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Geodermatophilaceae biofilm formation and rock recolonization

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BY

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ABSTRACT

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The Geodermatophilaceae genera—*Geodermatophilus, Blastococcus,* and *Modestobacter*—live on rock surfaces and create biofilms that deteriorate stone architecture and monuments. Their biofilms increase the rate of weathering and erosion but are not well studied. Recently, thirteen strains were isolated, and their genomes sequenced. These new Geodermatophilaceae isolates along with *Modestobacter marinus* strain BC501 and *Blastococcus saxobsidens* strain DD2 were used in this study to evaluate biofilm formation under different environmental factors including temperature, light, and salt and heavy metal exposure. Ten isolates were used for experiments to recolonize back onto the rock substrates from which the isolates were obtained. For most of the isolates, optimal temperature was 28^{o}C with light not influencing growth. These isolates exhibited salt tolerance and also showed elevated tolerance levels for heavy metals (cobalt, nickel, copper, lead, arsenate, and chromate). Two isolates—TF02A-26 and TF02A-35—were able to grow on the rock substrate after two months and one month, respectively in the absence of added nutrients. Determining how Geodermatophilaceae growth yields and biofilm adhesions are affected and effected by rock substrates could help preserve important stone structures from biodeterioration and help salty and heavy metal contaminated sites by bioremediation.

CHAPTER 1

INTRODUCTION

Rock Environment

Although rock surfaces are considered an extreme environment due to low levels of easily accessible nutrients, microbes have been living on rock surfaces for millennia (Gorbushina, 2007; Meslier et al., 2018). The physical shape and geochemistry of the rock substrate makes it more habitable than other extreme environments. Besides having an oligotrophic life style (Cutler & Viles, 2010), stone-dwelling microbes are exposed to variable levels of temperature, pH, radiation, heavy metals, and low water availability making rock substrates an extremely harsh place to live and thrive (Gorbushina, 2007). They are exposed to the widest range of temperatures from -45^{o}C to 60^{o}C in desert and arctic climates (Gorbushina, 2007; Meslier et al., 2018). Under desert conditions, these temperatures fluctuate widely from very hot in the daytime to extreme cold in the nighttime (Busarakam et al., 2016; Meslier et al., 2018). Although many other extreme environments maintain above average high or low temperatures, they do not exhibit daily, seasonal, or yearly fluctuations found with life on rock surfaces (Gorbushina, 2007). Temperature fluctuation is not the only dynamic parameter of the rock surface environment, as pH and salinity levels of rock substrates also vary widely, with water availably driving these fluctuations (Gorbushina, 2007). Sudden rainfall decreases salinity and pH levels fall allowing microbial growth. Under desiccation condition, these environments exhibit high salinity and pH levels. Thus, microbes need to tolerate long periods of desiccation and exposure to sudden and fast rehydration in order to survive on rock surfaces (Gorbushina, 2007; Meslier et al., 2018). In addition,

stone-dwelling microbes are exposed to radiation from the sun and atmosphere. Depending on the latitude and shading, rock surfaces may be exposed to the sun at all hours of the day (Cutler & Viles, 2010). Hence, microbes have to combat gamma, ultraviolet (UV), and ionizing radiation that damages cellular DNA (Busarakam et al., 2016; Sghaier et al., 2016).

Rock surfaces are an oligotrophic environment (Gorbushina, 2007) but rocks are a reservoir of heavy metals. Although some heavy metals are beneficial, many will impede microbial growth. Numerous metals essential for life—such as cobalt, nickel, and copper—are found in and on most rocks, but these metals are toxic at a certain threshold (Gadd, 2010). Other heavy metals and metalloids, for instance lead, arsenate, and chromate, can be potentially absorbed and used by microbes but tend to be mostly toxic to any organism (Gadd, 2010). Anthropogenic activities, such as industry and agriculture, increases the levels of heavy metals and metalloids on rock surfaces from aquatic (rain or runoff) or aerial (smog or fumes) sources (Gadd, 2010; Khanafari et al., 2008). Heavy metal distribution depends on the geochemistry of the rock substrates and is influenced by organism activity. Some microbes will grow and tolerate these toxic environments (Gadd, 2010).

Rock Deterioration

For millennia to withstand the test of time, humans have used rocks for buildings, monuments, and art. However, these rocks, as all rocks exposed at Earth's surface, deteriorate via weathering and biodeterioration. Many different environmental characteristics cause weathering and contribute to biodeterioration including climate (temperature, rainfall, humidity, wind and/or ice), the exposed surface area to volume of

rock, the chemical properties of the rock, and atmospheric chemistry (i.e. pollutants such as sulfur dioxides and nitrogen oxides) (Allen, El-Turki, Hallam, McLaughlin, & Stacey, 2000; Cutler & Viles, 2010). These physical and chemical factors polish what may start as a rough structure and can break down the rock eventually into dust-sized particles.

Climate plays an important role on rock weathering with the combination of temperature, water, wind and, in some instances, ice working together to slowly chip away at the rock surface (Negi & Sarethy, 2019). The most damaging factor, water (Mihajlovski, Seyer, Benamara, Bousta, & Martino, 2015), penetrates deep into the rock micropores and drives mechanical weathering and chemical reactions. At temperatures below 0^{o}C, water volume expands as it crystalizes and drives physical breakdown of the rock (Taber, 1929, 1930). In addition, water serves as a transportation venue through microfractures allowing many compounds and minerals inside the rock that cause chemical damages (Gomez-Alarcon & de La Torre, 1994; Taylor et al., 2007). This process changes a hard, durable structure into a less stable, fragile rock. Furthermore, many of these compounds are modified by microbial biogeochemical cycles (iron, sulfur, and carbon cycles) and stimulate biological growth, which leads to biodeterioration depending on the chemical property of the rock (Taylor et al., 2007). Besides having physically and chemically damaging properties, water is essential for life on rocks (Mihajlovski et al., 2015). Thus, the presence of water on rock surfaces leads to increased microbial activity and can accelerate bioweathering.

Elevated microbial and fungal growth causes an increase in biodegradation of rocks (Negi & Sarethy, 2019). Microorganisms cause structural damage to the rock

through biopitting and powdering (Chimienti et al., 2016). Biopitting is a geologic phenomenon in which the production of metabolites by endolithic microorganisms in and on rock surfaces results in the formation of pits, while powdering means to reduce the rock into fine particles (Lombardozzi et al., 2012). During the biopitting process, the environment inside the pit is more stable and better protected than the outer stone surfaces, thereby facilitating higher microbial growth rates and biofilm production (Lombardozzi et al., 2012). A biofilm is a community of microbes that adhere to each other and onto a surface. Therefore, the rock structure itself impacts the number and types of microbes able to live there. Biofilm formation also wreaks havoc on the rock surface through biofouling, which refers to altering the aesthetic of the rock surface but no mechanical damage (Cutler & Viles, 2010). Biofouling will result from microbial biofilms trapping air pollutants that stain the rock surface darker (Cutler & Viles, 2010). Furthermore, microbial biofilms cause biocorrosion (Negi & Sarethy, 2019) thorough the secretion of organic and inorganic acid metabolic byproducts from microbes (Mihajlovski et al., 2015; Negi & Sarethy, 2019). These secreted acids, along with nitrites and sulfur dioxides from air pollution, provide a nutrient source for nitrifying- and sulfur-oxidizing bacteria that speeds up the biocorrosion process by lowering the pH (Negi & Sarethy, 2019). However, it is not just acids that are being secreted, microbial exoenzymes degrade the rock for nutrients (Chimienti et al., 2016). Thus, microbes cause biodeterioration of rock surfaces through combined effects of biopitting, biofouling and biocorrosion.

This biodeterioration process is occurring worldwide to many historical and heritage sites. For example, Lascaux, home of the oldest human paintings, is being

damaged by cyanobacteria (Alonso, Dubost, Luis, Pommier, & Moënne-loccoz, 2017), while fungal mold (black crust) is destroying stone churches constructed in 1822 in Rio de Janeiro (Gaylarde, Baptista-neto, et al., 2017). As expected, physical and chemical environmental factors, especially in warm humid climates, accelerate microbial growth on rocks (Negi & Sarethy, 2019).

Primary Colonizers

A wide variety of microorganisms have been found on rock surfaces. Both heterotrophic and autotrophic organisms (fungi, lichens, algae, and bacteria) colonize rocks surfaces (Dhami et al., 2014; Mihajlovski et al., 2015). These organisms survive in the extreme rock environment and cause biodeterioration to the rock surfaces by physical or chemical damage (Dakal & Cameotra, 2012).

One of the most dominant group of microorganisms on rock surfaces are photoautotrophs like cyanobacteria and green algae (Chimienti et al., 2016). Microbial autotrophs are ubiquitous colonizers of rock substrates found in cold or hot deserts, and on the surface of buildings (Negi & Sarethy, 2019). These microbes dominate the Lascaux cave community when lights for tourism were installed and continue to be a major problem for this historic cave and other rock surfaces (Alonso et al., 2017; Chimienti et al., 2016). As autotrophs, cyanobacteria obtain energy from solar radiation via photosynthesis or other carbon dioxide fixation pathways as a strategy to overcome the poor nutrient environment characteristics of rock surfaces (Chimienti et al., 2016; Negi & Sarethy, 2019). Their autotrophic ability generates carbon compounds via carbon fixation that they use for cellular biomass or secrete into the environment. In the food web, autotrophic cyanobacteria provide organic carbon to heterotrophs (Chimienti

et al., 2016). Secreted polysaccharides allow them to adhere to the rock surfaces and provide a carbon source for secondary heterotrophic rock colonizers (Negi & Sarethy, 2019). Although green algae and cyanobacteria are mainly aquatic species, a few live on rock surfaces and are resistant to desiccation over long periods of time. After being exposed to water, these organisms quickly revitalize after a period of drought-induced dormancy (Cutler & Viles, 2010). While cyanobacteria and green algae dominate in high humidity locations (Cutler & Viles, 2010), they are also found in lower humidity conditions and help facilitate the growth of other less drought-adapted species by absorbing water in microscopic pores, thus keeping the surrounding community hydrated (Negi & Sarethy, 2019). Although hydration from rainfall provides nitrous oxides from anthropogenic activity, cyanobacteria are nitrogen fixers providing biologically available forms of nitrogen to the community (Cutler & Viles, 2010). In summary, autotrophs like cyanobacteria and green algae are suitable for survival in an oligotrophic environment and help pave the way for other microbes to colonize rock surfaces.

Fungi and lichen are similarly seen as dominant colonizers of rock surfaces and pose a definite problem for stone conservation efforts. Fungi produce a wide array of organic acids that can be detrimental to rock substrates and other organisms (Cutler & Viles, 2010; Negi & Sarethy, 2019). These organic acids are responsible for the biofouling stains seen on stone surfaces (Cutler & Viles, 2010; Negi & Sarethy, 2019). Stain formation occurs due to fungal exoenzymes used to digest their food (Cutler & Viles, 2010). In addition, lichens produce chemically damaging acids including carbonic acid and oxalic acid (Bjelland & Thorseth, 2002). The filamentous nature of fungi and

lichens can cause physical damage from chemical deterioration inside the rocks as opposed to just surface damage (Cutler & Viles, 2010; Mihajlovski et al., 2015; Negi & Sarethy, 2019). Fungal filamentous bodies transport water around the rock environment providing other microorganisms within the rock greater access to water (Gomez-Alarcon & de La Torre, 1994). Both the penetration of fungal or lichen hyphae and secretion of acids into rocks will potentially release heavy metals into the environment. While some metals are required for microbial growth, others are inhibitory to the growth of the rock microbial community (Bjelland & Thorseth, 2002; Cutler & Viles, 2010). In contrast to cyanobacteria and green algae, fungi and lichens are more resistant to desiccation and are found in arid climates (Bjelland & Thorseth, 2002; Cutler & Viles, 2010). Heterotrophic fungi can persist under oligotrophic conditions. Their hyphae scavenge nutrients from the atmosphere, and they are able to undergo long periods of suspended metabolism/dormancy (Cutler & Viles, 2010). Some fungi produce pigments that protect them from UV light (Cutler & Viles, 2010). Lichens are phototrophs that use sunlight for energy and are resistant to solar radiation (Bjelland & Thorseth, 2002). While fungi and lichens are resilient to the rock environment conditions and increase water availability, the secretion organic acids and exoenzymes creates problems including metal accumulation on the surface for other colonizers.

There are five major phyla of bacteria that grow in or on rocks: Actinobacteria, Firmicutes, *Deinococcus-Thermus*, Cyanobacteria and Chloroflexi (Sghaier et al., 2016). Actinobacteria are a key contributor to the biofilm and biodegradation to the rock surface (Gaylarde, Ogawa, Beech, & Kowalski, 2017). Heterotrophic bacteria in these groups may actually prefer low nutrients levels for growth (Gaylarde, Ogawa, et al.,

2017). The rock environment contains several nutrients in different forms of sulfur, nitrogen, and organic acids (Ranalli, Matteini, Tosini, Zanardini, & Sorlini, 2000). Actinobacteria, especially members of the family, Geodermatophilaceae, are resistant to higher levels of heavy metals found in the rock environment (Gaylarde, Ogawa, et al., 2017). Along with other microbes in these phyla, they exhibit increased levels of resistance to desiccation and salt. In order to survive long periods of low water activity and increased salt concentrations, some microbes may be halophilic or halotolerant. (Gaylarde, Ogawa, et al., 2017). Since many rock surface habitats are exposed to the sun, resistance to high levels of UV and ionizing radiation are particularly two important traits for survival under these conditions. One resistance mechanism occurs through production of pigments like melanin and carotenoids. Due to daily and seasonal changes, temperature is another important environmental factor these microbes face (Gaylarde, Ogawa, et al., 2017; Xiao et al., 2011). In subtropical climates, stone-dwelling bacteria generate higher biomass in the winter and spring seasons and show higher tolerance to temperature fluctuations than other organisms growing on the rock surfaces (Tayler & May, 1991; Warscheid, 2003). Filamentous Actinobacteria break into the rock similar to filamentous fungi (Cockell, Kelly, & Marteinsson, 2013) and produce spores that are resistant against environmental stress including acid production (Cockell et al., 2013; Mihajlovski et al., 2015). In the microbial rock community, Actinobacteria are key colonizers. Actinobacteria provide the most organic carbon source for other heterotrophic organisms and these bacteria, like Geodermatophilaceae, are able to degrade toxic metals (Gaylarde, Ogawa, et al., 2017). Actinobacteria will

precipitate heavy metals to accelerate their release from rocks which can be used as a defense mechanism against other organisms (Cockell et al., 2003).

To protect acid sensitive members of the community, certain microbes can help balance out the pH by creating an alkaline environment (Fortin, Ferris, & Beveridge, 1997). To reduce organic acid excretion, some bacteria protect themselves by controlling the fungal population through the production of antifungal compounds (Cutler & Viles, 2010). The diverse microbial community allows many different bacterial species to live and thrive on the surface by filling different niches, generating biofilm growth, and subsequent degradation of the rock.

Geodermatophilaceae

Among the phylum Actinobacteria, the Geodermatophilaceae family are known as the stone-dwelling colonizers (Meslier et al., 2018). These gram-positive bacteria (Chimienti et al., 2016) are resilient to drastic temperature changes, desiccation periods, and exposure to elevated levels of salt, heavy metals, UV and gamma radiation (Normand, Daffonchio, & Gtari, 2014). While resistant to heavy metals, the ability of Geodermatophilaceae to use some toxic metals as nutrients is unclear. These pigmented bacteria produce orange, yellow, pink, red, and black colonies on solid media (Gtari et al., 2012; Lechevalier, 1989; Mevs, Stackebrandt, Schumann, Gallikowski, & Hirsch, 2000; Urzi, Salamone, Schumann, Rohde, & Stackebrandt, 2004). The life cycle of Geodermatophilaceae bacteria consists of two forms: R-form (motile bud-forming rods) and C-form (sessile coccoid) (Ishiguro & Wolfe, 1970; Montero-Calasanz et al., 2017; Normand et al., 2014). The R-form is motile and dividing. The C-form consists of sporangia and vegetative cells and is considered the

resting form that will withstand adverse conditions. A supplementary thick fibrous layer is associated with the C-form that acts as a mortar to hold coccoid cells together. The trigger for the switching between these two phases is not clear, but an unidentified factor present in Difco Tryptose appears to be necessary to maintain the cells in the C-form as well as to trigger change from the R-form to the C-form. Both phases may grow through rocks and compromise the structure by forming biopits (Gaylarde, Ogawa, et al., 2017; Sghaier et al., 2016). These microbes travel by wind and are able to traverse thousands of kilometers to colonize a new rock surface (Sghaier et al., 2016). The Geodermatophilaceae family is classified into three taxonomically recognized genera: *Geodermatophilus, Blastococcus,* and *Modestobacter* (Normand et al., 2014).

