wastewater treatment facility were also looked at due to their ability to survive at both low pH and to be exposed to a high level of competition from other microorganisms in their environment (Zamora-Castro *et al*. 2008; Zhou *et al.* 2014; Mezzari *et al.* 2017).

The Sanger sequencing performed in this study allowed for each microalgal genus to be determined using BLAST (Basic Local Alignment Search Tool) analysis. Both WW1-1 and WW1-2 were found to be from the genus *Chlorella.* *Chlorella* have been extensively found in

regional lakes, known to survive under low pH conditions (Findlay & Kasian 1986; Huss et al. 2002; Germond *et al.* 2014) and have been successfully cultured in wastewater (Wang et al. 2014). The sequencing data has also been confirmed through the cell morphology (Figure 5), which displays the similar appearance of both samples of a coccoid shaped green microalgae (Luo *et al*. 2005).

Studies have been conducted using various genera and species of green microalgae for the purpose of bioremediation of either municipal or agricultural wastewater, often with a secondary goal of biomass production for animal feed or biodiesel production (Rawat *et al*. 2011; Sacristán de Alva *et al*. 2013; Zhou *et al*. 2014; Mezzari *et al*. 2017). In a previous study the growth of *Scenedesmus* spp., common freshwater green microalgae, in wastewater was shown to increase the production of secondary metabolites such as chlorophyll a, astaxanthin, and lutein between 2.1-30 fold (Kim *et al*. 2007). Furthermore, *Scenedesmus* spp. cells were shown to completely remove a multi drug resistant *Salmonella enterica* present in wastewater within 48 hours, however, since extracts were not used in this study it is unclear what was responsible for the decrease in *S. enterica* in this experiment (Mezzari *et al*. 2017).

This study investigated the ability for green microalgae originally found in a wastewater facility to produce secondary metabolites capable of inhibiting the growth of both gram-positive and gram-negative bacteria. Extracts from the wastewater algae samples were subsequently found to inhibit the growth of the gram-negative strains, *P. vulgaris, P. aeruginosa, S. sonnei* and *E. coli,* with *P. vulgaris* being resistant to the hexane extracts of both microalgae samples, and *S. enterica* resistant to both methanol and hexane extracts. Of the three species of gram-positive bacteria screened, two were found to be susceptible to both methanol and hexane extracts. The results are very promising compared to the study on the extract from a community

of *Coelastrum* sp., *Scenedesmus quadricauda* and *Selenastrum* sp. (Corona *et al*. 2017) Their extact exhibited antibacterial activity against only one of the screened bacterial species (the gram-positive *Serratia marcescens)* and showed no inhibition against gram-positive *Staphylococcus epidermidis,* and gram-negative *Pseudomonas fluorescens*.

The two other strains of green microalgae were isolated from low pH water found close in proximity to an abandoned and an active mine. The antibacterial activity of both samples varied based on the solvent used, which indicates a difference in the compounds producing the activity. A solvent such as hexane is more likely to isolate fatty acids and other non-polar compounds from microalgae and thus bacteria more susceptible to these compounds would have a higher rate of inhibition. In a previous study acidophilic green microalgae were screened against a range of bacteria and were extracted using many different solvents (Navarro *et al*. 2017). Their results showed variable levels of bacterial inhibition, but overall non-polar solvents such as hexane, diethyl ether, and chloroform were more effective (Navarro *et al*. 2017). However, in the current study, when extracts were tested against *B. cereus*, the methanol extract of LL2A was more active compared to the hexane extract, due to the different polarities of the bioactive molecules extracted with each solvent.

In the study by Senhorinho *et al* (2018), strains of green microalgae were similarly isolated from low pH environments and mining impacted sites (Senhorinho *et al*. 2018). Interestingly, the results in each study varied greatly in relation to which group of bacteria were susceptible to the microalgae extracts. In this study the extracts were highly active against the gram-positive *S. aureus*, and showed no activity against the gram-negative bacteria *E. coli* or *P. vulgaris* (Senhorinho *et al*. 2018). The opposite results were found in the current study with no inhibition against *S. aureus* and growth inhibition of both *E. coli* and *P. vulgaris*, even though

the bacterial strains tested were the same. The differences in results would seem to indicate that the microalgae screened produce different metabolites, which could be due to the difference in algal strains, or the differences in the original environments they were bioprospected from.

4.6 CONCLUSION

The results of this study suggested a great potential of green microalgae to produce antibacterial compounds that are active against a variety of pathogenic bacteria. All the green microalgae used in this study were bioprospected from high stress environments, including those impacted by mining operations resulting in low pH, and the highly competitive municipal wastewater. As the strains used were able to adapt to unfavorable environments, they are likely to have developed specific mechanisms to promote survival. This suggests the production of secondary metabolites with antibacterial activity as a result. Further research should be conducted to determine the chemical composition of the active extracts, and to then test specific components for antibacterial activity.

