

The Magnitude and Rate of Change of Cell Surface Functional Groups as a Function of Salinity:
Implications for Environments of Microbially-Facilitated Carbonate Formation

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Abstract

Microorganisms are an important component of many near surface aqueous environments with cell densities ranging between 10^4 - 10^7 cells ml^{-1} . While many environments are geochemically altered by microbial metabolism, their cell surfaces, complex structures hosting functional groups, also interact with dissolved species, mineral, and organic surfaces. These interactions can influence significantly mineral equilibria of minerals in a given aqueous environment through complexation of aqueous metals and surface stabilization of mineral nuclei.

While many studies have characterized the cell surface functionality of freshwater microorganisms, relatively few have focused on organisms that inhabit fluids ranging from brackish to marine. In this study, pure cultures of *Roseobacter litoralis* and *Halobacterium salinarum*, both organisms that inhabit saline environments, were grown at a range of ionic strengths and titrated to quantify surface functional groups. Multiple generations of growth at varied growth media ionic strengths (0.5M-3.0M) were analyzed to evaluate the magnitude and rate of change in cell surface functional groups.

Roseobacter litoralis and *Halobacterium salinarum* both displayed changes in average cell surface functional groups after growth in increased or decreased ionic strength growth media. Carboxyl groups increased by as much as 30% and 29.5% and decreased by as much as 63% and 9.8% respectively in *R. litoralis* and *H. salinarum*. Responses to various growth media ionic strength were observed rapidly, after 6 generations of growth in both microorganisms. Data produced was evaluated in relation to previous work to provide a framework for predicting microbial functional group density at various ionic strengths.

In environments such as hypersaline lagoons microorganisms have more surface functional groups, and therefore may play an integral role in mineral nucleation and metal

adsorption. Microorganisms that inhabit environments with fluctuating ionic strengths rapidly adapt their surface functional groups. A model that can predict the density of functional groups at given ionic strength environments can be valuable in metal adsorption for the purpose of bioremediation and mineral nucleation. Organisms capable of producing a functional group density above a threshold may potentially be able to facilitate mineral precipitation without regard to the specific organism.

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1) Introduction

Microorganisms are found in nearly all surface and near surface environments. In aqueous environments cell densities can range between 10^4 - 10^7 cells ml^{-1} (Whitman et. al., 1998). The presence of abundant prokaryotes in all environments requires careful consideration of the effects that these microorganisms can have on an environment. The metabolism of microorganisms alter the geochemistry of environments and can promote dissolution and precipitation of minerals and clays. Mineral equilibria are impacted by microbial metabolisms that transform metal redox state, change solution pH, and cycle carbon (Konhouser, 2006). The interaction of microorganisms with their environment and the role that they play in geochemical processes should be considered to properly characterize metal speciation and kinetics of water:rock interactions.

1.1 Cell Surface Functionality

The cell surface of bacteria are complex structures with two primary physiologic structures, Gram positive and Gram negative. Gram positive microorganisms contain a thick multi-layered peptidoglycan layer (Madigan et. al., 2010). This layer is the outermost layer in gram positive cells, and contain biomolecules, including lipids and lipoproteins, exposed to the environment they inhabit. In gram negative microorganisms the peptidoglycan layer is single layered and thin. Surrounding the thin peptidoglycan layer is an outer membrane with lipids, lipopolysaccharides, and lipoproteins attached. In both gram negative and gram positive cells there can be an S-layer present consisting of repeating units of glycoproteins. S-layers are most commonly found in archaea.

Archaea and bacteria have differences in metabolic pathways and physical structure. The major difference relevant to this study lies in the structure of the cell membranes. Archaea have only a single cell membrane where bacteria have a double layered membrane. Archaea also lack

a peptidoglycan layer. Most archaea have an S-layer present made up of glycoproteins. Archaea are of particular interest to this study because of the environments they are found. Archaea are commonly found in environments with very high salinities (Dawson et. al., 2012).