Because it is challenging to obtain the appropriate growth conditions in the laboratory without losing an isolate to competition due to their slow growth, the genus *Geodermatophilus* has been poorly studied under laboratory conditions (Montero-Calasanz et al., 2013), but recently many novel species have been isolated (Hezbri et al., 2017; Hezbri, Louati, Nouioui, Gtari, Rohde, Spröer, Schumann, Klenk, Ghodhbane-Gtari, et al., 2016; Trujillo, Goodfellow, Busarakam, & Riesco, 2015). This genus is found in arid desert soils (like Sahara Desert sand), on the surface of rocks, and some in rhizosphere soil (Hezbri et al., 2015; Montero-Calasanz et al., 2013). From its name, ‘Ge’ (stone) ‘derma’ (skin) ‘philus’ (loving) this genus is mainly found on the surface layer of rocks (Normand & Benson, 2012). Although all members of the Geodermatophilaceae family have a higher than average level of resistance to UV and gamma radiation, *Geodermatophilus* has the highest level of resistance among the three genera (Gtari et al., 2012). *Geodermatophilus* also has a high level of resistance

to oxidative stress, desiccation and high temperatures (18^{o}C to 40^{o}C) (Gtari et al., 2012; Hezbri et al., 2015, 2017). However, their heavy metal tolerance levels are the lowest among the three Geodermatophilaceae genera, but is still higher than the average bacteria (Gtari et al., 2012). In their non-motile form, these cocci bacteria will form a thick fibrous mass which helps keep the cells together and aggregate similar to a sporangium (Philippe Normand et al., 2014). Some strains produce the esterase enzymes that are resistant to their harsh environment as well (Hezbri et al., 2015).

Members of the genus *Blastococcus* are inhabitants of the interior of rocks, in marine sediments 20 meters below the surface, and as endophytes on plant surfaces (Chouaia et al., 2012, Hezbri et al., 2016, Normand et al., 2014). The C-form of *Blastococcus* consists of cocci cells that aggregate to form tetrads and buds, while the R-form is produced from the buds and has a variety of shapes including motile rods and vibrio cells (Urzi et al., 2004). *Blastococcus* has a higher level of resistance to heavy metals than *Geodermatophilus* and *Modestobacter* (Chouaia et al., 2012; Gtari et al., 2012). Since *Blastococcus* is found in the interior of rocks, these properties are not unexpected. It is unclear if this genus can use some heavy metals as an energy source or if they are just resistant to them. However, *Blastococcus* has a lower level of resistance to gamma and UV radiation and to oxidative stress compared to the other two Geodermatophilaceae genera (Chouaia et al., 2012; Gtari et al., 2012). In addition, *Blastococcus* species are both aerobic and microaerophilic and able to grow under a wider range of temperatures (3^{o}C to 40^{o}C) than *Geodermatophilus* (Gtari et al., 2012; Philippe Normand et al., 2014; Urzi et al., 2004). *Blastococcus* produces thermostable esterases similar to *Geodermatophilus* (Chouaia et al., 2012). Strains isolated from

limestone and marble surfaces use a wider array of organic compounds than those isolated from marine sediments (Normand et al., 2014). These properties of high heavy metal tolerance, aerobic and microaerophilic nature, and a wider growth temperature range including lower temperatures may help explain why *Blastococcus* out of the three Geodermatophilaceae genera is more suitable for life inside rock.

The last genera of the Geodermatophilaceae family is *Modestobacter* (Normand et al., 2014). *Modestobacter* are found on surfaces containing low available organic carbon, rock surfaces, desert soils, and deep-sea sediments (Busarakam et al., 2016, Xiao et al., 2011). In contrast to the other two Geodermatophilaceae genera, *Modestobacter* are psychrotolerant or even psychrophilic meaning they can grow around 0ºC, but they can grow at higher temperatures up to 35ºC. (Mevs et al., 2000; Xiao et al., 2011). This genus is highly pigmented producing a pink color that turns to a dark melanin-like pigment over time (Busarakam et al., 2016, Xiao et al., 2011). *Modestobacter* forms cocci shapes along with short rods that can aggregate as well and form short filaments (Mevs et al., 2000; Xiao et al., 2011). These cells divide by budding like *Blastococcus* (Philippe Normand et al., 2014). Like *Geodermatophilus*, all *Modestobacter* species are aerobic (Gtari et al., 2012). *Modestobacter* exhibits resistance levels to heavy metals and UV and gamma irradiation that are in the middle of the levels found for the other two Geodermatophilaceae genera supporting the hypothesis that *Modestobacter* lives physically in between the other two genera in the environment (Gtari et al., 2012; Normand et al., 2014).

Biofilms

Rock surfaces are an extreme environment to live on. One strategy to boost microbial growth is for them to adhere together and onto a surface as a community in a biofilm (Sivadon, Barnier, & Urios, 2019). A biofilm community provides protection, nutrients, and water to its occupants (Cutler & Viles, 2010; Sivadon et al., 2019). While starting off as a single bacterial cell may be hard, the formation of a biofilm will help provide the resources necessary to grow and thrive and consequently damage a rock surface.

Regardless of which organism colonizes the rock surface first, it is important for the primary colonizers to adhere to the rock and start to form an Extracellular Polymeric Substance (EPS) (Negi & Sarethy, 2019; Rosenberg, 1989). Cyanobacteria and green algae are an example of organisms that adhere by secreting an EPS and providing heterotrophic organisms with a carbon source (Negi & Sarethy, 2019). Geodermatophilaceae bacteria will naturally clump together on the rock surfaces or even inside the rock and form biofilms even in low nutrient conditions (Chimienti et al., 2016; Gaylarde, Ogawa, et al., 2017). EPS consists of different substances including sugars, nucleic acids, pigments, enzymes, lipids, dead cells, and airborne particles (Negi & Sarethy, 2019; Sivadon et al., 2019). Pigments produced are used as a sun block for the members of the biofilm community and protects less resistant cells to the harmful UV radiation (Cutler & Viles, 2010). Importantly, the EPS will help the community retain and absorb water from the humidity in the atmosphere (Gorbushina, 2007). This water retention property is seen when EPS is produced under periods of high humidity, but shrinks under periods of desiccation (Gorbushina, 2007). Besides protecting against water fluctuation, the biofilm structure helps control temperature

fluctuations for occupants inside the biofilm (Cutler & Viles, 2010). Although a source of nutrients and water, the EPS also functions to holds quorum sensing signals involved in cell-to-cell communication among members of the biofilm community (Sivadon et al., 2019). Biofilms cause an increase in antibiotic resistance and exoenzyme production that are involved to extract nutrients from the rock environment (Sivadon et al., 2019). Thus, community members of a biofilm are more protected and have more available water and nutrients than a single planktonic cell.

While biofilms are critically important for microbes, they will cause significant damage to the rock surfaces by biodeterioration (Chimienti et al., 2016; Gaylarde, Ogawa, et al., 2017). This deterioration results in unsightly spots and holes that damage the structure and aesthetic integrity of stones. Additionally, pigments that protect biofilms from UV light result in the formation of crusts of green, black or other colors (green for algae, black for fungi, black and other pigments of different actinobacteria) that aesthetically damage—biofoul—stone surfaces (Gaylarde, Ogawa, et al., 2017). Small fractures on the rock surfaces allow Geodermatophilaceae species to move into the rock and solubilize minerals (Kinner et al., 2005). The most common mineral elements—carbonates, silicates, and phosphates—present in rocks are solubilized by microbes (Negi & Sarethy, 2019). All of these mineral elements are solubilized at low pH by organic acids (fungi and bacteria), mineral acids (from microbial cycles), and carbonic acid formed form cellular respiration (Gorbushina, 2007). Acids leave behind dark stains instead of a crust on the rock surface (Gorbushina, 2007). Beside acids, microbes can damage minerals via redox process and reducing metals for adsorption and use in cellular processes (Gadd, 2010). During biofilm response to

desiccation and hydration, the shrinking periods and growth periods wear down the rock mechanically (Gadd, 2010). Overall, rock weathering will increase as biofilms dig-in causing physical changes to the surface along with the rock dissolving due to pH changes (Gorbushina, 2007).

Clearly, biofilms provide microbes with better growth conditions in oligotrophic environments. Members of the biofilm community will share nutrients, water, and protection. However, biofilms are the main cause of stone biodeterioration through enhancing weathering by mechanical damage, chemically eroding the rock, and causing aesthetic harm.

Research Goals

Most of our current knowledge on members of the Geodermatophilaceae species have focused on growth parameters including the range and optimal growth conditions for temperature, pH, salt, and oxygen (Gtari et al., 2012; Philippe Normand et al., 2014; Sghaier et al., 2016; Xiao et al., 2011). There is limited research on carbohydrate utilization and levels of resistance to heavy metals, desiccation, UV and gamma radiation by Geodermatophilaceae species (Gtari et al., 2012; Mevs et al., 2000; Urzi et al., 2004). Additionally, there is little information on biofilm formation by the various species in the Geodermatophilaceae family and the mechanisms responsible for their ability to attach to rock surfaces for prolonged periods. Several novel species of Geodermatophilaceae were isolated recently and partially physiologically characterized (Ennis, 2018). The genomes for these *Blastococcus* and *Geodermatophilus* isolates were sequenced providing a valuable database that is available on Joint Genome Institute/Integrated Microbial Genomes (JGI/IMG) (https://img.jgi.doe.gov/) for this

study. Although these bacteria were isolated from rocks, the ability to recolonize the rock substrates has not been tested. One hypothesis is that members of the Geodermatophilaceae family are the primary rock colonizers.

The overall goal of this project was to determine how effective Geodermatophilaceae species are at creating biofilms for colonization. Specific objectives were (1) to characterize growth concentration and biofilm formation of these 13 Geodermatophilaceae isolates for their tolerance to salt and heavy metals and (2) to assess these 13 isolates ability to recolonize rock surfaces. First environmental optimization would occur to determine optimal time period, temperature, and light conditions for growth and biofilm formation. Emulating *in vivo* conditions showed the impact of growth and biofilm stability among the different genera. Two environment parameters—salt tolerance and heavy metal tolerance—were explored to test how effective their biofilm adherence would be on rock surfaces. Finally, this work tests the hypothesis that members of the Geodermatophilaceae family are primary rock colonizers to recolonize on to rock surfaces *in vitro*. To get the best possible results for recolonizing, this study was performed with the rocks from which the bacterial isolates were obtained.

CHAPTER 2

METHODS

Bacterial Strains and Growth Conditions

All bacteria strains used in this study are listed in Table 2.1. Eleven Geodermatophilaceae strains were isolated from rock samples obtained from India or New England (Table 2.1). *Modestobacter marinus* strain BC501 and *Blastococcus saxobsidens* strain DD2 were used as a baseline for studies on the novel Geodermatophilaceae species as they have been fully sequenced and have multiple studies performed on them (Chouaia et al., 2012; Philippe Normand et al., 2012).

Bacterial strains were streaked from glycerol frozen stock cultures on Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910) agar plates and allowed to grow for two weeks at 28^{o}C. Czapek-Dox medium was composed of the following: 15 g/l of sucrose, 2 g/l sodium nitrate, 0.5 g/l dipotassium phosphate, 0.5 g/l magnesium sulfate, 0.5 potassium chloride, and 0.01 iron (II) sulfate. To provide working cultures, Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910) broth medium was inoculated from the agar plates and were incubated for two weeks with shaking at 28^{o}C. These working broth stock cultures were subcultured monthly.

Table 2.1. Bacteria used in this study.

Geodermatophilaceae

Isolate ID	Genomic Sequencing ID	Location of Isolate	Rock Type	Source or Reference
DD2	*Blastococcus saxobsidens* DD2	Cagliari, Sardinia	Calcarenite	Normand et al., 2012
BC501	*Modestobacter marinus* strain BC501	Carrara, Tuscany, Italy	Calcareous	Chouaia et al., 2012
DF01-2	*Geodermatophilus* sp.	Dindigul Fort	Granite	Ennis, 2018
TF02-6	*Geodermatophilus* sp.	Tiruchirappalli Rockfort	Granite	Ennis, 2018
TF02-8	*Blastococcus* sp.			Ennis, 2018
TF02-9	*Blastococcus* sp.			Ennis, 2018
TBT05-19	*Blastococcus* sp.	Thanjavur Brihadeshwara Temple	Granite	Ennis, 2018
TF02A-26	*Blastococcus* sp.	Tiruchirappalli Rockfort	Granite	Ennis, 2018
TF02A-30	*Blastococcus* sp.			Ennis, 2018
TF02A-35	*Blastococcus* sp.			Ennis, 2018
GayMR16	*Blastococcus* sp.	Gay City, CT	Granite	Ennis, 2018
GayMR19	*Blastococcus* sp.			Ennis, 2018
GayMR20	*Blastococcus* sp.			Ennis, 2018

Other Isolates

Isolate	Genomic Sequencing ID	Source or Reference
168	*Bacillus subtilis*	Lab Stock
MG1655	*Escherichia coli*	Lab Stock
TF02A-27[1]	*Microvirga* sp.	Ennis, 2018
PS03-16[1]	*Mycobacterium* sp.	Ennis, 2018

[1] Both were dropped from experiment after they were fully sequenced to be non Geodermatophilaceae species

Biofilm Adhesion Assay

Biofilm formation was determined by use of the polystyrene microtiter plate assay (O'Toole & Kolter, 1998). Isolates were grown for more than a week in liquid Czapek-Dox medium supplemented with yeast extract (DSMZ medium 130; Dox, 1910). Isolates were diluted to an optical density of 595nm (OD_{595}) of 0.1 in fresh Czapek-Dox medium. A 96-well microtiter plate was inoculated with 200µl (for the temperature and light assays) to 100µl (for the salt and heavy metal assay). The plates were sealed with parafilm (Bemis, Oshkosh, WI) to retain moisture and were incubated at 28^{o}C for seven days. Both incubation temperatures and lengths of the incubation were varied to determine the optimal conditions for further experiments.

For the temperature and light experiments, a row of 12 wells of the 96-well microtiter plate was inoculated with a single isolate so that each plate tested eight isolates. For the heavy metal experiments, a 96-well microtiter plate was inoculated with a single isolate with the top four rows belonging to one heavy metal and the bottom four rows belonging to a different metal. Each column was testing a different salt or heavy metal concentration with replicates of four. The salt experiments had similar setup, but the top four rows had a different isolate from the bottom four rows. After incubation, cell growth was determined by measuring OD_{595} on a Tecan Infinite 200 plate reader with Magellan software (Tecan Group, Ltd., Switzerland). This measurement was used to compare the effect the treatment had on bacterial growth. The unbound cells were removed by inverting the microtiter plate over a reservoir and the plate was incubated at 80^{o}C for 30 minutes to fix biofilms to the wells. Following the heat fixing, the bound cells were stained with 200 µl of 0.01% crystal violet for 20

minutes at room temperature and rinsed with distilled water by submersion. Water and unbound stain were removed by inversion and microtiter plate was tapped dry over a paper towel for one minute. The bound stain was incubated with 200µl of destain solution (80% ethanol, 20% acetone; v/v) for 15 minutes at room temperature to solubilize the dye. After 10 seconds of shaking, the amount of solubilized dye present in each well was measured at absorbance of 590nm (A_{595}).

The means and standard errors of the OD_{595} and A_{595} measurements were calculated by Microsoft Excel (Microsoft, Redmond, WA). Significant differences in the means of the environmental optimizations between the three temperatures and between 12- hours of light versus 0-hours tested were determined using Analysis of Variance (ANOVA) and the T-Test in Microsoft Excel. The OD_{595} and A_{595} values verses time of incubation periods was put in a bar graph and compared to determine the optimal incubation time and the optimal lighting conditions to grow the biofilms for the rest of this study. Graphs of the means with error bars that represented the standard error were all generated using Microsoft Excel

Environmental Optimization of Biofilm Assays

To determine the effect of temperature and length of incubation periods, microtiter plates were incubated at 21^{o}C (room temperature), 28^{o}C, and 37^{o}C. For each temperature tested, seven microtiter plates were set up to allow for daily measurement of biofilms. Every 24 hours for one week, one of the seven microtiter plates were removed and measured as described above. For these experiments *M.*

marinus BC501 and isolates TF02-8, TF02-9, PS03-16, *B. saxobsidens* DD2, TF02-6, TF02A-26, and TF02A-27 were used.

The effect of light was tested in a manner similar to the above conditions. To emulate 0-hours (complete darkness), the microtiter plates were individually wrapped in aluminum foil and incubated at 28^{o}C. To test the effect light exposure, microtiter plates were incubated at 28^{o}C under lights timed to be on for 12-hours. Plates were sampled every 24 hours for seven days to measure growth and biofilm adhesion.

Salt Tolerance

The effect of different salt concentrations on biofilm formation and adhesion was determined by the above biofilm adhesion assay. Sterile Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910) was used which contains 0.01 M of NaCl. Each well on the 96 well microtiter plates were inoculated with a 100µl of the growth medium containing 0.1 OD_{595} suspension of each isolate. For each row of 12, another 100µl of distilled water or different concentrations (0.1, 0.25, 0.5, 0.6, 0.75, 1.0, 1.25, 1.5, 2.0, and 2.5 M) of NaCl were added to the wells. The first two columns contained water. Each microtiter plate contained two isolate and for replicate measurements of the concentrations. For these experiments *M. marinus* BC501, B. This assay was performed using the *M. marinus* BC501*, Blastococcus saxobsidens* DD2, and isolates DF01-2, TF02-8, TF02-9, TBT05-19, TF02A-26, TF02A-30, TF02A-35, GayMR16, GayMR19, and GayMR20 were tested *Escherichia coli* MG1655, and *Bacillus subtilis* 168 were also tested and used as non-halotolerant controls. The Geodermatophilaceae strains were incubated for one and two weeks. The control *E.*

coli MG1655 and *B. subtilis* 168 were incubated for 48 hours. Incubation was done at 28^{o}C in twelve hours of light per day.