CHAPTER 5: CONCLUSIONS

Antibiotic resistance has become an increasingly dangerous worldwide threat to the lives of people. Antibiotic discovery has, however, experienced a fall in the 21st century, with the result that multidrug resistant infections are expected to increase (Fernandes 2006). To avoid the potential of entering the pre-antibiotic era, there is urgent need for research that looks for novel compounds, particularly from little explored natural sources. Most of the currently marketed antibiotics originated from fungi or bacteria, however, compounds from photosynthetic green microalgae have been seldom investigated and identified.

Green microalgae are capable of surviving in a wide range of environments, including those that are considered extreme (Gimmler 2001a). In order to survive in these extreme environments microalgae need to have survival mechanisms, often through the use of secondary metabolism and proteins related to stress response(Malik 1980; Baviskar & Khandelwal 2015; Senhorinho *et al*. 2015; Corona *et al*. 2017).

A limited number of previous studies have shown promising antibacterial activity against human pathogens by bioprospected microalgal strains from extreme environments (Corona *et al*. 2017; Navarro *et al*. 2017; Senhorinho *et al*. 2018).This potential was confirmed with the strains used in this study, which were bioprospected from wastewater facilities and water bodies displaying low pH. All four strains tested exhibited activity against both gram-positive and gram-negative bacteria. As the intracellular compounds were extracted and antibacterial activity observed from both, hexane and methanolic fractions, it is likely that the activity is not originating from fatty acids alone, which is the compound most commonly previously credited

for the antibacterial activity from microalgae (Desbois & Smith 2010; Catarina Guedes *et al*. 2011).

Looking at natural sources, including green microalgae from extreme environments, offers a new pipeline of potentially novel compounds to be discovered and investigated. Further studies including the fractionation of extracts, and subsequent identification and purification of compounds will aid in the understanding of the roles that specific compounds can play, and there future as potential antibiotic drugs.

CHAPTER 6: FUTURE EXPERIMENTS

As the bioprospected microalgae exhibit significant antibacterial activity, further research is warranted. It is recommended that the first steps for the next stage are determining minimum inhibitory concentrations (MICs) and fractionation of the extracts to elucidate the active components.

Minimum Inhibitory Concentration (MIC)

As a follow-on to this work, the minimum inhibitory concentrations (MICs) of extracts that inhibit bacteria should be determined. For example, using 96 well plates, microalgal extracts would be prepared in a two-fold dilution (0.5μg/mL to 1020 μg/mL) in Müller-Hinton broth and added into sterile wells. Each plate will include six negative control wells of bacteria and broth only, and bacteria in 2% DMSO. Six wells containing 2% DMSO in Müller-Hinton broth would be used as a blank, and six wells containing broth, bacteria and ampicillin (and an antibiotic active against Pseudomonas) would be used as positive controls. The plates will be incubated at 37°C for 18 hours and the MIC will be defined as the minimum concentration of either extract or ampicillin (and the other antibiotic) that inhibit bacterial growth. Three biological replicates will be tested.

Fractionation of Extracts

It is also important to determine what specific fraction of the extract is responsible for antibacterial activity. This could be carried out using UPLC-based hydrophilic interaction liquid chromatography (HILIC) (Paglia et al. 2012; Paglia et al. 2014). The acidic conditions utilize a BEH Amide column (2.1 x 150 mm, 1.7µm), with mobile phase A (acetonitrile + 0.1% formic acid), and mobile phase B (H_2O + 0.1% formic acid) and an injected sample volume of 3.5µL. The basic conditions utilize a BEH Amide column (2.1 x 150 mm, 1.7µm), with mobile phase A (acetonitrile 95% -ammonium bicarbonate 10mM 5% (pH9)), and mobile phase B (acetonitrile 5% -ammonium bicarbonate 10mM 95% (pH 9)) and an injected sample volume of 3.5µL. The elution gradient will be as follows for acidic conditions: 0 min, 99% A, 1% B; 0.1 min, 99% A, 1% B; 7 min, 30% A, 70% B; 7.1 min, 99% A, 1% B; and 10 min, 99% A, 1% B, with a flow rate of 0.4mL/min. The elution gradient will be as follows for basic conditions: 0 min, 99% A, 1% B; 0.1 min, 99% A, 1% B; 6 min, 30% A, 70% B; 6.5 min, 99% A, 1% B; and 10 min, 99% A, 1% B, with a flow rate of 0.4mL/min.

Following fractionation, the samples could be analyzed using Q-Tof (quadrupole time-of-flight) MS to determine the chemical identity of each fraction. Fractions showing antibacterial activity should be analyzed on the mass spectrometer using electron impact ionization as per Desbois *et al.* (2009) (Desbois et al. 2009). Ten microliters of the sample should be injected into negative ionization mode, at 3kV and peak detection between 50-1500 m/z. The spectrum should be recorded at 300 MHz, with chemical shifts measured with respect to tetramethylsilane in parts per million.