Microbial cell surface biomolecules, whether bacterial or archaeal, contain a suite of functional groups that interact with the environment they inhabit. The pH and ionic strength of their environments equilibrate with these surfaces by adsorption or desorption of H^+ ions and cations, and in turn control their net surface charge. The reactivity of these functional groups can be constrained through acid base titrations and in turn modeled using deprotonation constants (pKas) relating to carboxyl, phosphate, and hydroxyl groups (pKas =4.82 +/- 0.14, 6.9 +/- 0.5, and 9.4 +/- 0.2, respectively; Fein et al, 1997; Fowle and Fein, 2000)(Zhao et. al., 2015). In most natural systems the pH of water ranges between ~ 6-8 (Brezonik and Arnold, 2011) and under these conditions the most numerous of these reactive moieties, the carboxyl groups, are deprotonated.

Upon deprotonation, these functional groups may assume a new role in their aqueous environment through surface complexation with positively charged ions or metals. This in turn can, for example, sequester toxic metals in aqueous systems and/or potentially mobilize them through colloidal transport in the environment (Turner and Fein, 2007). Once associated with a surface, the metal-microbe complex can act as an initial activation energy lowering step to initiate mineral formation (Konhauser et. al., 1993; Douglas and Beveridge, 1998). Precipitation of minerals requires the dehydration of ions to first be overcome so that they can form the covalent or ionic bonds into the crystal structure of the mineral (Zhang et. al. 2012). Ions bound to the surface of microorganisms may form precursors to the mineral phase that can serve as

nuclei for mineral maturation and ripening as seen in barite formation (Torres-Crespo et. al., 2015).

Adsorption of metals from an aqueous environment is not an active process carried out by microorganisms. Ions such as Cd, Cu, and Pb bind depending upon equilibrium kinetics, and their electrostatic properties (Fowle and Fein, 1999). Although not actively sought by microorganisms, metals show competitive sorption, and thus has been described using a distribution coefficient, K_D , that gives a ratio of metal sorbed to metal in solution (Equation 1; Fowle and Fein, 1999). This coefficient, however, does not describe the equilibrium between available sites for adsorption, and available ions. Equation 2 describes the electrostatic reaction between a charged surface carboxyl group and a metal ion from solution.

$$K_D = \frac{[metal\ adsorbed]}{[metal\ in\ solution]} \quad (1)$$



Furthermore, equation 2 demonstrates the reversibility of metal adsorption (Fowle and Fein, 2000), and that metal adsorption at equilibrium is predictable given sites available and concentration of aqueous metal ions available.

Environmental salinity or growth medium salinity plays a role in the physiology of microorganisms beyond electrostatic considerations. Cell membranes of microorganisms are permeable to water, and therefore are subject to osmotic stresses. Fluctuation in the ionic strength of growth media or environments produces a need for microorganisms to adapt to prevent their cells from rupturing. To maintain an osmotic balance organisms will accumulate or expel ions, such as potassium and calcium, to maintain an isotonic environment (Orjen, 2002). Some organisms will produce organic or inorganic solutes at a high energetic cost to reduce osmotic stresses (Orjen, 2002).

Titration of carboxyl group densities of many common freshwater microorganisms has revealed similarity in functional group densities between species (Yee and Fein, 2001), however recent work by Kinnebrew (2012), Kenward et al. (2013) and Voegerl (2014) suggest that surface carboxyl group density increases with increasing ionic strength of growth media. More surface functional groups contribute to a greater overall surface charge of microbes, and thus can sorb greater quantities of metals to their surface. In fact Kenward (2013) describes a threshold for surface carboxyl group sites in order for precipitation to occur in the mineral dolomite.