The means (n=4) and standard errors of the OD_{595} and A_{595} measurements were calculated by Microsoft Excel (Microsoft, Redmond, WA). The initial OD_{595} and resulting biofilm A_{595} were used to evaluate bacterial growth and biofilm adhesion. To evaluate the levels of resistance, two parameters were determined: Minimum Inhibitory Concentration (MIC) and Maximum Tolerable Concentration (MTC). The MTC is highest concentration of salt, which does not affect growth or biofilm production (Richards, Krumholz, Chval, & Tisa, 2002). The MIC values represents the minimum salt concentration inhibiting bacterial growth or biofilm production. These values were determined by inspection after plotting the growth (OD_{595}) or biofilm production (A_{595}) as a function of the log of the salt concentration. The MIC was given by the intersection of the survival curve with the horizontal axis

Heavy Metal Tolerance

The effect of heavy metal concentration on biofilm formation and adhesion was determined in a similar matter to salt tolerance measurements described above. Six heavy metals were tested: cobalt ($CoCl_2$), nickel (NiCl), copper $CuSO_4$), lead ($Pb(NO_3)_2$), arsenate ($KAsO_4$), and chromium (K_2CrO_4). Because they are considered essential metals for growth but toxic at elevated levels, cobalt, nickel, and copper were selected. Lead, arsenate, and chromate are potentially used by microbes, but are considered mostly toxic. Sterile aqueous solutions of $CoCl_2$, NiCl, and $CuSO_4$ were prepared to finial concentrations (mM) of 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5.0, and

6.0. Sterile aqueous solutions of $Pb(NO_3)_2$ were prepared to finial concentrations (mM) of 1.0, 2.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, and 30.0. Sterile aqueous solutions of $KAsO_4$ were prepared for finial concentrations (mM) of 1.0, 2.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, and 50.0. Sterile aqueous K_2CrO_4, prepared to final concentrations (mM) of 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 5.0, 7.5, 10.0, and 20.0. Two microtiter plates for each isolate were incubated for one and two weeks. The control *E. coli* MG1655 and *B. subtilis* 168 plates were incubated for 48 hours. Incubation was done at 28^{o}C in twelve hours of light per day until the biofilm adhesion was measured.

The MIC and MTC values were determined as described previously for the salt tolerance tests. Significant differences in the means of the different heavy metal concentrations tested were determined the T-Test in Microsoft Excel.

Recolonizing Rock Substrate Experiments

Recolonizing Geodermatophilaceae isolates onto rock surface was performed using a modified biofilm adhesion assay. For each isolate, the same rocks from which the strain was isolated was used in this procedure (*i.e.* DF01-2 used sample rock from Dindigul Fort) (Table 2.1). Aliquots (<1.00 g) of gravel-sized rock chips were first autoclaved for sterilization and then placed into a 24-well microtiter plate.

To ensure that the isolates would adhere to the rock surface, the assay was first performed with growth medium. Isolates DF01-2, TF02-8, TF02-9, TBT05-19, TF02A-26, TF02A-30, TF02A-35, GayMR16, GayMR19, and GayMR20 were used for this adhesion assay. The isolates were grown for one week at 28^{o}C shaking incubation in Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910 and diluted in sterile Czapek-Dox supplemented with yeast extract to an OD_{595} of 0.1. To

each well, 1 ml of diluted culture were added. Each isolate was performed in triplicate and contained their corresponding sterile rock. For controls, one well contained 1 ml of diluted culture for the isolate without a rock chip and another well contained a rock chip with 1 ml of sterile Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910). Sterile rock chips were first placed in the well and 1 ml of diluted isolate or blank medium was added. Microtiter plates were sealed with parafilm (Bemis, Oshkosh, WI) and incubated at 28^{o}C under twelve hours of light. The two 24-well microtiter plates were incubated for two weeks and one month. After two weeks and one month of incubation, the rock substrates were removed from the microtiter plate using a flame sterilized forceps and placed into a new sterile microtiter plate. Pictures of the rock substrates were taken before and after leaving the incubated microtiter plate to allow for visualization of the adhesion to the rock surfaces. The original microtiter plate with isolates was read at OD_{595} on a Tecan Infinite 200 plate reader with Magellan software (Tecan Group, Ltd., Switzerland). This measurement determined bacterial growth. The same procedure described above for the biofilm adhesion assay was used except that 1 ml of the stain and de-stain was instead of 200µl. The A_{595} values for biofilm adhesion values were measured. This value provided a measurement of how cells adhered to the well verses rock.

To determine the adhesion to the rock surface, a modified biofilm adhesion assay was used. Samples were heat fixed by incubating at 80^{o}C for 30 minutes. After being heat fixed, the rock chips are stained with 1 ml of the 0.01% crystal violet stain. After 20 minutes, excess stain is rinsed off the rock substrates by submerging them in water with forceps until the excess is off the rock chips. The rock chips were returned to a new 24-

well microtiter plate and 1 ml of de-stain solution was added and incubated for 15 minutes. The rock substrates were removed from the microtiter plate and the A_{595} of the plate was read to determine biofilm adhesion values. The A_{595} values were compared the values for blank rock substates. These values for the isolates were compared among each other to determine the five isolates that adhered the best to the rock surface. The values were compared the values obtained from the original plate to quantify how many bacteria were lost to the wells. Additional blank rock chips went through the adapted version of the biofilm adhesion assay and added more values. The biofilm value (A_{595}) was standardized to the weight of the rock sample and the mean values were calculated with standard error.

Once the five isolates that adhered best to the rock surface (see results) were determined, they were used to recolonize rock substrates in phosphate buffered saline solution (PBS). Isolates TF02-8, TBT05-19, TF02A-26, TF02A-35, and GayMR20 were used for this assay. The experiment was set up similarly to the procedure described above. Cultures were grown for one week at 28^{o}C shaking incubation in Czapek-Dox supplemented with yeast extract (DSMZ medium 130; Dox, 1910). The cultures were centrifuged to the collect the cells and resuspended in PBS at OD_{595} of 0.1. Similar to the biofilm adhesion assay, 1 ml of culture suspended in PBS were added to the wells. Controls consisted of 1 ml culture PBS in two samples, one with and one without a rock substrate. These microtiter plates were incubated one and two months because of the absence of growth medium. Under these conditions, cells were using the rock substrates as the sole source of nutrients. Both the OD_{595} and A_{595} measurements were determined as described previously. The biofilm measurement (A_{595}) was

standardized based on rock substrate mass. However, only growth measurement values were compared the blank rock substrate values.

The means and standard errors for OD_{595} and corrected A_{595} were calculated by Microsoft Excel (Microsoft, Redmond, WA). The T-Test was used to determine significant differences in the means of the rock substrate recolonization between those that had isolate growth and blank rock substrates. Bar graphs of the means with error bars that represented the standard error were all generated using Microsoft Excel.

CHAPTER 3

RESULTS

Environmental Optimization and Biofilm Adhesion Assays

Incubation Period and Temperature Optimization

Because of the absence of baseline information on biofilm formation by Geodermatophilaceae, optimal environmental conditions were determined for eight isolates. Over a 7-day time period, growth was determined by bacterial concentration (OD_{595}) and biofilm adhesion (A_{595}) (Figure 3.1). All bacterial concentration peaked or leveled off by the seventh day of incubation. Each isolate followed the growth patterns found on Figure 3.1 (A, B, and C). Biofilm adhesion showed similar results with peaks or leveling off on the seventh day (Figure 3.1 D, E, and F). The only exception being isolates TF02A-26, TF02A-27 and TF02-8 that peaked on the fourth day at 28^{o}C and 37^{o}C. However, the results show that bacterial concentration and biofilm adhesion conditions were optimal on the seventh day. Therefore, all further experiments used the optimal condition of seven days incubation.

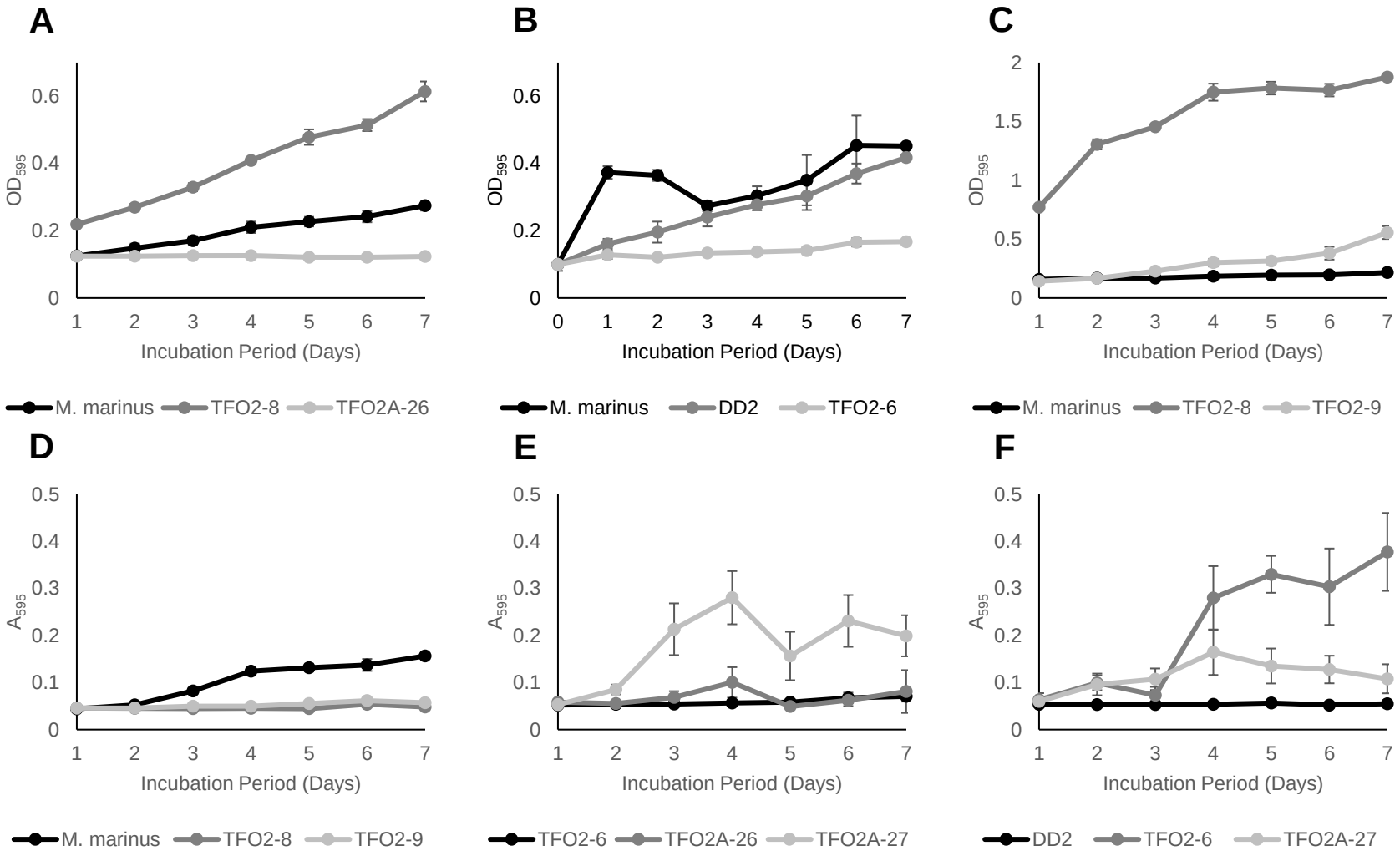


Figure 3.1. Seven-day growth curves and biofilm adhesion assays for three representational isolates. Each isolate represents a pattern of high, intermediate, and low bacterial concentration that was measured based on the OD_{595} (A, B, and C) and for biofilm adhesion A_{595} (D, E, and F). A and D were incubated at 21ºC. B and E were incubated at 28ºC. C and F were incubated at 37ºC. Error bars represent standard deviation.

The optimal growth temperature (*i.e.* 21ºC, 28ºC, and 37ºC) was determined a similar manner as the incubation time-period. Since a 7-day incubation period was already determined optimal for these isolates, the effect of incubation temperature was determined for the Geodermatophilaceae isolates (Figure 3.2). Among the conditions tested, all isolates had a significantly higher bacterial concentration at 28ºC than at 21ºC (Figure 3.2; Table S1). Between 28ºC and 37ºC, the isolates were split on the optimal temperature. *M. marnius* BC501, *B. saxobsidens* DD2, PS02-16, and TF02A-27 showed significant higher bacterial concentrations at 28ºC (Table S1), while isolates TF02-6, TF02-8, TF02-9 and TF02A-26 have significant higher bacterial concentrations at 37ºC (Table S1). For biofilm adhesion, *M. marinus* BC501 was the only isolate to have a significant optimal temperature at 21ºC (Figure 3.2). Isolates TF02-9 and TF02A-27 also have their optimal biofilm adhesion temperature at 21ºC, but this value was not significant ($P>0.1$). At 28ºC, *B. saxobsidens* DD2 was the only isolate to have optimal biofilm adhesion at that temperature, but optimum was not significantly different from the other temperatures ($P>0.05$). Isolates TF02-6, TF02-8, PS03-16, and TF02A-26 were significantly optimal for biofilm production at 37ºC (Table S1).

Among the temperatures tested, 28ºC was chosen to be used as the optimal temperature. At 37ºC there was no significant optimal bacterial concentration with four isolates having significant optimal biofilm adhesion. However, at 37ºC there was a decrease in media volume from evaporation that could dry out the samples and fixed the isolates to the well, so 37ºC was not chosen. At 21ºC, there was no significant optimal bacterial concentration with only one isolate (*M. marinus* BC501) having significant biofilm adhesion. Despite having no significant optimal biofilm adhesion,

28^{o}C was considered the optimal temperature because all eight isolate bacterial concentrations were significantly optimal. As bacterial concentration is the indicator for growth, 28^{o}C was used for all further experiments.

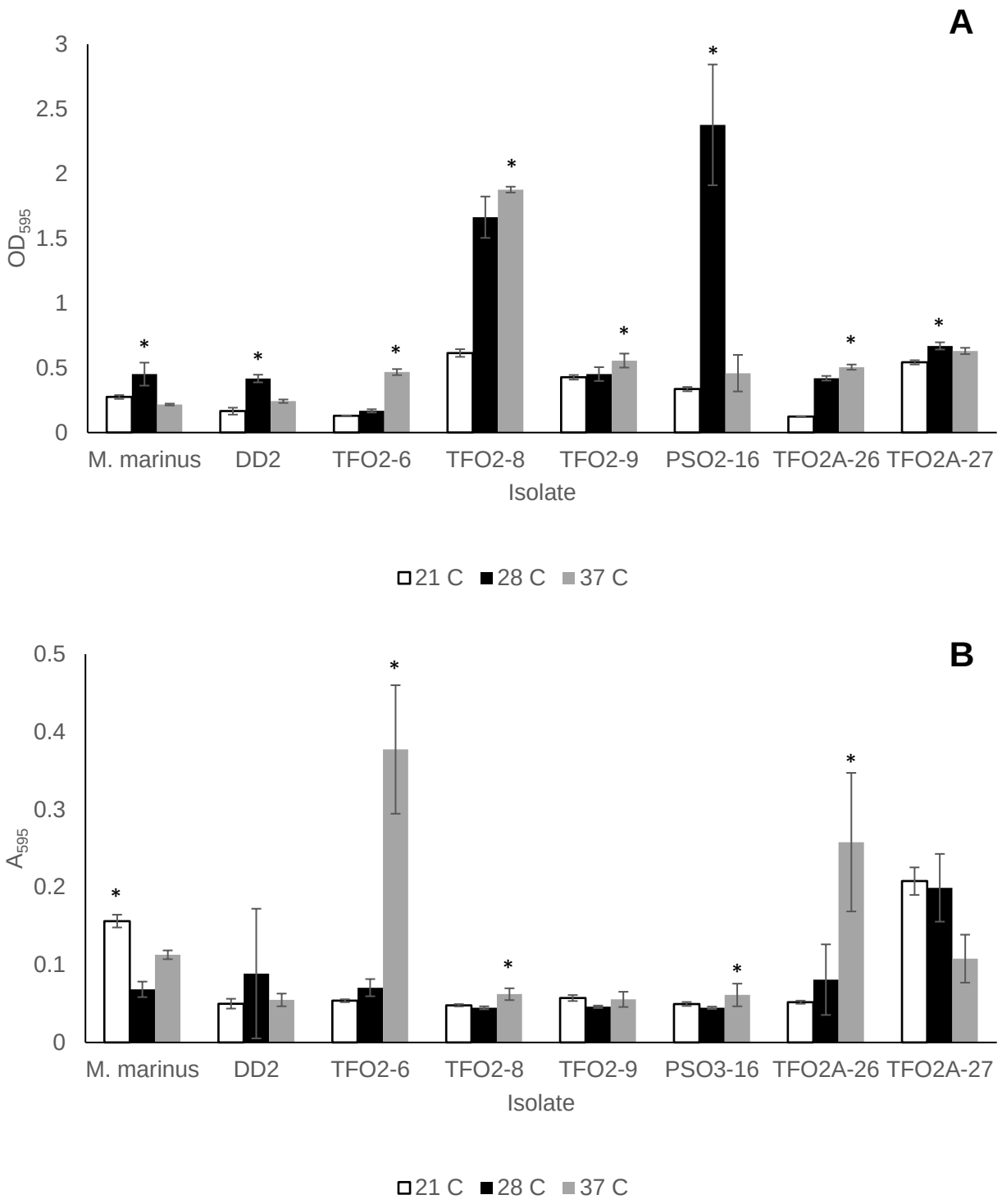


Figure 3.2. The effect of temperature on growth yield and biofilm adhesion for Geodermatophilaceae species. Cultures were incubated for seven days at three temperatures. (A) mean bacterial growth yield as measured by OD_{595} and (B) mean biofilm adhesion value measured by A_{595}. Error bars represent the standard deviation of the mean. Asterisks represents significant optimal temperature ($P<0.01$).