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APPENDICIES

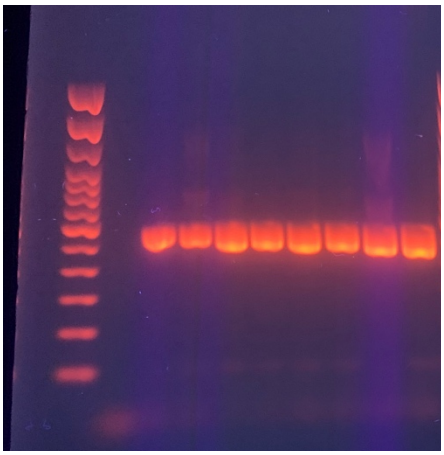

Figure S1: Agarose gel before PCR purification.

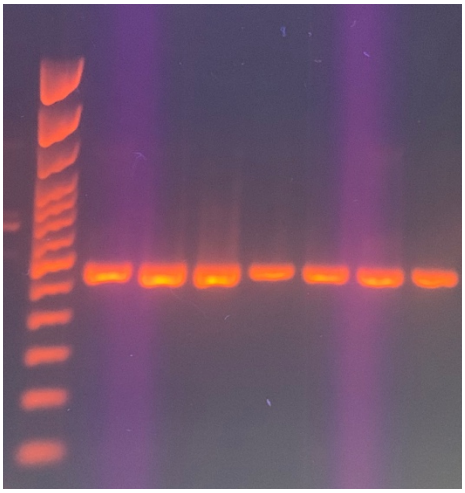

Figure S2: Agarose gel after PCR purification.

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Scenedesmus sp. KMMCC 1047 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spac	1160	1160	53%	0.0	98.92%	JQ315792.1
☑	Chlamydomonas sp. CCAP 11/168 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection	1158	1158	53%	0.0	98.92%	FR865562.1
☑	Chlamydomonas sp. CCAP 11/167 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection	1158	1158	53%	0.0	98.92%	FR865561.1
☑	Chlamydomonas sp. CCAP 11/143 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection	1158	1158	53%	0.0	98.92%	FR865538.1
☑	Chlamydomonas sp. CCAP 11/142 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection	1158	1158	53%	0.0	98.92%	FR865537.1

Figure S3: LL2A 18S BLAST results.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Chloroidium lichenum strain CCAP 211/33 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcri	924	924	35%	0.0	100.00%	MH551518.1
Chloroidium lichenum strain SAG 2295 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal	924	924	35%	0.0	100.00%	MH551517.1
Chloroidium lichenum strain CCAP 464/1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosom	924	924	35%	0.0	100.00%	MH551516.1
Chloroidium lichenum strain SAG 2041 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal	924	924	35%	0.0	100.00%	MH551515.1
Chloroidium lichenum strain CAUP H1999-1 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transc	924	924	35%	0.0	100.00%	MH551514.1
Chloroidium lichenum strain ISBAL-008 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal	924	924	35%	0.0	100.00%	MH551513.1
Chloroidium lichenum strain SAG 2141 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribec	924	924	35%	0.0	100.00%	MH551512.1
Chloroidium lichenum strain AB-98017 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal	924	924	35%	0.0	100.00%	MH551511.1
Chloroidium lichenum strain SAG 2293 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribec	924	924	35%	0.0	100.00%	MH551510.1

Figure S4: CC 18S BLAST results.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Chlorella sp. BR2 18S ribosomal RNA gene, partial sequence	931	931	36%	0.0	99.61%	JQ423156.1
Chlorella sp. Y9 18S ribosomal RNA gene, partial sequence	929	929	36%	0.0	99.23%	JF950558.1
Micractinium sp. IPOME-2 18S ribosomal RNA gene, partial sequence	928	928	36%	0.0	99.42%	KR936170.1
Chlorella sp. NIES-4385 gene for 18S ribosomal RNA, partial sequence	926	926	35%	0.0	100.00%	LC535350.1
Chlorella sp. isolate NJUST-1 small subunit ribosomal RNA gene, partial sequence	926	926	35%	0.0	100.00%	MN906179.1
Chlorella sorokiniana NKH18 gene for 18S ribosomal RNA, partial sequence	926	926	35%	0.0	100.00%	LC505550.1
Chlorella sorokiniana NKH6 gene for 18S ribosomal RNA, partial sequence	926	926	35%	0.0	100.00%	LC505542.1
Micractinium sp. KSF0094 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	926	926	35%	0.0	100.00%	MN414469.1

Figure S5: WW1-1 18S BLAST results.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Chlorella sp. isolate Daqing Salt Lake 1 small subunit ribosomal RNA gene, partial sequence	926	926	36%	0.0	100.00%	MK764913.1
'Chlorella' luteoviridis genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, culture collection CCAP 211/5B	926	926	36%	0.0	100.00%	FR865678.1
Chlorella sp. KMMCC EC-35 18S ribosomal RNA gene, partial sequence	926	926	36%	0.0	100.00%	HQ702282.1

Figure S6: WW1-2 18S BLAST results.