1.2 Metal Adsorption

Metal adsorption onto any surface is influenced by the population or cloud of ions associated with the charged interfaces (electrostatic double layer or EDL)(Figure 16). This layer is the zone between the surface of the mineral or microorganism and the bulk solution. Interactions between a surface and metal ions occur within this zone. Reduction in the thickness of the EDL increases the likelihood of interactions between charged surface functional groups and oppositely charged metal ions. Increasing solution ionic strength typically increases metal adsorption to bacterial surfaces by compression of the EDL and easing the approach of the metal of interest (Shaw, 1976). The compression of the EDL has a limit and can cease to exist if compressed enough, thereby effecting adsorption (Mills et. al., 1994). For example, metal-bacteria adsorption experiments using cadmium, lead, and strontium resulted in slight increases at first, followed by decreases in adsorption across increasing ionic strengths (Borrok and Fein, 2005) perhaps due to site saturation or competition.

Although the EDL has been shown to be important in forming surface-metal complexes, equilibrium between metal ion concentration and available sites plays a large role as well. In adsorption experiments performed using Cd, Ca, and *Bacillus subtilis*, it was found that regardless of contact time or beginning conditions of saturation, the final concentration of sorbed metal was the same (Fowle, 2000). Regardless of whether the metal in solution was originally

bound to the microorganism or started in solution, the equilibrium model effectively described the resulting fractionation between sorbed and free ions in solution. The same study also showed that this process is rapid, and therefore is likely a surficial interaction between the microorganism and metal ion.

In a study investigating the ability of halophilic archaea to adsorb metals it was found that *Haloferax sulfurifontis* had a large capacity to adsorb metals at environmental pH conditions (Kinnebrew, 2012). In the study it was found that Pb^{2+} was rapidly adsorbed onto cell surfaces under high ionic strength conditions. Adsorption by microorganisms in undersaturated and oversaturated conditions will occur, but precipitation of solid phases are not observed in undersaturated aqueous solutions (Fowle and Fein, 2001). Under fluctuating ionic strength conditions, such as in an evaporative environment (e.g. sabkha), supersaturation with respect to solid phases can be induced. Adaptations by microorganisms (i.e. functional group site increases) and increased metal ion concentration from mixing or evaporation may greatly influence the rate at which metal sorption and precipitation of minerals may occur.

1.3 Mineral Nucleation

Precipitation of minerals from aqueous solution can be influenced by microbial surfaces and processes. Microbes alter the geochemistry of environments by many metabolic processes that include changing metal redox state, fluctuating solution pH, and cycling of carbon. Microbes also passively affect environment geochemistry. The surface of microbes can bind and stabilize nuclei of minerals (Konhauser, 2006).

For all minerals there are kinetic barriers to their formation that must be overcome. In aqueous environments, due to the charge of ions and the polarity of water molecules, a hydration sheath is formed by interactions between positive and negative charges. The presence of a hydration sheath stabilizes metal ions in solution, slowing their diffusion in the fluid, reducing

the likelihood of abiotic complexation. Under natural conditions the barrier to precipitation is too great to proceed unaided. Charged surface functional groups of microorganisms have been shown to be capable of dehydrating ions, and thus the kinetic barrier to mineral formation.

Microorganisms often facilitate precipitation of clay and carbonate minerals beyond abiotic rock:water interaction. Biofilms containing EPS are capable of binding and stabilizing nuclei promoting further precipitation. Microbial metabolism can influence solution geochemistry by altering redox states and saturation state with respect to clay and carbonate minerals. Binding of ions from solution due to charges on functional groups will also serve as a nucleation point for mineral formation.

Microbially facilitated clay formation has been well studied, and is commonly linked with iron sorption. In many studies clay mineral precipitation in the presence of microorganisms was associated with high aqueous iron content. Iron bound to the surface of microorganisms can then interact with aqueous aluminum and silica. EPS has also been linked to clay mineral nucleation (Konhauser and Urrutia, 1999). Konhauser and Urrutia (1999) suggest that clay mineral formation facilitated by microorganisms begin as iron rich aggregates that become increasingly mineralized. Microorganisms have also been shown to enhance precipitation of clay minerals by adhering to already existing clay mineral surfaces (Walker et. al., 1989). In doing so, surface molecules attract more aqueous ions necessary for further precipitation to occur.