Light Optimization

After determining the optimal temperature to be 28^{o}C, the effect light on Geodermatophilaceae species growth and biofilm adhesion was tested. Although isolates PS03-16 and TF02A-27 were later determined not to be Geodermatophilaceae species (Ennis, 2018), they were included in the light experiment. *Modestobacter marinus* BC501*, B. saxobsidens* DD2, and PS03-16 had significantly higher bacterial concentrations with exposure to light (Table S2), while isolates TF02-6, TF02-8, TF02A-26, and TF02A-27 had significantly higher bacterial concentration without exposure to light (Figure 3.3); (Table S2). Isolate TF02-9 did not show any significant difference for either condition. For biofilm adhesion, *B. saxobsidens* DD2, TF02-6, TF02-8, and TF02-27 showed a significantly higher adhesion rate without exposure to light (Figure 3.3; Table S2). Isolate PS03-16 showed significant biofilm adhesion with 12-hour exposure to light. *Modestobacter marinus* BC501, TF02-9, and TF02A-26 did not show any significant difference between 12-hour and 0-hour exposure to light. Based on these results, a 12-hours of light exposure period was chosen for future experiments to emulate *in vivo* conditions.

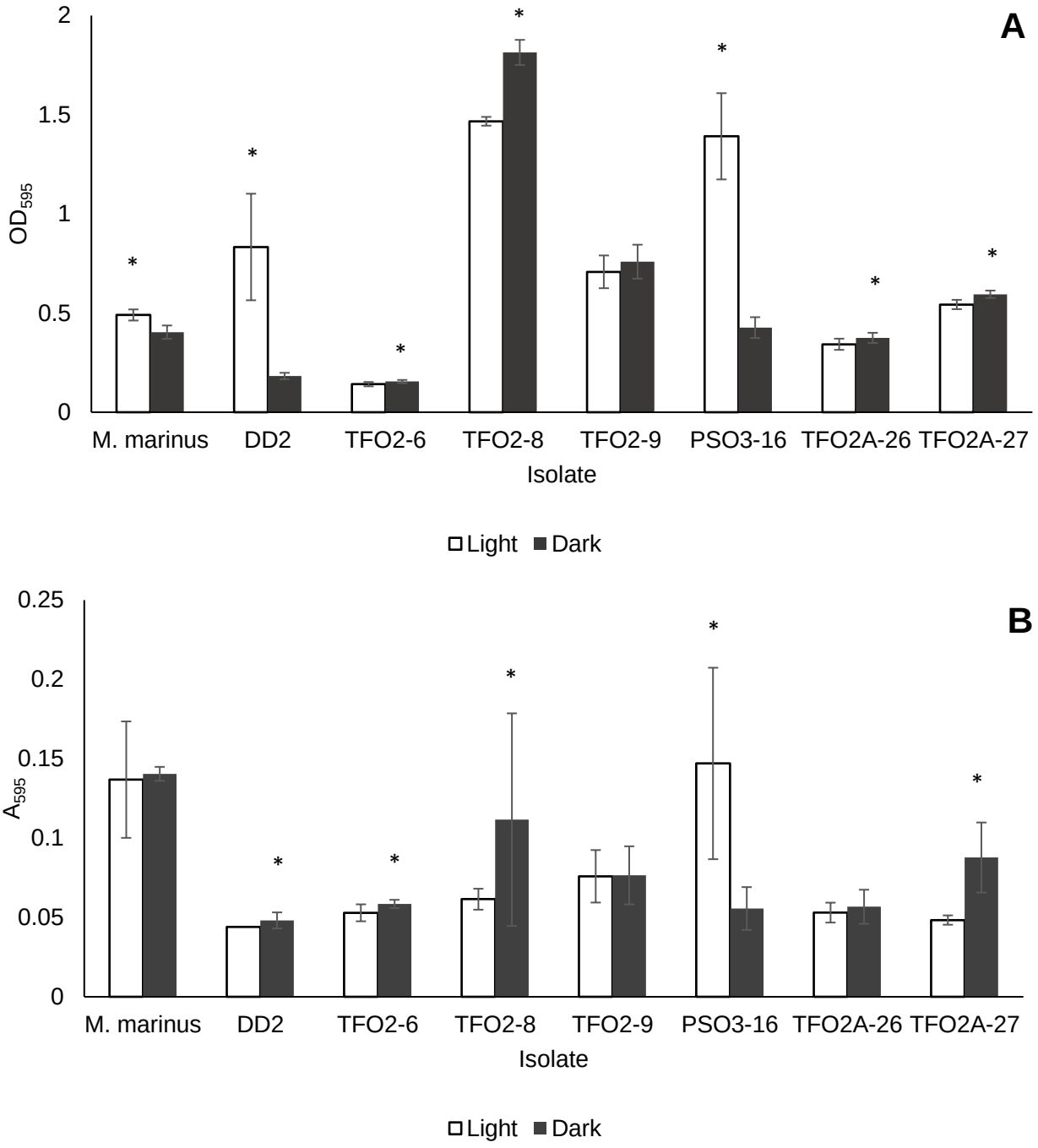


Figure 3.3. The effect of light on bacterial concentration and biofilm adhesion for Geodermatophilaceae species. Cultures were incubated at 28°C for seven days and exposed to 12-hours of light or 0-hours. (A) Mean growth yield as measured by OD_{595} and (B) mean biofilm adhesion rate as measured by A_{595}. Error bars represent the standard deviation of the mean. Asterisks represents significant optimal light condition ($P<0.01$).

Salt Tolerance

For further experiments, twelve full Geodermatophilaceae isolates—*M. marinus* BC501*, B. saxobsidens* DD2, DF01-2, TF02-8, TF02-9, TBT05-19, TF02A-26, TF02A-30, TF02A-35, GayMR16, GayMR19, and GayMR20 were chosen. The genomes of these isolates were completely sequenced and all of them grow well within a week (Ennis 2018). The effect of salt stress on these twelve Geodermatophilaceae isolates were tested for bacterial growth yield (OD_{595}) and biofilm adhesion ability (A_{595}). The growth medium used in the optimization experiments contained 0.01 M NaCl. Figure 3.4 shows representative patterns for the effect of salt stress on growth yield and the biofilm adhesion ability. Isolate TF02A-30 shows a salt-resistant pattern with high MTC and MIC values, while M. *marinus* BC501 show a salt-sensitive pattern with no MTC value and a lower MIC value. Isolate TF02A-35 shows an intermediate pattern. As controls, *E. coli* MG1655 and *B. subtilis* 168 were also included in this experiment but, showed similar patterns to *B. saxobsidens* DD2 (Table 3.1). *Blastococcus saxobsidens* DD2 and isolate TBT05-19 had high bacterial growth yields and low biofilm adherence (A_{595}) to the microtiter plate. DF01-2 and TF02-9 showed low bacterial growth yields and high biofilm adherence.

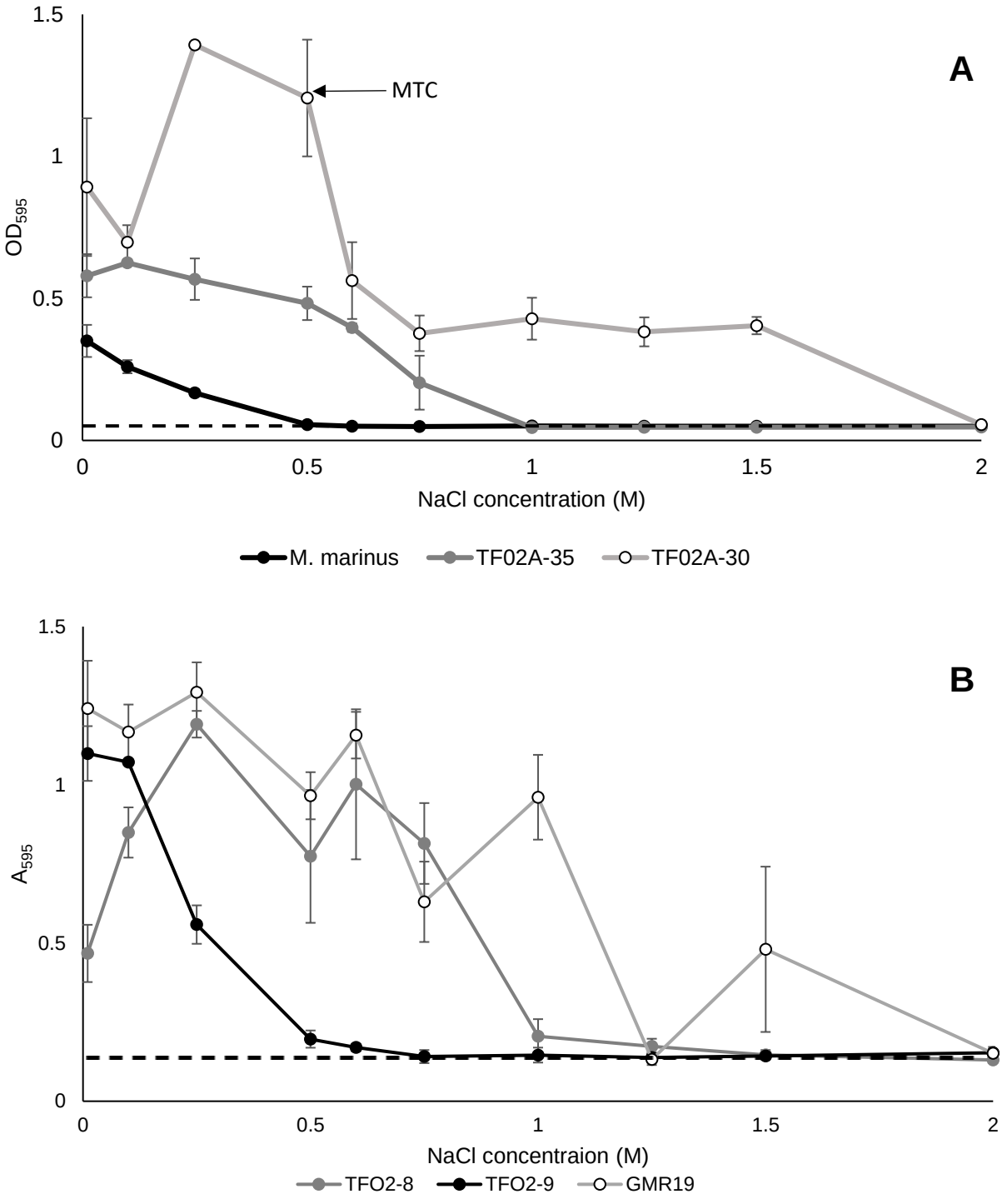


Figure 3.4. Geodermatophilaceae isolate (A) growth yield measured by OD_{595} and (B) biofilm adhesion measured by A_{595} of a resistant (TF02A-30 and GayMR19), intermediate (TF02A-35 and TF02-9) and sensitive (*M. marinus* BC501 and TF02-8) by NaCl concentration (M). Cultures were incubated at 28ºC for seven days and exposed to twelve hours of light. Error bars represent standard deviation and maximum tolerance concentration of TF02A-30 is marked by MTC (n=4). Dashed line represents the threshold value.

Three isolates, TF02-9, TF02A-30 (Figure 3.4), and GayMR19, showed an increase growth yield with the addition of 0.10 to 0.50 M NaCl compared to the control. Additionally, four isolates, *B. saxobsidens* DD2, DF01-2, TF02-8, and TBT05-19, had an increase of bacterial adhesion compared to their growth at 0.01 M NaCl.

The MTC and MIC values were determined as described in the methods and are presented in Table 3.1. Four isolates had high MTC values for growth yields between 0.50 M NaCl and 0.75 M NaCl: GayMR19, TF02A-30, TF02A-35, and GayMR20. GayMR19 had the highest MTC for growth yield and was not affected by the salt concentration until 0.75 M NaCl (Table 3.1). TF02A-30 had the second highest MTC and was only affected at 0.6 M NaCl. TF02A-35 and GayMR20 had the third highest MTC at 0.50 M NaCl (Table 3.1). Looking at MTCs for biofilm adhesion, GayMR19 also had the highest MTC at 0.75 M along with TF02-8 (Table 3.1). The third highest MTC for biofilm adhesion was TBT05-19 at 0.60 M (Table 3.1). There were three isolates the intermediate growth yield range of MTCs at 0.25 being *B. saxobsidens* DD2, TF02-8, and TF02A-26 (Table 3.1). For biofilm adhesion, the range was between 0.10 M and 0.50 M which included five isolates. *B. saxobsidens DD2* had an MTC at 0.50 M and DF01-2 had an MTC at 0.25 M (Table 3.1). The other three isolates, *M. marinus* BC501, TF02-9, and GayMR20 had an MTC at 0.10 M (Table 3.1). A sensitive growth yield MTC in this group was determined to be from 0.10 M and below. The low MTC group consisted of *M. marinus* BC501, DF01-2, TF02-9, TBT05-19, and GayMR16 (Table 3.1). *M. marinus* BC501, DF01-2 and TF02-9 having an MTC of 0.10 M NaCl and TBT05-19 and GayMR16 having an MTC of 0.01 M NaCl (Table 3.1). The sensitive

biofilm adhesion MTC was at 0.01 M. Those isolates were TF02A-26, TF02A-30, TF02A-35, and GayMR16 (Table 3.1).

Turning to MICs, *B. saxobsidens* DD2, TF02A-30, and GayMR19 exhibited high resistance growth yields to salt and had MICs at 1.5 M to 2.0 M (Table 3.1). TF02A-30 tolerated the highest concentration with an MIC at 2.0 M. For biofilm adhesion, GayMR19 had the highest MIC at 1.25 M. The intermediate growth yield tolerance to salt was between 0.75 M and 1.00 M. Species that exhibited MICs between that included, *M. marinus* BC501, DF01-2, TF02-8, TF02-9, TBT05-19, TF02A-35, and GayMR20 (Table 3.1). Again, the same range was true for biofilm adhesion that includes seven isolates. *B. saxobsidens* DD2 had the MIC of 0.60 M (Table 3.1). Both *M. marinus* BC501 and DF01-2 had the MIC of 0.75 M (Table 3.1). Four isolates, TF02-8, TBT05-19, TF02A-35, and GayMR20 had an MIC of 1.00 M (Table 3.1). On the sensitive growth yield MIC side were *M. marinus* BC501, DF01-2, TF02A-26, and GayMR16 that was below 0.50 M. DF01-2 and GayMR16 had an MIC of 0.10 M, and *M. marinus* BC501 and TF02A-26 had an MIC of 0.50 M (Table 3.1). The sensitive biofilm adhesion range was the same and included TF02-9, TF02A-26, TF02A-30, and GayMR16. GayMR16 having the lowest MIC for biofilm adhesion at 0.01 M (Table 3.1). The second most sensitive was TF02A-30 at an MIC of 0.25 M (Table 3.1). TF02-9 and TF02A-26 had the same MIC at 0.50 M (Table 3.1).

Growth yields expressed higher or equal MICs than biofilm adhesion except for *M. marinus* BC501 and DF01-2. Neither growth yield nor biofilm adhesion had higher MTCs than the other. Looking at specific isolates, all except for one species (GayMR16) demonstrates an elevated tolerance of salt. The two that are the most

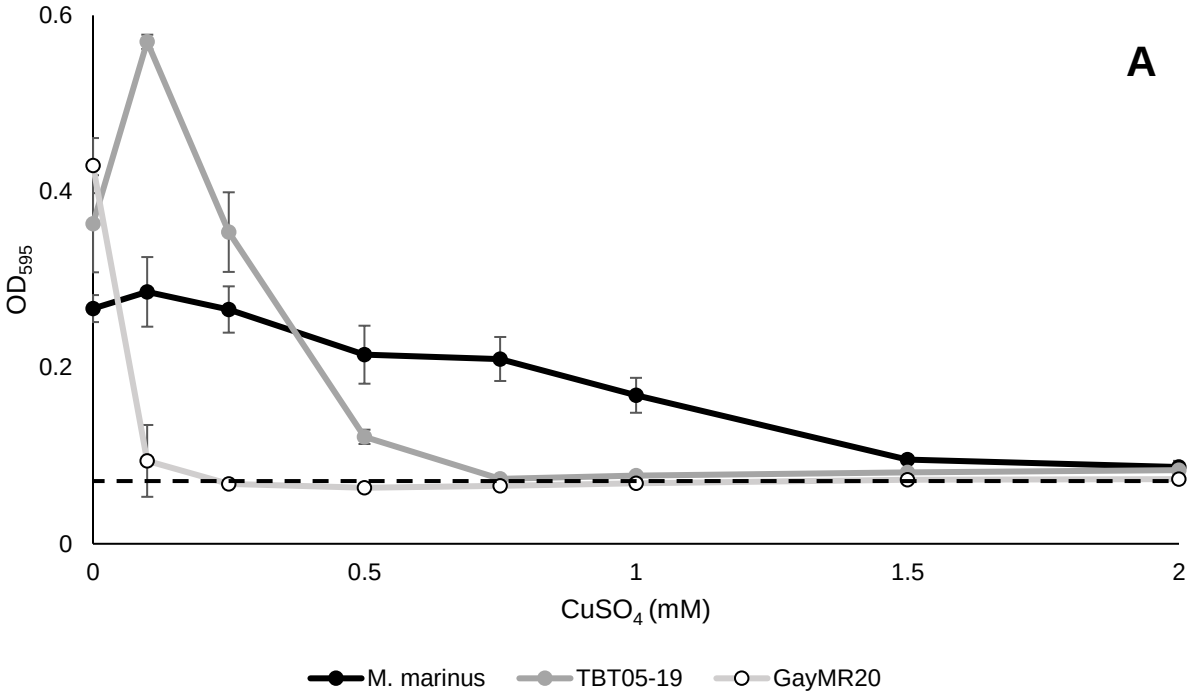
resistant are TF02A-30 and GayMR19. The one that was immediately affected by a higher salt concentration was GayMR16 (Table 3.1). GayMR16 is the most sensitive to higher salt concentrations as it also has a sensitive growth yield MIC (0.10 M). There seems to be different methods of tolerances as a couple hit a threshold and were unable to grow after it while some slow down growth and still grow at higher concentrations (Table 3.1).

Table 3.1. MTC and MIC of NaCl for twelve Geodermatophilaceae. Cultures were incubated for two-week incubation at 28°C with twelve hours of light exposure. The MTC and MIC were determined and the mean MTC and MIC (n=4) values are presented.

Isolate	NaCl concentration (M)			
	Growth Yield		Biofilm Adhesion	
	MIC	MTC	MIC	MTC
M. marinus BC501	0.50	0.01	0.75	0.10
B. saxobsidens DD2	1.50	0.25	0.60	0.50
DF01-2	0.10	0.10	0.75	0.25
TF02-8	1.00	0.25	1.00	0.75
TF02-9	0.75	0.10	0.50	0.10
TBT05-19	1.00	0.01	1.00	0.60
TF02A-26	0.50	0.25	0.50	0.01
TF02A-30	2.00	0.60	0.25	0.01
TF02A-35	1.00	0.50	1.00	0.01
GayMR16	0.10	0.01	0.01	0.01
GayMR19	1.50	0.75	1.25	0.75
GayMR20	1.00	0.50	1.00	0.10
B. subtilis	2.00	0.10	0.25	0.10
E. coli	1.50	0.25	0.50	0.10

Heavy Metal Tolerance

Because rock surfaces are composed of different metals, the heavy metal sensitivities of the twelve Geodermatophilaceae isolates, along with *E. coli* MG1655 and *B. subtilis* 168 control, were tested for their effects on growth yield (OD_{595}) and biofilm adhesion (A_{595}) (Figure 3.5). Resistance to heavy metals was determined by the biofilm adhesion assay using both A_{595} and OD_{595} to determine the MTC and MIC (Table 3.2 and Table 3.3). The MICs and MTCs of the isolates to the six heavy metals is described below.



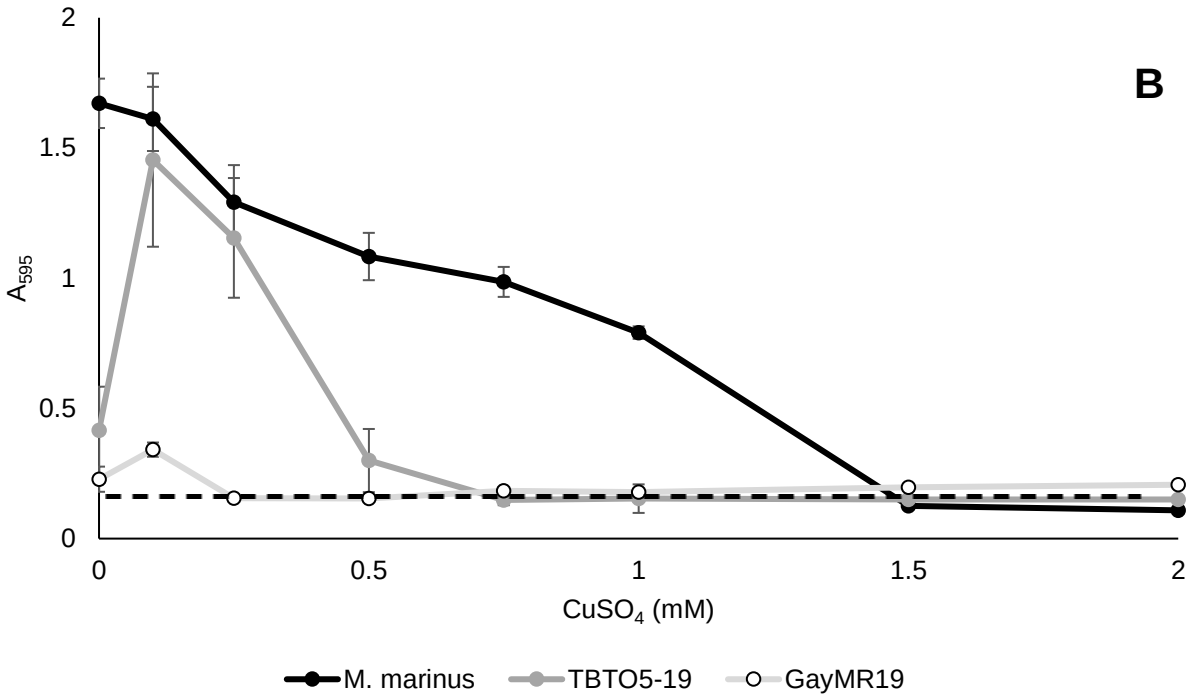


Figure 3.5. The effect of copper sulfate on (A) growth yield measured by OD_{595} and (B) biofilm adhesion as measured by A_{595}, of a copper-resistant isolate (*M. marinus* BC501), intermediate-resistant strain (TBT05-19) and sensitive-strain (GayMR20 and GayMR19). Cultures were incubated at 28°C for seven days and exposed to twelve hours of light. Error bars represent standard deviation. Dashed line represents threshold value.

Table 3.2. Heavy metal MIC values (mM) for twelve Geodermatophilaceae isolates using growth yield (OD_{595}) and biofilm adhesion (A_{595}). Cultures were incubated for two weeks at 28°C with twelve hours of light exposure.

Isolate	MIC (mM)											
	Growth Yield						Biofilm Adhesion					
	$CoCl_2$	NiCl	$CuSO_4$	$Pb(NO_3)_2$	$KAsO_4$	CrO_4	$CoCl_2$	NiCl	$CuSO_4$	$Pb(NO_3)_2$	$KAsO_4$	CrO_4
M. marinus BC501	0.25	1.00	1.50	2.5	>50	1.0	0.75	1.50	1.50	2.5	>50	5.0
B. saxobsidens DD2	0.50	0.50	0.75	2.0	2.5	0.25	0.50	0.50	0.75	2.0	2.5	0.50
DF01-2	<0.1	0.10	<0.1	<1	>50	<0.1	<0.1	0.10	<0.1	<1	>50	<0.1
TF02-8	0.50	0.50	1.00	2.5	50	1.5	0.50	0.50	1.00	5.0	50	1.5
TF02-9	0.25	0.50	0.50	<1	20	<0.1	0.25	0.25	0.50	2.5	25	10
TBT05-19	0.25	0.25	0.75	1.0	>50	0.50	0.25	0.25	0.75	2.0	>50	10
TF02A-26	0.10	0.25	0.50	1.0	>50	<0.1	0.10	0.25	0.50	1.0	>50	>20
TF02A-30	0.75	0.50	0.50	<1	>50	0.50	0.75	0.50	0.50	<1	>50	>20
TF02A-35	0.10	0.25	0.50	<1	5.0	<0.1	0.25	0.25	0.50	1.0	2.5	20
GayMR16	0.1	0.25	<0.1	<1	20	<0.1	0.10	0.25	<0.1	<1	20	7.5
GayMR19	<0.1	0.10	0.25	<1	20	0.25	<0.1	<0.1	0.25	<1	50	1.5
GayMR20	<0.1	0.10	0.25	1.0	>50	0.50	0.10	0.25	0.50	7.5	>50	2.0
B. subtilis	0.50	1.00	0.50	<1	25	1.0	0.25	1.00	0.50	5.0	15	5.0
E. coli	0.50	0.75	0.50	2.5	15	0.50	0.25	0.75	1.50	5.0	10	2.5

Table 3.3. Heavy metal MTC values (mM) for twelve Geodermatophilaceae isolates using growth yield (OD_{595}) and biofilm adhesion (A_{595}). Cultures were incubated for two weeks at 28°C with twelve hours of light exposure.

Isolate	MTC (mM)											
	Growth Yield						Biofilm Adhesion					
	$CoCl_2$	NiCl	$CuSO_4$	$Pb(NO_3)_2$	$KAsO_4$	CrO_4	$CoCl_2$	NiCl	$CuSO_4$	$Pb(NO_3)_2$	$KAsO_4$	CrO_4
M. marinus BC501	0.10	0.10	0.25	1.0	<1	<0.1	<0.1	<0.10	0.10	<1	25	0.25
B. saxobsidens DD2	<0.1	0.10	<0.1	1.0	<1	<0.1	<0.1	0.10	0.50	1.0	2.0	<0.1
DF01-2	<0.1	<0.1	<0.1	<1	>50	<0.1	<0.1	<0.1	<0.1	<1	>50	<0.1
TF02-8	<0.1	<0.1	<0.1	<1	<1	<0.1	0.10	<0.1	0.75	<1	7.5	0.50
TF02-9	<0.1	0.10	<0.1	<1	5.0	<0.1	<0.1	0.10	0.10	<1	1.0	<0.1
TBT05-19	0.10	0.10	0.25	<1	<1	<0.1	0.25	0.10	0.25	1.0	<1	0.50
TF02A-26	<0.1	0.10	<0.1	<1	>50	<0.1	<0.1	0.10	<0.1	<1	>50	<0.1
TF02A-30	<0.1	<0.1	0.25	<1	<1	<0.1	<0.1	<0.1	0.10	<1	<1	<0.1
TF02A-35	<0.1	<0.1	0.25	<1	<1	<0.1	0.10	0.10	0.25	<1	<1	2.0
GayMR16	<0.1	<0.1	<0.1	<1	10	<0.1	<0.1	<0.1	<0.1	<1	15	<0.1
GayMR19	<0.1	<0.1	0.10	<1	<1	<0.1	<0.1	<0.1	0.10	<1	10	0.50
GayMR20	<0.1	<0.1	<0.1	<1	2.0	<0.1	<0.1	<0.1	<0.1	<1	5.0	<0.1
B. subtilis	0.25	0.5	<0.1	1.0	2.5	0.25	0.10	0.10	0.25	2.5	2.0	<0.1
E. coli	<0.1	0.10	<0.1	<1	<1	<0.1	0.10	0.10	<0.1	<1	<1	<0.1

Cobalt chloride

For two isolates (TF02-8 and TBT05-19), additional (0.10 mM) cobalt increased biofilm adhesion over the control (0 mM). Isolate TF02-8 had a significantly higher adhesion value going from a mean A_{595} of 1.02 to 1.44 (P=0.034), while isolate TBT05-19 also had a significant increase going from a mean A_{595} of 0.32 to 0.58 (P<0.001).

DF01-2 and GayMR19 were sensitive to cobalt and did not grow in the presence of this heavy metal (Table 3.2). They all had no MTC and MIC values in growth yield or biofilm adhesion (Table 3.2 and Table 3.3).

There were no isolates that had a high resistance to cobalt for MTC for growth yields, however, TBT05-19 did have a high MTC for biofilm adhesion at 0.25 mM (Table 3.3). Two isolates had an intermediate MTC for growth yields. *M. marinus* BC501 and TBT05-19 had an MTC at 0.10 mM (Table 3.3). There were also two isolates that had an intermediate MTC for cobalt for biofilm adhesion at 0.10 mM that were TF02-8 and TF02A-35 (Table 3.3). Lastly, there were eight isolates, *B. saxobsidens* DD2, TF02-8, TF02-9, TF02A-26, TF02A-30, TF02A-35, GayMR16, and GayMR20, that had sensitive growth yields to cobalt and did not have an MTC (Table 3.3). For biofilm adhesion, there were seven isolates that did not have an MTC which included *M. marinus* BC501, *B. saxobsidens* DD2, TF02-9, TF02A-26, TF02-30, GayMR16, and GayMR20 (Table 3.3). These isolates were also cobalt sensitive according to biofilm adhesion. However, *B. subtilis* 168 had a high growth yield MTC for cobalt at 0.25 mM and *E. coli* MG1655 did not have a growth yield MTC. At 0.10 mM, both *B. subtilis* 168 and *E. coli* MG1655 had an intermediate biofilm MTC.

At high growth yield MIC, TF02A-30 had an MIC at 0.75 mM meaning resistance to cobalt (Table 3.2). For biofilm adhesion, two isolates, *M. marinus* BC501 and TF02A-35 had high MICs at 0.75 mM (Table 3.2). The intermediate growth yield range for cobalt fell from 0.25 mM and 0.50 mM of five isolates—*M. marinus* BC501, *B. saxobsidens* DD2, TF02-8, TF02-9, and TBT05-19. Three of those isolates, *M. marinus* BC501, TF02-9, and TBT05-19 had a growth yield MIC at 0.25 mM, while *B. saxobsidens* DD2 and TF02-8 were at 0.50 mM (Table 3.2). Biofilm adhesion had the same intermediate cobalt resistance range that includes five isolates as well. At 0.25 mM MIC were TF02-9, TBT05-19, and TF02A-35 (Table 3.2). The other two, *B. saxobsidens* DD2 and TF02-8 had a MIC at 0.50 mM (Table 3.2). The sensitive cobalt growth yield was at 0.10 mM and below that includes TF02A-26, TF02A-35, GayMR16, and GayMR20 (GayMR20 being the only one that did not have an MIC) (Table 3.2). For biofilm adhesion, TF02A-26, GayMR16, and GayMR20 all had sensitive cobalt MICs at 0.10 mM (Table 3.2). Both *E. coli* MG1655 and *B. subtilis* 168 had intermediate growth yield MICs at 0.50 mM and biofilm adhesion at 0.25 mM (Table 3.2).

Nickel chloride

Nickel resistance had similar results to cobalt, however, the isolates showed a slightly higher resistance. There was no high resistance to nickel for growth yield and biofilm adhesion MTCs. There were five isolates that had an intermediate growth yield MTC for nickel, *M. marinus* BC501, *B. saxobsidens* DD2, TF02-9, TBT05-19, and TF02A-26, at 0.10 mM (Table 3.3). The same is true for biofilm adhesion, except for *M. marinus* BC501, TF02A-35 took its place (Table 3.3). Seven isolates did not have a growth yield MTC that includes DF01-2, TF02-8, TF02A-30, TF02A-35, GayMR16,

GayMR19 and GayMR20 that were sensitive to nickel (Table 3.3). There were also seven isolates sensitive to nickel that did not have biofilm adhesion MTCs: *M. marinus* BC501, DF01-2, TF02-8, TF02A-30, GayMR16, GayMR19, and GayMR20 (Table 3.3). *M. marinus* BC501 and TF02A-35 were the isolates that had MTCs for either growth yields or biofilm adhesion. In the intermediate nickel resistance range for growth yield MIC, *E. coli* MG1655 was at 0.10 mM (Table 3.3). *B. subtilis* 168 had the highest MTC for growth yields at 0.50 mM (Table 3.3). Both *E. coli* MG1655 and *B. subtilis* 168 had intermediate biofilm adhesion MTCs at 0.10 mM (Table 3.3).