Microorganisms can have a profound effect on the precipitation of carbonate minerals such as calcium carbonate. In a study by Kandiandis et. al. (2008) a natural hotspring was used to measure calcium carbonate precipitation rates relative to microbial biomass present. It was found that in the absence of microbial biomass rates of calcium carbonate precipitation were greatly reduced. A reduction in biomass of 45% resulted in a change in rate of precipitation by an order

of magnitude (Kandiandis et. al., 2008). The viability of microorganisms was found to have little effect on the rate of precipitation of CaCO₃, suggesting that metabolic activity in this system did not impact precipitation rate.

Microbially induced calcium carbonate precipitation (MICP) has been applied to a wide range of geological and engineering uses. For example in high permeability cap rocks, injection of fluids that promote biofilm growth can reduce permeability by 2 to 4 orders of magnitude (Phillips et. al., 2013), and can reduce CO₂ leakage. In another study, sedimentary microbial ecosystems were described that show carbonate precipitation in the presence of microbial mats (Farias, 2014). In these systems, the researchers describe increasing salinity conditions that induces mineral precipitation. EPS within microbial mats are responsible for binding calcium required for carbonate precipitation.

Unlike calcium carbonate, dolomite is kinetically inhibited even though saturation with respect to dolomite may be reached. In the sedimentary record dolomite is often found in abundance, but when investigating modern deposits dolomite is seldom found. Dolomite formation is dependent upon factors such as saturation level with respect to dolomite, Mg/Ca ratio, and pH. Though these conditions may all be met, dolomite commonly does not nucleate. It has been observed that in order to precipitate dolomite at earth surface conditions, high temperatures are required in order to reach the activation energy needed to begin formation. Temperatures in the range of 100-200° C were found necessary in a previous study to invoke enough precipitation to determine a rate constant for the reaction (Arvidson and Mackenzie, 1999). The large activation energy required for dolomite formation, thus, plays a large role in its absence in recent sedimentary records.

Formation of abiotic dolomite in lab experiments have been successful but only at temperatures upwards of 80°C, and up to 250°C (Roberts et al. 2013). High temperatures provide the necessary energy to overcome the kinetic barrier for formation to occur. However, in recent studies, microorganisms have been shown to facilitate the nucleation of dolomite. Kenward et al., (2013) linked a high carboxyl group density (8.1×10^{-4} mol g⁻¹ for disordered, and 1.6×10^{-3} mol g⁻¹ for ordered) to the nucleation of low temperature dolomite in dolomite-supersaturated solutions. To understand how dolomite is naturally formed at Earth surface conditions it is essential to investigate such kinetics, and to investigate means by which they may be overcome or altered in natural systems.

The purpose of this study is to test the hypothesis that with increasing ionic strength of growth medium carboxyl group density will increase on cell surfaces. The goals of this study are to (1) quantify surface functional group site densities and pKas of two marine microorganisms, and (2) evaluate the rate at which these microorganisms change surface functional group density in response to changing growth medium ionic strength. To evaluate these factors microbial surfaces were characterized over several generations by a series of titrations and computer analysis. This study has implications in the fate and transport of metals by interaction with microbial surfaces. Here we utilize microorganisms native to seawater ionic strengths, a group of microorganisms whose surface properties are scarcely investigated. Better understanding the response of microorganisms to changes in environment ionic strength can lead to a model that predicts where modern or unknown carbonate mineral deposits, including dolomite, may occur, and thus potential hydrocarbon reservoirs.

2. Methods

The bacteria chosen for this research, *Halobacterium salinarum* and *Roseobacter litoralis*, were chosen due to their optimal growth salinities (0.5-2.5M) and cell structures. Each bacterium was initially cultured at prescribed optimal growth conditions, evaluated to determine the point of mid-exponential growth, enumerated, harvested, and titrated to quantify surface functional groups. Harvest at mid-exponential growth was chosen to maximize the health of cells in each culture. For each, this process was performed over multiple growth medium ionic strengths.

2.1 Culturing

2.1.2 *Halobacterium salinarum* (ATCC® 700922™)

H. salinarum was chosen due to its natural hypersaline growth environment (1.5-3.0M). *H. salinarum* is an obligate aerobic heterotrophic haloarchaeon isolated from fluid inclusions of the Badwater salt pan deposits, a very saline environment (Mormile et. al., 2003). Salt deposits of the Badwater Basin are in an extremely evaporitic environment. This organism is also found in hypersaline lakes, salterns, and salted fish. Optimal growth for *H. salinarum* occurs at 2.5M ionic strength and 37°C. A protein found in *H. salinarum*, bacteriorhodopsin, allows for the organism to produce energy by a light driven proton pump, however for this study cultures were grown in the dark. This microorganism is gram-negative, and contains an S-layer or surface layer. This is the outermost layer in cells that they are present, consisting of repeating units of glycoproteins. The S-layer makes up a large percentage of the lipids and proteins of the cell envelope (Mescher and Strominger, 1976).

Methods for culturing were followed as recommended by the American Type Culture Collection (ATCC) (Table 1, Appendix A), and the medium formulation was subsequently

manipulated to raise and lower ionic strength (Table 2, Appendix A) by addition or removal of NaCl. Oxygen is the primary terminal electron acceptor for *H. salinarum*. Growth curves were obtained by spectrophotometry using optical density measurements on a Thermo Spectronic Genesys 20 spectrophotometer at 600 nm to determine mid-exponential growth (Widdel, 2010). Absorbance readings were collected over 100 hours after which the rate of change slowed. Cultures were placed into sterile 50 mL tubes, and were allowed to grow with the cap loosely placed on top to allow the sample to remain in the presence of oxygen for growth. New cultures were inoculated from previous cultures at mid-exponential phase in a 5:20 mL ratio for a total volume of 25 mL. Cultures were incubated at 37° C on a shaker at 140 RPM in the dark for 6 generations. Samples were taken from generation 1, 3, and 6 for titration.

2.1.2 *Roseobacter litoralis* (ATCC® 49566™)

R. litoralis is a marine organism isolated from seaweed. This organism is gram-negative and has a thin peptidoglycan layer surrounded by an outer membrane containing lipopolysaccharides on its surface. The *Roseobacter* genus is a pink-pigmented, aerobic photoheterotroph, closely related to the *Erythrobacter* genus, containing chlorophyll a, allowing for phototrophic growth in addition to heterotrophy (Shiba, 1991). For this experiment cells were cultured aerobically with oxygen as the terminal electron acceptor. Yeast extract provided a carbon source. *R. litoralis* will only produce bacteriochlorophyll a when grown aerobically (Shiba 1991), and so was cultured as such for this study. This genus of bacteria are Gram-negative (Zong and Jiao, 2012), and thus have a thin peptidoglycan layer surrounded by an outer membrane containing proteins and lipopolysaccharides.

Methods for culturing were followed as described by the ATCC. Media were prepared according to the recipe as described by the ATCC (Table 3, Appendix A), and subsequently

manipulated to raise and lower ionic strength (Table 4, Appendix A) by addition of NaCl. Growth curves were obtained by spectrophotometry using a Thermo Spectronic Genesys 20 spectrophotometer at 600 nm to determine mid-exponential growth (Widdel, 2010). New cultures were inoculated from previous cultures at mid-exponential phase by transferring 5 mL of culture to 25 mL of fresh media in sterile 50 mL tubes. Cultures were incubated aerobically at 26° C on a shaker at 140 RPM under a light bank producing light at 2500 lux. Cultures of *R. litoralis* were exposed to light or dark during growth on a 12-hour schedule to simulate diurnal cycles. Cultures were placed into sterile 50 mL tubes, and were allowed to grow with the cap loosely placed on top to allow the sample to remain in the presence of oxygen for growth. In total, 9 generations of growth were produced for each growth medium ionic strength. From generations 1, 3, 6 and, 9 samples were taken to perform titrations.