The isolate with the highest growth yield MIC at 1.00 mM NiCl was *M. marinus* BC501 (Table 3.2). *M. marinus* BC501 also had the highest nickel resistance MIC biofilm adhesion at 1.50 mM (Table 3.2). The intermediate nickel resistance growth yield range was from 0.25 mM and 0.50 mM that included eight isolates. Half of the isolates had an MIC at 0.50 mM which were *B. saxobsidens* DD2, TF02-8, TF02-9, and TF02A-30 (Table 3.2). The other half of the intermediate range at 0.25 mM were TBT05-19, TF02A-26, TF02A-35, and GayMR16 (Table 3.2). The intermediate nickel resistance biofilm adhesion range was the same from 0.25 mM to 0.50 mM, but there were nine isolates that belonged in this range. Only three isolates, *B. saxobsidens* DD2, TF02-8, and TF02A-30, had an MIC of 0.50 mM (Table 3.2). The other six, TF02-9, TBT05-19, TF02A-26, TF02A-35, GayMR16, and GayMR20 had an MIC of 0.25 mM (Table 3.2). The sensitive to nickel growth yield MIC was at 0.10 mM that includes DF01-2, GayMR19, and GayMR20 (Table 3.2). For biofilm adhesion there were only two nickel sensitive isolates. DF01-2 had an MIC at 0.10 and GayMR19 did not have a biofilm adhesion MIC (Table 3.2). *E. coli* MG1655 and *B. subtilis* 168 had high growth

yield and biofilm adhesion MICs at 0.75 mM and 1.00 mM for both respectively (Table 3.2).

Copper sulfate

Bacterial growth and adhesion with copper and without showed some isolates grew better with copper. TBT05-19 and TF02A-35 had a higher bacterial concentration at 0.10 mM than at 0 mM. TBT05-19 has a mean OD_{595} of 0.36 at 0 mM which significantly increase to 0.57 at 0.10 mM (t_5=-55.08, P<0.001). TF02A-35 significantly increased from a mean OD_{595} of 0.17 at 0 mM to 0.39 at 0.10 mM (t_4=-15.91, P<0.001). In addition, TBT05-19 and TF02A-35 along with GayMR19 had a higher biofilm adhesion at 0.10 mM than at 0 mM. TBT05-19 had a mean A_{595} of 0.42 at 0 mM which significantly increased to 1.45 at 0.10 mM (t_4=-6.28, P=0.002) and stayed at a high mean A_{595} of 1.15 at 0.25 mM until it dropped off. TF02A-35 significantly increased from a mean A_{595} of 0.82 at 0 mM to 2.19 at 0.10 mM (t_2=-108.70, P<0.001) and stayed at a high mean A_{595} of 1.47 0.25 mM until dropping off. Lastly, GayMR19 significantly increased in mean A_{595} going from 0.23 at 0 mM to 0.34 at 0.10 mM (t_6=-3.55, P=0.006).

Next, the Geodermatophilaceae isolates showed a resistance to copper. Only two isolates, DF01-2 and GayMR16, were too sensitive to grow in the presence of copper therefore having no MICs or MTCs for growth yields and biofilm adhesion. Two other isolates did not have an MTC for both growth yield and biofilm adhesion, TF02A-26 and GayMR20 (not including DF01-2 and GayMR16) and were affected by copper immediately (Table 3.3).

For growth yield, those that had a high copper resistant growth yield MTC of 0.25 mM included, *M. marinus* BC501, TBT05-19, TF02A-30, and TF02A-35 (Table 3.3). The highest copper resistance MTC for biofilm adhesion was TF02-8 at 0.75 mM (Table 3.3). Back to growth yield, GayMR19 fell into intermediate copper resistance MTC with a value of 0.10 mM (Table 3.3). The biofilm adhesion MTC intermediate copper resistance range fell between 0.25 mM and 0.50 mM. The isolates that had an MTC at 0.25 mM were TBT05-19 and TF02A-35 and at 0.50 mM was *B. saxobsidens* DD2 (Table 3.3). Lastly for growth yield MTCs, there were three copper sensitive isolates that did not have an MTC the included *B. saxobsidens* DD2, TF02-8, and TF02-9 (Table 3.3). Those isolates that had copper sensitive MTCs for biofilm adhesion had an MTC at 0.10 mM that included, *M. marinus* BC501, TF02-9, TF02A-30, and GayMR19 (Table 3.3). *E. coli* MG1655 did not have an MTC for growth yield nor for biofilm adhesion (Table 3.3). Additionally, *B. subtilis* 168 did not have an MTC for growth yields but did have an intermediate MTC for biofilm adhesion at 0.25 mM (Table 3.3).

Next to MICs, four copper resistant growth yield isolates: *M. marinus* BC501, *B. saxobsidens* DD2, TF02-8, and TBT05-19 had a high MIC range of 0.75 mM to 1.50 mM (Table 3.2). *M. marinus* BC501 having the highest copper resistance with an MIC at 1.50 mM followed by TF02-8 at 1.00 mM, then *B. saxobsidens* DD2 and TBT05-19 at 0.75 mM (Table 3.2). The same was exactly true for the four copper resistant biofilm adhesion MIC as it was for the growth yields; in fact, the only difference in MICs between growth yield and biofilm adhesion was GayMR20 that had a higher biofilm adhesion resistance. Starting with the intermediate copper resistance for growth yield was in the range of 0.25 mM to 0.50 mM which contained six isolates: TF02-9, TF02A-

26, TF02A-30, TF02A-35, GayMR19 and GayMR20 (Table 3.2). The intermediate copper resistance range for biofilm adhesion MICs had six isolates as well between 0.25 mM and 0.50 mM. At 0.25 mM was GayMR19 and the other five at 0.50 mM were TF02-9, TF02A-26, TF02A-30, TF02A-35, and GayMR20 (Table 3.2). The two copper sensitive isolates for both growth yield and biofilm adhesion were below 0.10 mM and were stated previously. At 0.50 mM for growth yields, *E. coli* MG1655 and *B. subtilis* 168 had intermediate MICs (Table 3.2). For biofilm adhesion, *B. subtilis* 168 was again in the intermediate MIC at 0.50 mM, but *E. coli* MG1655 was highly resistant at 1.50 mM (Table 3.2).

Lead nitrate

Two isolates had better biofilm adhesion with lead present than without lead. *B. saxobsidens* DD2 and GayMR19 peak biofilm adhesion was not at 0 mM. *B. saxobsidens* DD2 had a significantly higher biofilm adhesion at 1.0 mM than at 0 mM—going from a mean A_{595} of 0.24 to 0.41 (t_3=-3.70, P=0.02). GayMR19 had peak biofilm adhesion at 5.0 mM significantly increasing from a mean A_{595} of 0.24 at 0 mM to 0.47 at 5.0 mM (t_5=-11.08, P<0.001).

Lead resistance had a similar result to cobalt as well. Two out of all the isolates, *M. marinus* BC501 and *B. saxobsidens* DD2, had a growth yield MTC at 1.0 mM and the rest had no MTCs showing growth hinderance because of lead (Table 3.3). For biofilm adhesion MTC, there, again, were only two isolates that had an MTC at 1.0 mM: *B. saxobsidens* DD2 (again) and TBT05-19 (Table 3.3). Four isolates—DF01-2, TF02A-30, GayMR16, and GayMR19—were unable to grow in the presence of lead having no MICs or MTCs for growth yields or for biofilm adhesion (Table 3.2). For

growth yield and biofilm adhesion, *E. coli* MG1655 also did not have an MTC. However, *B. subtilis* 168 did for growth yield the MTC was at 1.0 mM and the biofilm adhesion was high at 2.5 mM (Table 3.3).

There was no isolate growth yield that had a high resistant MIC. The high lead resistance biofilm adhesion MIC range was between 5.0 mM and 7.5 mM. At 5.0 mM was TF02-8 and GayMR20 was the most resistant to lead with an MIC at 7.5 mM (Table 3.2). The intermediate lead resistance growth yield MIC range was between 2.0 mM and 2.5 mM which three isolates: *M. marinus* BC501 (2.5 mM), *B. saxobsidens* DD2 (2.0 mM), and TF02-8 (2.5 mM) (Table 3.2). The same range of 2.0 mM to 2.5 mM was used for the intermediate lead resistance biofilm adhesion MIC. Four isolates were in the range which were *B. saxobsidens* DD2 and TBT05-19 at 2.0 mM and *M. marinus* BC501 and TF02-9 at 2.5 mM (Table 3.2). The lead sensitive range for growth yield was 0.10 mM and below. Two isolates (besides the four that did not grow at all) were below 0.10 mM which were TF02-9 and TF02A-35 (Table 3.2). There are three isolates, TBT05-19, TF02A-26 and GayMR20 that had MICs at 0.10 mM (Table 3.2). For biofilm adhesion, there were two isolates, TF02A-26 and TF02A-35, that had a lead sensitive MIC of 1.0 mM (Table 3.2). For growth yield MIC, *B. subtilis* 168 was sensitive to lead and did not have an MIC while *E. coli* MG1655 had an intermediate MIC of 2.5 mM (Table 3.2). For biofilm adhesion, they both had a high MIC of 5.0 mM (Table 3.2).

Arsenate oxide

There was one isolate the grew better with arsenate than without and four that had higher biofilm adhesion with arsenate than the without it. TF02A-26 had the highest bacterial concentration at 50.0 mM with a mean OD_{595} of 0.20 a significant increase of a

mean OD_{595} of 0.08 at 0 mM (t_3=-6.89, P=0.003). TF02A-26 did not have the highest bacterial concentration at 0 mM. In addition, TF02A-26 along with *M. marinus* BC501, DF01-2, and GayMR16 did not have the highest biofilm adhesion at 0 mM. TF02A-26 significantly increased from a mean A_{595} of 0.35 at 0 mM to 0.88 at 50.0 mM (t_4=-14.17, P<0.001). *M. marinus* BC501 significantly increased from a mean A_{595} of 1.10 at 0 mM to 1.54 at 2.0 mM (t_2=-10.87, P=0.004). DF01-2 significantly increased from a mean A_{595} of 0.2 at 0 mM to 0.47 at 10.0 mM (t_4=-9.09, P<0.001). GayMR16 significantly increased from a mean A_{595} of 0.44 at 0 mM to 1.00 at 7.5 mM (t_3=-4.48, P=0.01).

In contrast to the other heavy metals, Geodermatophilaceae showed a high resistance to arsenate. The two isolates that had a high arsenate resistance growth yield and biofilm adhesion MTC that grew above 50 mM. Both DF01-2 and TF02A-26 had an MTC above 50 mM (Table 3.3). The intermediate arsenate resistance growth yield MTC range was wide going from 2.0 mM to 10 mM and consisted of three isolates, TF02-9 (5.0 mM), GayMR16 (10 mM), and GayMR20 (2.0 mM) (Table 3.3). The intermediate arsenate resistance range for biofilm adhesion MTC was much higher and wider than growth yield going from 7.5 mM to 25 mM. There were four isolates in that range: TF02-8 at 7.5 mM, GayMR19 at 10 mM, GayMR16 at 15 mM and *M. marinus* BC501 at 25 mM (Table 3.3). Seven isolates were sensitive to arsenate had a growth yield MTC below 1.0 mM. The sensitive isolates include, *M. marinus* BC501, *B. saxobsidens* DD2, TF02-8, TBT05-19, TF02A-30, TF02A-35, and GayMR19 (Table 3.3). For biofilm adhesion, the arsenate sensitive MTC range was wider going from below 1.0 mM to 5.0 mM for six isolates. There were only three isolates, TBT05-19, TF02A-30, and TF02A-35, that were below 1.0 mM (Table 3.3). The other isolates

biofilm adhesion MTC were TF02-9 at 1.0 mM, *B. saxobsidens* DD2 at 2.0 mM, and GayMR20 at 5.0 mM (Table 3.3). For *E. coli* MG1655, the growth yield and biofilm adhesion MTC were the as it was below 1.0 mM for both (Table 3.3). Looking at *B. subtilis* 168, it had a growth yield MTC of 2.5 mM in the intermediate range and in the biofilm adhesion range it was in the sensitive range at 2.0 mM (Table 3.3).

Looking at MICs, this time six isolates had a high resistance to arsenate growth yield and biofilm adhesion MIC that went above 50 mM. Isolates, *M. marinus* BC501, DF01-2, TBT05-19, TF02A-26, TF02A-30 and GayMR20 had an MIC above 50 mM (Table 3.2). The MIC intermediate arsenate resistance growth yield range was at 20 mM to 50 mM and consisted of four isolates. Three isolates had an MIC of 20 mM which were TF02-9, GayMR16, and GayMR19 (Table 3.2). The other one, TF02-8, had a MIC at 50 mM (Table 3.2). The intermediate arsenate resistance range for biofilm adhesion was also from 20 mM to 50 mM included four isolates. One isolate, GayMR16 was at 20 mM and another one, TF02-9, was at 25 mM (Table 3.2). The other two isolates, TF02-8 and GayMR19, had an MIC of 50 mM (Table 3.2). Two isolates had a sensitive arsenate growth yield MIC range from 2.5 mM to 5.0 mM. At 2.5 Mm was *B. saxobsidens* DD2 and TF02A-35 was at 5.0 mM (Table 3.2). For biofilm adhesion, there were two isolates, *B. saxobsidens* DD2 and TF02A-35, that were sensitive to arsenate with an MIC of 2.5 mM (Table 3.2). For growth yield, both *B. subtilis* 168 (at 25 mM) and *E. coli* MG1655 (at 15 mM) were in the lower end of the intermediate range MIC (Table 3.2). The same was true for biofilm adhesion MIC, for *B. subtilis* 168 was at 15 mM and *E. coli* MG1655 was at 10 mM (Table 3.2).

Chromate oxide

Four isolates had better biofilm adhesion with chromate present than without it. TF02-8, TBT05-19, TF02A-26, and TF02A-35 peak biofilm adhesion was not at 0 mM. TF02-8 significantly increased from a mean A_{595} of 0.37 at 0 mM to 0.53 at 0.5 mM (t_4=-5.38, P=0.002). TBT05-19 significantly increased from a mean A_{595} of 0.59 at 0 mM to 1.49 at 0.1 mM (t_2=-19.26, P=0.001). TF02A-26 significantly increased from a mean A_{595} of 0.33 at 0 mM to 0.43 at 2.0 mM (t_5=-2.45, P=0.03). Lastly, TF02A-35 significantly increased from a mean A_{595} of 0.28 at 0 mM to 0.37 at 1.5 mM (t_4=-4.74, P=0.005).

One isolates could not grow in the presences of chromate. The sensitive isolate being DF01-2 that had no MICs or MTCs for growth yield or for biofilm adhesion (Table 3.2 and Table 3.3). The rest of the Geodermatophilaceae isolates had a wide range of resistance.

All the Geodermatophilaceae isolates had growth yield MTC below 0.1 mM (Table 3.3). The isolates were sensitive to chromate. Biofilm adhesion was affected differently as there were some isolates that were able to attach to the well. One isolate, TF02A-35 had the highest chromate resistance biofilm adhesion MTC of 2.0 mM (Table 3.3). The intermediate chromate resistance range for biofilm adhesion MTC was between 0.25 mM and 0.50 mM. One isolate, *M. marinus* BC501, had an MTC of 0.25 (Table 3.3). The other three had an MTC of 0.50 mM which were TF02-8, TBT05-19, GayMR19 (Table 3.3). There were seven isolates that had an MTC below 0.1 mM which included *B. saxobsidens* DD2, DF01-2, TF02-9, TF02A-26, TF02A-30, GayMR16, and GayMR20 (Table 3.3). Unlike all the other isolates, including *E. coli* MG1655, *B.*

subtilis 168 had a growth yield MTC at 0.50 mM instead of being below 0.1 mM (Table 3.3).

On the high chromate resistant end of growth yield MIC, *M. marinus* BC501 had an MIC of 1.0 mM, and TF02-8 was the most resistant to chromate with an MIC of 1.5 mM (Table 3.2). For biofilm adhesion, there were three isolates with an MIC of 20 mM and above. At 20 mM was TF02A-35 and two isolates, TF02A-26 and TF02A-30, did not have their MIC reach and went above 20 mM (Table 3.2). The intermediate chromate resistance growth yield MIC range was from 0.25 mM to 5.0 mM that included five isolates, *B. saxobsidens* DD2 (0.25 mM), TBT05-19 (0.50 mM), TF02A-30 (0.50 mM), GayMR19 (0.25 mM), and GayMR20 (0.50 mM) (Table 3.2). The intermediate chromate resistance biofilm adhesion MIC range was a lot wider going from 1.5 mM to 10 mM which included seven isolates. There were two isolates at 1.5 mM that were TF02-8 and GayMR19 and one isolate, GayMR20, at 2.0 mM (Table 3.2). At 5.0 mM was *M. marinus* BC501 and then at 7.5 mM was GayMR16 (Table 3.2). Lastly at the high end of the intermediate biofilm adhesion MIC range was TF02-9 and TBT05-19 that had an MIC of 10 mM (Table 3.2). The chromate sensitive growth yield MIC was below 0.1 mM that included DF01-2, TF02-9, TF02A-26, TF02A-35, GayMR16 (Table 3.2). The chromate sensitive biofilm adhesion range wen from 0.5 mM and below. Isolate DF01-2 had an MIC below 0.1 mM and *B. saxobsidens* DD2 was at 0.50 mM (Table 3.2). For growth yield, *B. subtilis* 168 had a high MIC of 1.0 mM and *E. coli* MG1655 had an MIC in the intermediate range at 0.5 mM (Table 3.2). Then on the biofilm adhesion end, *B. subtilis* 168 and *E. coli* MG1655 were in the intermediate MIC range at 5.0 mM and 2.5 mM, respectively (Table 3.2).