2.2 Native Marine Consortium

A native microbial consortium from seawater collected in Puerto Rico at Crashboat Beach (Figure 1) (GPS= 18.4583° N, 67.1638° W) was analyzed to determine surface functional groups as a field check to in lab values. Seawater was collected in a 5 gallon plastic carboy. The carboy was first rinsed with sea water, then filled to prevent the inclusion of any contaminant/residual particles. The carboy was filled in approximately 4 feet of water (approximately 13 feet from the shoreline) to minimize the amount of sand being collected. The carboy was then capped, put in a cooler (no ice or coolant was added), and shipped back to Lawrence, Kansas. There were no preservatives added. Upon arrival at The University of Kansas, the carboy was transferred to a refrigerated cooler until the time of analysis. Fluid samples were placed into sterile 50 mL tubes and centrifuged to separate out microorganisms.

The sample was washed three times with 0.1M NaCl. After washing, microorganisms were resuspended in 0.1M NaCl to a concentration of 1 g L⁻¹ microorganism to electrolyte.

2.3 Harvesting

Upon reaching mid-exponential growth bacteria were placed into pre-weighed centrifuge tubes and centrifuged for 10 mins at 9000 RPM to form a pellet. The supernatant was removed and the pellet was washed three times with a 0.1M NaCl solution. The NaCl solution had been gassed with 100% N₂ for 1 hour prior to washing or suspension of bacteria to remove any dissolved CO₂. The pellet was weighed then resuspended in the N₂-bubbled 0.1M NaCl solution to a concentration of 1 g L⁻¹ bacteria to electrolyte for titration.

2.4 Titration

Harvested bacteria suspended in 0.1M NaCl were titrated under nitrogen to determine carboxyl group densities. Titrations were performed on a Metrohm Titrando 832 autotitrator using Tiamo software to record the titrations. Titrations were performed using calibrated 0.1M NaOH and 0.1M HCl manufactured by Arcos Organics. Samples were titrated up to pH 8, down to a pH of 3.2, up to a pH of 8, then back down again to 3.2 to ensure that carboxyl groups could be accurately and reversibly quantified. Titrations were performed for multiple generations (generations 1, 3, 6, and 9) of each ionic strength.

2.5 ProtoFit Analysis of Titrations

ProtoFit GUI Version 2.1 was used to analyze data produced from each titration (Turner and Fein, 2006). For each generation of bacteria for all ionic strengths, pH measurements, volume of acid, number of measurements, mass of pellet, valence of background ions, temperature, surface area, and normality of acid were input to determine pKa values and functional group site densities. ProtoFit software calculates an *adsorbent proton buffering*

function that describes the proton buffering capacity of the surface being investigated as a function of pH (Turner and Fein, 2006). Data output from ProtoFit were compiled to determine average carboxyl group density from generations 1, 3, 6, and 9 for *R. littoralis* and generations 1, 3, and 6 for *H. salinarum* at each ionic strength.

3. Results

3.1 Roseobacter littoralis

3.1.1 Growth Curve

For *R. littoralis* a mid-exponential point was determined to be at approximately 24 hours with an optical density measured at 600 nm of 1.14 abs. After approximate 35-40 hours growth slowed and near an optical density of approximately 1.6 (Figure 2).

3.1.2 Titration

Titration data was exported from the Tiamo software and plotted in Microsoft Excel to compare total acid volumes required to reach a pH of 3 (Figure 3) for each organism. Three titrations were performed for each generation at each ionic strength. Titrations of *R. littoralis* cultured at the optimal ionic strength of 0.7M required 0.42 mL of acid. Cultures grown in increased ionic strength medium of 1.0M required 0.459 mL of acid, slightly more than the optimal growth conditions. Further increasing the ionic strength of growth media to 1.5M yielded a titration that required 0.597 mL of acid. When decreasing the ionic strength of growth medium to 0.5M the amount of acid required was reduced to 0.227 mL.

3.1.3 ProtoFit Analysis

Titration data obtained from experiments were put into ProtoFit GUI Version 2.1 software to obtain pKa and site density values. Results from this analysis showed increased

carboxyl and total site density for increased growth media ionic strengths. It also showed decreased total and carboxyl site densities when organisms were grown in decreased growth media ionic strength.

Initial (0.7M growth media ionic strength) average carboxyl and total site densities were found to be $0.60 \pm 0.15 \text{ mol kg}^{-1}$ and $1.37 \pm 0.21 \text{ mol kg}^{-1}$ respectively. When grown in 0.5M ionic strength media carboxyl groups decreased to $0.22 \pm 0.05 \text{ mol kg}^{-1}$, a 63% decrease. Total site density decreased to $0.90 \pm 0.17 \text{ mol kg}^{-1}$, a 34% decrease. When increasing the growth media ionic strength to 1.0M, carboxyl group density increased to $0.78 \pm 0.08 \text{ mol kg}^{-1}$, a 30% increase. Average total site density for 1.0M also increased to a value of 1.56 ± 0.09 , a 14% increase. The error in these results includes error from each data value included in the average value. Average values were produced from titrations of three pellets from the same culture. When increasing the growth media ionic strength to 1.5M, average carboxyl and total site densities increased 19% and 7% from optimal respectively to values of 0.71 ± 0.04 , and $1.47 \pm 0.15 \text{ mol kg}^{-1}$. *R. litoralis* ProtoFit results for average site densities are shown in Figure 4, and percent changes in Figure 5.

For all cultures an overall decrease in both total and carboxyl groups was observed across each generation, however for cultures grown in increased ionic strength media a decrease was first observed followed by an increase (Figures 6, 7). The greatest response to increased ionic strength was seen at ionic strengths within 0.5M of the optimal ionic strength media. All ProtoFit outputs are shown in Table 4.

3.2 *Halobacterium salinarum*

3.2.1 Growth Curve

Mid-exponential growth was observed to occur at 48 hours of growth in *H. salinarum* cultures. At 600 nm an optical density of approximately 0.95 was measured for cultures. After approximately 75 hours the optical density became relatively constant near 2 (Figure 8).

3.2.2 Titration

Titrations of *H. salinarum* showed similar results in acid volume requirements for varied ionic strength media (Figure 9). The optimal Ionic strength of 2.5M required 0.815 mL of acid to reach a pH of 3. Increasing the media ionic strength to 3.0 a volume of 1.65 mL of acid was required. Decreasing media ionic strength to 2.0M yielded a titration that required 0.636 mL of acid.

3.2.3 ProtoFit Analysis

Initial (2.5M growth media ionic strength) carboxyl and total site densities for *H. salinarum* were found to be 0.53 ± 0.06 and 2.27 ± 0.10 mol kg⁻¹ respectively. An increase in growth media ionic strength to 3.0 yielded an increase in carboxyl site density of 29.5% to 0.69 ± 0.10 mol kg⁻¹, and a 10% increase in total site density to 2.50 ± 0.09 mol kg⁻¹. Decreasing the growth media ionic strength to 2.0M resulted in a 9.8% decrease in carboxyl site density to 0.48 ± 0.07 mol kg⁻¹, and a 7.2% decrease in total site density to 2.11 ± 0.21 mol kg⁻¹. Results for average carboxyl and average total site density are shown graphically in Figure 10, and percent changes in Figure 11.