Overall, it appears the *M. marinus* BC501 had the highest resistance to the six heavy metals tested in terms of MIC growth yield and biofilm adhesion with cobalt, lead, and chromate being exceptions for the growth yield and lead and chromate being the exception for the biofilm adhesion (Table 3.2). Isolate *M. marinus* BC501 also had the highest resistance to the six heavy metals in terms of MTC for growth yield except for arsenate (Table 3.3). There was no isolate that stood out of having the highest resistance MTC for biofilm adhesion (Table 3.3). Isolate DF01-2 had the lowest resistance to all six heavy metals in terms of MIC growth yield and biofilm adhesion with arsenate being an exception for the growth yield and biofilm adhesion (Table 3.2). Isolate TF02-8 had the least resistance to the six heavy metals in terms of MTC for growth yield having not registered one on any heavy metal (Table 3.3). Isolate TF02A-30 had the least resistance to the six heavy metals in terms of MTC for biofilm adhesion apart from copper (Table 3.3).

Recolonizing Rock Substrates

Adhesion to the Rock Substrate

Ten Geodermatophilaceae species were used to test the ability to recolonize onto rock surfaces. For the initial experiments, the rock samples and bacteria were incubated with growth media. Figure 3.6 shows the results for the biofilm adhesion (A_{595}) for these isolates and the mean values for the control rock substrates without bacteria. Visual observations of the samples after one month are presented in Figure 3.7. Statistics are from after one month of growth.

Two (DF01-2 and TF02-9) of the seven Indian isolates did not significantly adhere to the rock surface ($P>0.10$) and exhibited values close to the control. The four

remaining isolates showed significantly higher biofilm adhesion to the rock surfaces than the control rock substrates. Isolate TF02-8 did not exhibit significant adhesion ($P>0.10$) in the assay but showed visible growth on the surface forming a small floret (Figure 3.7). Isolate TBT05-19 had significant adhesion only after one month ($P<0.008$) and showed some observable growth (Figure 3.7). The TF02As isolates had moderate levels of adhesion, isolate TF02A-26 had high levels of adhesion ($P=0.067$) (Figure 3.6) but did not show much visible growth (Figure 3.7). Both isolates TF02A-30 ($P=0.017$) and TF02A-35 ($P=0.042$) showed visible growth on a several rock surfaces, and TF02A-35 produced a large floret attached to one of the rock surfaces (Figure 3.7). All the three New England isolates (GayMR16, GAYMR19 and GayMR20) had moderate levels of adhesion that increased with time. No visible growth was observed with GayMR16 ($P=0.078$). Isolates GayMR19 and GayMR20 had significant adhesion after one month ($P<0.001$). GayMR20 was the only New England isolate to show visible growth on the rock surface, changing its color from a white to an off-color white (Figure 3.7).

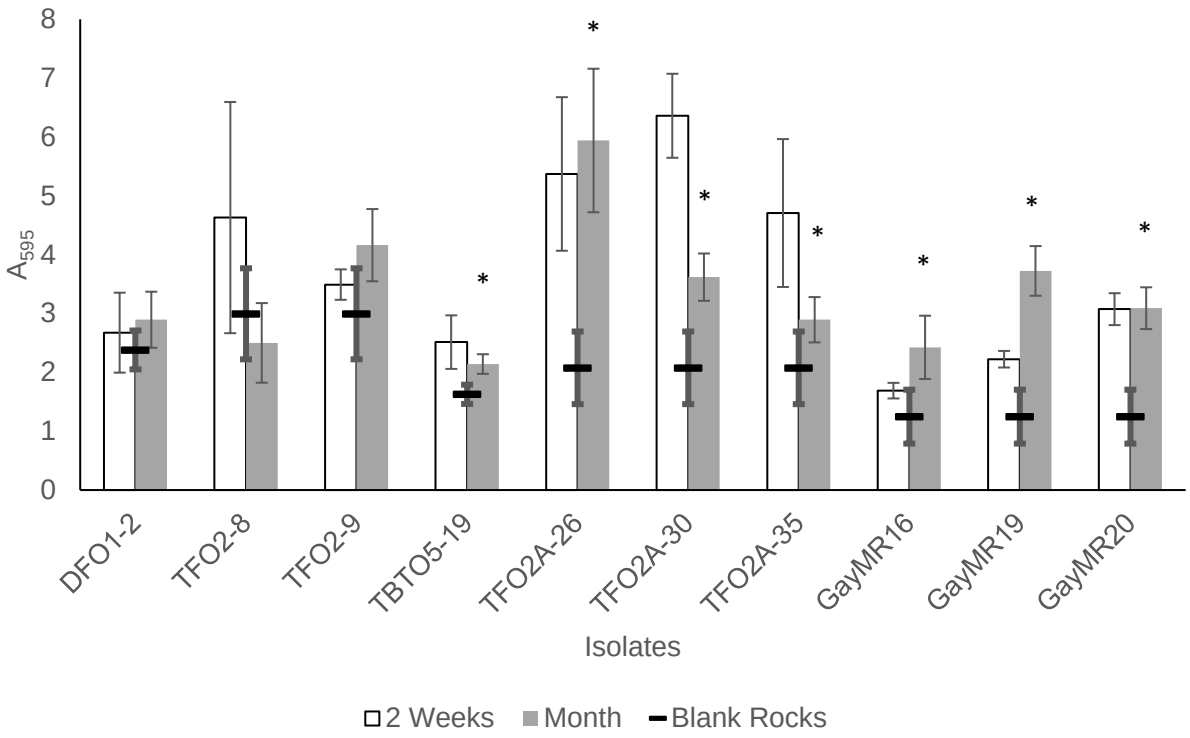


Figure 3.6. Mean biofilm adhesion values for Geodermatophilaceae isolates. Biofilm adhesion values (A_{595}) were for standardized by rock weight. Bacteria incubated with rock substrates and Czapek medium with yeast extract at 28^{o}C under twelve hours of light. Samples were assayed at two-weeks (open bars) and one-month (filled bars) incubation. Black bar represents A_{595} values of blank rock substrates. Error bars represent the standard deviation of the mean. Asterisks represents significant difference ($P<0.10$) only at month isolate growth and blank rock substrates.

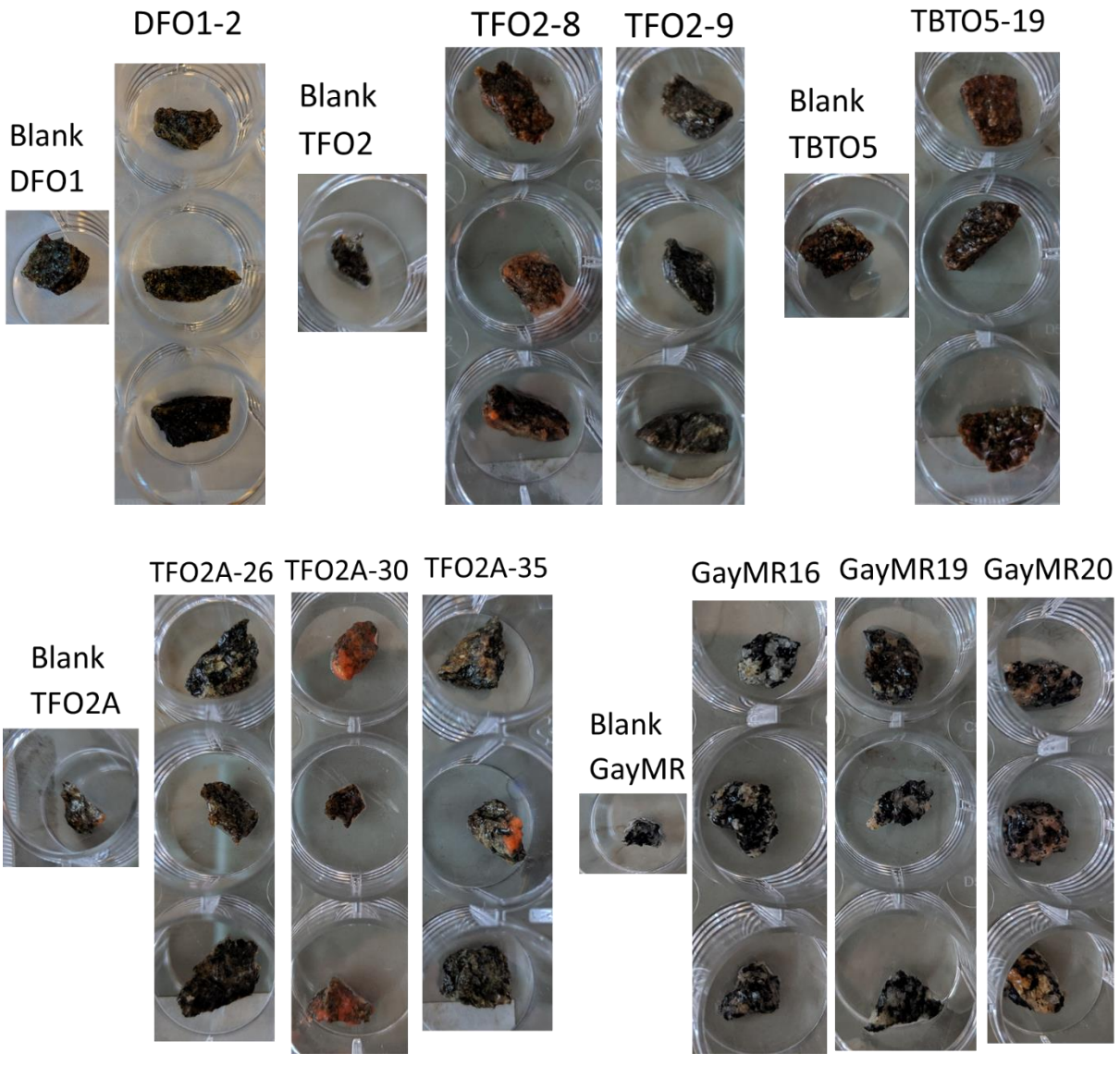


Figure 3.7. Photographs of rock surfaces after incubation with Geodermatophilaceae isolates. Bacteria incubated with rock substrates and Czapek medium with yeast extract at 28^{o}C under twelve hours of light for one month.

Recolonizing Rock Substrates in Phosphate Buffer Saline (PBS)

Since the bacteria will bind to rock substrates in the presence of nutrients, the ability of Geodermatophilaceae isolates to bind to rock surfaces without added nutrients was tested. Geochemical analysis of the rock substrates is presented in Table 3.11 (modified from Ennis 2018). Based on the previous results, isolates TF02-8, TBT05-19, TF02A-26, TF02A-35, and GayMR20 were chosen for further experimentation. Although isolate TF02-8 did not show significant adhesion, it was chosen because it visually had the highest bacterial concentration (Figure 3.7).

The incubation of isolates TF02-8 TBT05-19, TF02A-26, TF02A-35, and GayMR20 with rock substrates without added nutrients failed result in visible growth, but adhesion was measured after one- and two-months incubation. After one-month incubation only isolates TF02A-26 and TF02A-35 showed significant adhesion to the rock surfaces ($P=0.08$ and $P<0.05$, respectively) (Figure 3.8). After two months incubation, GayMR20 showed an increase in adhesion to rock surfaces. Isolates TF02-8 and TF02A-26 showed no changes in adhesion to rock surface after another month of incubation. Isolate TF05-19 had a slight increase in adhesion to the rock surface, isolate TF02-35 showed a decrease in adhesion value.

Table 3.11. Geochemical analysis of Indian and New England stone samples modified from Ennis (2018).

Sample	Region	Oxides (%)										
		SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	Total
TF	India	73.40	0.07	14.24	1.07	0.03	0.05	0.52	2.80	7.23	0.09	99.50
TB		70.94	0.65	11.78	4.96	0.09	0.56	2.78	2.35	4.38	0.66	99.15
TFA		76.71	0.285	13.115	1.6	0.01	0.065	0.43	2.06	5.695	0.08	100.05
CT-GayMR	New England	56.87	0.49	15.31	10.34	0.23	4.37	8.15	2.83	1.00	0.09	99.68

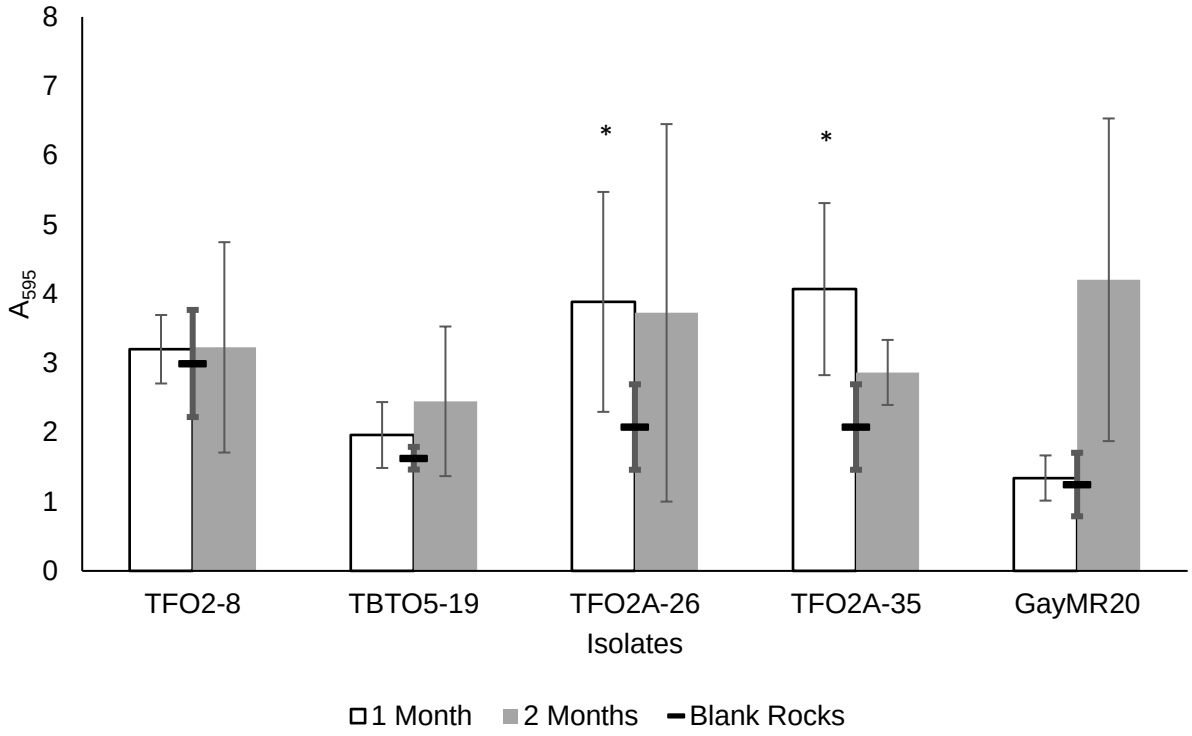


Figure 3.8. Mean biofilm adhesion values for Geodermatophilaceae isolates. Biofilm adhesion values (A_{595}) were for standardized by rock substrate weight. Bacteria incubated with rock substrates and PBS at 28^{o}C under twelve hours of light. Samples were assayed at one- (open bars) and two-month (filled bars) incubation. Black bar represents A_{595} values of blank rock substrates. Error bars represent the standard deviation of the mean. Asterisks represents significant difference ($P<0.10$) between isolate growth and blank rock substrates.

CHAPTER 4

DISCUSSION

The results show that these Geodermatophilaceae isolates grew best at 28oC for one week with light having no effect on growth. Overall, these isolates exhibt tolerance for salt and heavy metals in growth concentration or biofilm adhesion. Two isolates—TF02A-26 and TF02A-35—were able to grow on the rock substrate after two months and one month, respectively in the absence of added nutrients.

Environmental, Salt, and Heavy Metal Tolerance Differences among the Genera

Environmental Optimization

Species in the Geodermatophilaceae have previously been found to grow at 21^{o}C, 28^{o}C, and 37^{o}C (Hezbri et al., 2015; Philippe Normand et al., 2014; Xiao et al., 2011). The general consensus being that they grow best at temperatures in the mid to low 20^{o}C (Philippe Normand et al., 2014). However, results suggest that 37^{o}C was optimal for isolates TF02-6, TF02-8, and TF02A-26, but it was not ideal. The 37^{o}C incubation treatment led to evaporation of the growth medium in the microtiter plate wells even with parafilm so, isolates were already fixed to the wells before peak growth yields were observed. In addition, other experiments needed to be run longer than one week thus 37^{o}C was not acceptable. The optimal temperature for *Modestobacter* is between 19^{o}C and 21^{o}C (Philippe Normand et al., 2014) and it is not surprising that the optimal growth for *M. marinus* BC501 turned out to be at 21^{o}C. These bacteria performed better at 37^{o}C than 28^{o}C, but, again, it may be due to evaporation of the medium. For *Geodermatophilus*, the optimal range is between 24^{o}C and 28^{o}C (Philippe Normand et al., 2014). Even though TF02-6 was considered a slow grower (and not

included in subsequent experiments), the optimal temperature was 28ºC which also aligns with reports in the literature. Finally, *Blastococcus* has the optimal temperature of 25ºC (Philippe Normand et al., 2014). There was not significant difference between the *Blastococcus* isolates tested at 21ºC and 28ºC. Since both temperatures have not been described as optimal for this genus, it makes sense that the temperatures tested did not influence biofilm adherence in this study. Although looking at OD, *B. saxobsidens* DD2, TF02-8, and TF02A-26 were significantly higher at 28ºC than 21ºC. Considering 28ºC is closer to 25ºC, it is not unexpected.

For all temperatures, almost all the isolates' growth yields peaked or leveled off by the seventh day. Those that did peak could likely keep growing and did not reach stationary phase. Geodermatophilaceae is a slow grower family, so the peak may be another week or even a month later. Thus, measuring on the seventh day was the best cut off because some did level off and any longer could count as time loss for the experiments. Isolate TF02-6 did not have any substantial bacterial concentration as it was the slowest grower. Therefore, TF02-6 was left out the following experiments because at a week of optimal conditions it did not show any increased concentration. Only TF02A-26, TF02A-27, and TF02-8 peaked on the fourth day at 28ºC and 37ºC. Isolate TF02A-27 was found not to be a Geodermatophilaceae isolate so it explains the higher growth rate. At 37ºC, it may be due to the decreased media volume, and these three isolates were unable to increase the bacterial concentration because there was not enough growth media. At 28ºC, it may have been the optimal temperature for these bacteria and entered stationary phase relatively fast for this family then died down after

the fourth day. The following experiments with salt tolerance and heavy metal tolerance may elongate the isolates' exponential phases, so two weeks were used.

Between 0-hours and 12-hours exposure to light, there was no clear optimal condition. Since there was no difference in biofilm adhesion between the two conditions, it seems that the pigments are not useful for nutrients or beneficial beyond protection again solar radiation for a biofilm. Although the growth yield results of *M. marinus BC501* and TF02-8 were significantly different between 12-hours of light than 0-hours, it might just be the slight dilution difference they started at between the two parameters. When using the data from 28^{o}C, the ODs fall in between the two conditions so it may just be due to the different dilutions.

Growing in 0-hours versus 12-hours of light showed that the pigments are not necessary for growth but are likely used for protection and do not have to be induced for growth. Organisms of this family present many different colors of pigments which is used as a 'sunblock' against solar radiation (Busarakam et al., 2016). Gtari et al.'s study (2012) indicated a link between UV and ionization protection and the pigments produced but further studies are still needed for a complete overview of Geodermatophilaceae and their response to light.

Salt Tolerance

Geodermatophilaceae are halotolerant species of actinobacteria. Although these organisms were deemed halotolerant, they are not considered to be halophilic species because they were unable to grow at 2.5 M NaCl as observed in this study (Table 3.1) (Margesin & Schinner, 2001). They have been seen to grow in ocean level salinity (0.6 M NaCl) (Hezbri et al., 2015; Xiao et al., 2011). All but two species were able to grow at

or above 0.6 M. Isolates TF02A-26 and GayMR16 did not grow past the concentration of 0.50 M of NaCl, which demonstrates a low salt tolerance for either growth yield or biofilm adhesion. Alternatively looking at growth yields and biofilm adhesion, *M. marinus* BC501*,* DF01-2 and TF02-9 are halotolerant but unable to grow above 0.75 M and TF02-8, TBT05-19, TF02A-35, and GayMR20 do not grow above 1.0 M, but both values are well within the range most Geodermatophilaceae species can grow (Hezbri et al., 2015; Philippe Normand et al., 2014; Xiao et al., 2011). Isolates *B. saxobsidens* DD2, TF02A-30, and GayMR19 grew above that concentration that the other Geodermatophilaceae isolates tested could not tolerate (1 M). Isolate TF02A-30 had a growth yield of 2 M demonstrates the highest tolerance of the novel species tested. However, all of the novel species tested were within the tolerance threshold (>1.4 M) of other Geodermatophilaceae species that were not involved in this experiment (Busarakam et al., 2016). Both *E. coli* MG1655 and *B. subtilis* 168 were also included in this experiment, but the bacterial concentration and biofilm adhesion values were abnormally high for what they had in the past. This could be due to having 48 hours of growth instead of 24 hours. They survived the around the same salt concentrations as *B. saxobsidens* DD2 which also may be due to the longer incubation time.

Heavy Metal Tolerance

Geodermatophilaceae have a high resistance to heavy metals. Three of the six heavy metals that were tested were cobalt, nickel, and copper because they are essential for growth, but can be toxic at a certain threshold. The other three heavy metals tested—lead, arsenate, and chromate—were because they can potentially be

used by microbes but are mostly toxic (Gadd, 2010). Surprisingly, expect for nickel, there was at least one isolate that had better growth yield or biofilm adhesion with a small concentration of each of the heavy metals. This means that possibly all these heavy metals at the right concentration could improve growth of Geodermatophilaceae species.

Overall, DF01-2 had the least resistance to heavy metals for MIC. Since DF01-2 is the only *Geodermatophilus* species, it makes sense because they are considered to have the least resistance to heavy metals out of the three genera (Philippe Normand et al., 2014). Isolate DF01-2 has low resistance than what has been discovered in other research for *Geodermatophilus* (Gtari et al., 2012). This isolate is extra sensitive compared to other *Geodermatophilus* species. *Blastococcus* has been shown to have the highest heavy metal resistance of the three genera (Philippe Normand et al., 2014). This, however, was not reflected in the study as *M. marinus* BC501 had the highest MICs overall for growth yield and biofilm adhesion. In fact, this isolate had higher MIC resistance to arsenate and copper for growth yield and biofilm adhesion compared to previous research, but *M. marinus* BC501 had a lower resistance to lead in comparison to the *Modestobacter* species—*Modestobacter multiseptatus* BC501—tested in (Gtari et al., 2012). The different species could explain the differences in resistance. This studies *M. marinus* BC501 is within the same type of range as the other *Modestobacter*. For the other metals, the growth yield and biofilm adhesion MIC were about the same. In addition, while *Blastococcus* isolates, TF02-8 and TF02A-30, did not have the lowest resistance (MIC), they did have the lowest MTCs for growth yield and biofilm adhesion, respectively. These *Blastococcus* isolates must transport out heavy metals as a

response to toxic levels that decreases their ability to grow, replicate, and form a durable biofilm.

In addition, *E. coli* MG1655 and *B. subtilis* 168 did have higher MICs than most of the *Blastococcus* isolates but was lower than *M. marinus* BC501 for cobalt, nickel, and lead. However, *E. coli* MG1655 MTCs was the same or lower than most of the *Blastococcus* isolates for all the heavy metals. It could mean that *E. coli* MG1655 was replicating faster than heavy metal toxicity could kill them for cobalt, nickel, and lead. A 48-hour incubation could have been too long for *E. coli* MG1655 as the MICs might be lower after a 24-hour incubation. *B. subtilis* 168 had highest MTCs for cobalt, nickel, and chromate. This may also be attributed to replication time along with *B. subtilis* 168 able to produce spores which could resist the toxicity longer and still contribute to the OD_{595}. Furthermore, *B. subtilis* 168 has shown in previous experiments to have a lower MICs and MTCs for cobalt, nickel, lead, arsenate, and chromate (Richards et al., 2002). In that experiment, a different *E. coli* strain was used that had lower MICs for those heavy metals as well. It is reasonable to conclude that *E. coli* MG1655 and *B. subtilis* 168 had higher MICs and MTCs than what they should have. Their short replication time over 48-hours probably created the higher MICs and MTCs recorded in this experiment compared to Richards et al. (2002). This experiment should be run again but using 24-hour incubation for *E. coli* MG1655 and *B. subtilis* 168 to ensure it is not faster replication that created higher MICs.

Furthermore, differences between *Blastococcus* MICs could be explained in the difference in experimental set up. Gtari et al. (2012) tested *B. saxobsidens* DD2's resistance to arsenate, cobalt, chromate, copper, nickel, and lead. Except for cobalt,

their study's MICs were higher, for *B. saxobsidens* DD2 could survive 85 mM arsenate, 20 mM chromate, 3 mM copper, 1 mM nickel, and 30 mM lead (Gtari et al., 2012). Their study used agar plates for growth and a four-week incubation period (Gtari et al., 2012). There are two possible reasons for the differences in MICs: (1) the *Blastococcus* did not have enough time to grow in high heavy metal concentrations and (2) the differences in media type may have caused differences in the microbes' form. On agar plates, Geodermatophilaceae grew in aggregates form while in liquid culture Geodermatophilaceae stayed more in the motile phase. It is possible that both conclusions go hand in hand, that both Geodermatophilaceae species needed more time to shift from the motile phase into the aggregate phase and there was enough time. In addition, *M. marinus* BC501 is a marine isolate and is used to growing in liquid environments while the *Blastococcus* isolates are not as readily adapted. It is possible that if the experiment went longer or was on agar plates it could have gone in a different direction. The differences in *B. saxobsidens* DD2 would also be explained, but further testing is needed.

Recolonizing Rock Substrates

This study shows that it is possible to recolonize the Geodermatophilaceae family on rock substrates. Using a growth media to help stimulate bacterial concentration, it was shown that this family could adhere to the rock surface based on absorbance numbers. Florets appeared on the interface of the water, which highlights the fact that this family grows better with oxygen (P Normand, 2006). It is interesting to note that the bacteria that created florets on the rock surface did not have significant absorbance values (e.g. TF02A-35 had floret but a $P>0.05$). It is possible that the extra polymeric

substance (EPS) that the biofilm creates could stop the stain from setting into the biofilm and thus making an artificially smaller number even though it is clear by the floret that the bacteria are attached. A different stain could be used next time to combat the EPS but, considering that this experiment was to determine only adherence to the rock substrates, observations gave the full picture of attachment if values did not reflect adherence. Add on that, TF02-8 and TF02A-30 in Figure 3.7 look like they adhered well to the rock substrate, it was more due to lifting the rock chip into the top biofilm layer on the media surface than actual adherence to the rock substrate (as the values of TF02-8 reflect). Those values could be actual reflection on adherence because the preparation of the rock chips would remove bacteria not fully attached to the rock surface.

The isolates that grew in PBS had a large standard error probably due to having growth on two out of the three rock substrates, but one not growing contributed to a large standard error. It may have taken more time for the bacteria to grow on all of the rock substrates or that the shape of the rock substrates contributed to adhesion, so bacteria were not able to attach as well to a flat surface than a rough one. In addition, all rock substrates in the PBS assay were below the water interface and losing exposure to the atmosphere may have slowed down growth as opposed to the isolates that grew a floret from before. Isolate TF02A-26 had bacterial adhesion to the rock substrate after one month, but after two months there was no longer a significant value to show adhesion. This could mean that the isolate ran out of nutrients after two months and died out or it could mean that the isolate changed from the aggregate phase into the motile phase and thus was no longer attached to the rock substrate. Since there was no visible biofilm, an EPS most likely did not contribute to a low

adhesion OD. In contrast, GayMR20 had no growth after one month but demonstrated growth after two months most likely growing off of the iron and calcium found in the rock substrate (Gadd, 2010). This isolate shows that this family can grow on rock substrates even if it takes a long time. Furthermore, to ensure that all these isolates can grow on rock substrates, the experiment could be run again but for a longer period of time may even up to a year to see if the other isolates needed more time to grow. The last isolate to show growth, TF02A-35, did not wildly change values from one month to two months showing a leveled growth. TF02A-35 reached a threshold growth it could grow at with the nutrients supplied from the rock substrate. Since there was no observable biofilm on the rock surface, it is possible that this isolate, and possibly Geodermatophilaceae, are unable to form a large biofilm community without other rock colonizers to add to the EPS. The rock geochemistry (Table 3.11) eluded that this isolate could be living off of silica or potassium as they are found in abundance (Gadd, 2010). Using other rock substrates with comparable geochemistry could help identify what elements the isolates need most for optimal growth.

Implications and Future Directions

Additional studies on these isolates will provide a means to deconvolve further links between this family, their hosting rock substrate, and their environmental conditions. Looking at light conditions, these Geodermatophilaceae showed no significant differences between 0-hours of light versus 12-hours of light thus the pigments these isolates have may not be necessary for growth. Their pigments are likely used for protection against solar radiation (Busarakam et al., 2016).

Habitats, particularly gradients from freshwater to saline environments, could be expanded as well. Notably, DF01-2, a *Geodermatophilus* isolate, can grow better in higher salt concentration—based on the MTC—than *M. marinus* BC501 (a marine species), suggesting that the family Geodermatophilaceae can inhabit marine environments. This notion is consistent with their growth in the liquid media used in the biofilm assay in this experiment. Geodermatophilaceae could live in a surrounding rocky environment, like tide pools, although *Geodermatophilus* growth may be hampered by lower, colder conditions outside of the *Geodermatophilus* optimal temperature growth range for extended periods of time. However, it is completely possible for these novel Geodermatophilaceae species to be living nearer to the ocean coast than where they were discovered. Considering TF02A-30 was not affected by a high salt concentration until after 0.60 M and can grow in salt concentrations to around 2.0 M, *Blastococcus* species could live at the coast. It is possible they live there now but are drowned in competition when sampling occurs because they have a slow growth rate. This maybe the reason they are mostly found in desert climates. They may also have been left over from dried out seas and probably could be found in soil salinization sites or sites endanger of desertification. Wind dispersal carries these organisms across the desert and into the ocean where they are found to mainly inhabit.

The experiments were conducted with the goal of capturing *in vivo* conditions as much as possible, but it is possible that environment factored substantively in heavy metal resistance. The isolate position on the rock surfaces from which these isolates were harvested could influence how resistant these isolates are compared to bacteria in this study and other studies. Less resistant isolates may not be as challenged as the

one in other studies while more resistant isolates have been challenged more. Future work could entail sampling from heavy metal contaminated rocks for other Geodermatophilaceae species and compare their resistance to other isolates.

Additional future work could include recolonizing a couple of isolates, TF02A-30 and GayMR19, that were not chosen for PBS recolonization that may grow just as well as TF02A-35 and GayMR20 did with no added nutrients. Since TF02A-30 showed relatively the same absorbance values as TF02A-35, TF02A-30 may grow just as well as TF02A-35 did. This might also be true for GayMR19 as it also reflected GayMR20, the other isolate that showed growth in PBS. The next step would be to recolonize the other isolates or at least TF02A-30 and GayMR19 having to use only a rock substrate for nutrients. The geochemistry combined with the heavy metal data can also be used to get a better understanding of the concentration of heavy metals needed to thrive and the possible challenges the isolates are experiencing in the environment. Other future research experiments could look at the surface of a recolonized rocks with a scanning electron microscope to determine how attached these isolates are and what damage they are doing to the rock surface. It may also be worth it to ensure that the rock substrates that are being recolonized are partially exposed to the atmosphere to perhaps induce more florets.

Now that it has been proven that the Geodermatophilaceae family can recolonize onto rock substrates as primary colonizers *in vivo*, experiments can be run to hinder rock deterioration or enable bioremediation. Inhibiting these primary colonizers from attaching could prevent a large biofilm community from forming and damaging rock surfaces. Meaning buildings and other structures could last longer. On the other hand,

encouraging these biofilms to form could potentially bring about bioremediation to environments contaminated with heavy metals or salt by providing the nutrients and water from the EPS for secondary colonizer organisms to grow. Soil salinization could be bioremediated by the Geodermatophilaceae family.

APPENDIX

Table S1. Temperature ANOVA Table: Two-factor with replication table between temperature groups (ºC) for bacterial growth yields and biofilm adhesion to optimize Geodermatophilaceae isolates.

Type	Isolates	Temperature groups	Degrees of freedom	F	P-value
Bacterial Growth Yields	*M. marinus* BC501 *B. saxobsidens* DD2 TF02-6 TF02-8 TF02-9 PS03-16 TF02A-26 TF02A-27	21, 28	7, 240	245	2.10×10^{-105}
	M. marinus BC501 *B. saxobsidens* DD2 PS03-16 TF02A-27	28, 37	3, 72	95.1	5.47×10^{-25}
	TF02-6 TF02-8 TF02-9 TF02A-26	28, 37	3, 120	19.8	1.77×10^{-10}
Biofilm adhesion	TF02-6 TF02-8 PS03-16 TF02A-26	21, 28, 37	6, 108	46.9	6.57×10^{-28}

Table S2. Light ANOVA Table: Two-factor with replication table between 12-hour and 0-hour light exposure for bacterial growth yields and biofilm adhesion to optimize Geodermatophilaceae isolates.

Type	Isolates	Degrees of freedom	F	P-value
Bacterial Growth Yields	*M. marinus* BC501 *B. saxobsidens* DD2 PS03-16	2, 42	76.4	1.01×10^{-14}
	TF02-6 TF02-8 TF02A-26 TF02A-27	3, 104	194	1.80×10^{-42}
Biofilm adhesion	*B. saxobsidens* DD2 TF02-6 TF02-8 TF02A-27	3, 120	6.03	7.34×10^{-4}

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