After 3 generations of growth for all ionic strength media a decrease was observed in *H. salinarum* cultures. In 2.0M ionic strength media after 6 generations a decrease was continued to

be observed, while an increase was observed after 6 generations of growth in 3.0M ionic strength media (Figures 12, 13).

3.3 Native Marine Consortium

Titration data from a native seawater consortium collected in Puerto Rico was collected and input into ProtoFit software. The consortium was found to have a carboxyl group site density of $0.76 \pm 0.16 \text{ mol kg}^{-1}$. The total site density was $2.01 \pm 0.23 \text{ mol kg}^{-1}$.

4. Discussion

The suite of functional groups found on the surfaces of microorganisms are vital to the survival of cells in changing environments. The charge of functional groups in natural systems allows for oppositely charged molecules in the environment to interact. Nutrients needed by the organism can be adsorbed and taken in as a result of functional group interactions with other molecules. They can also play a role in providing the cell with structure or fluidity in response to environmental changes. In addition, passage of molecules in or out of the cell can be regulated by surface molecules allowing for the cell to maintain a constant internal chemistry for survival. The results of this study provide valuable insight into how microorganisms respond physiologically to environmental changes. Specifically the results expand our knowledge of how microbial surfaces respond to changes in ionic strength in their growth environments. Increases and decreases of surface functional groups can have implications in both sequestration and transport of metals, and mineral nucleation and formation. Understanding the physiological changes of microorganisms in response to ionic strength, and the implications of those changes are useful in providing a better understanding of both modern and ancient carbonate deposits.

4.1 Physiological Response to High Ionic Strength

High salinity environments create a stressful environment for organisms inhabiting it by creating osmotic differences (hypertonic or hypotonic environments in the cytoplasm of cell) (Dawson et. al., 2012). However, most highly saline and alkaline environments are inhabited by halophilic algae, bacteria, and archaea (Dawson et. al., 2012). In high stress environments organisms must adapt their physiology to maintain survival. In recent years several physiological adaptations have been shown to occur in microorganisms. Microorganisms have been shown to adapt to high salinity environments through the uptake of osmolytes. *Bacillus licheniformis* was found to increase uptake of glycine, betaine, and proline in response to increased salt concentrations in their growth environment, allowing the cell to maintain an isotonic environment without interfering with metabolism (Sangeeta et. al., 2015). Membrane lipids have also been shown to change structure in response to salinity changes. The degree of unsaturation (as double bonds) in membrane lipids were higher in microorganisms with high optimal growth salinities and increased with increasing salinity (Dawson et. al., 2012). Unsaturation in membrane lipids increases membrane stability and can reduce permeability, however fully unsaturated phytanyl chains increase permeability (Dannenmuller et. al., 2000). Another study suggests that cell walls develop a higher site density of surface functional groups in higher ionic strength fluids as a defense against high metal loads (Kinnebrew, 2012). Results of this study suggest that salt concentrations alone are sufficient to influence a change in site densities, and that the structure of a microorganism's cell surface changes with growth medium ionic strength by increasing or decreasing surface functional groups. Perhaps the influence of increased surface functional groups promotes membrane strength and altered permeability necessary for passage or

retention of osmolytes due to changes in salinity in their growth environment, as a response to stressed caused by fluctuating ionic strength.

H. salinarum, when cultured in lower ionic strength than optimal, showed a 10% decrease in surface carboxyl groups and a 7% decrease in total functional groups. In a lower ionic strength environment the stress placed on the cells becomes less. Metabolically, to produce more functional groups requires more energy, therefore the decrease in functional groups in lower ionic strength environments could be a result of the organism attempting to achieve metabolic efficiency. However, previous studies (Sangeeta et. al., 2015; Dawson et. al., 2012; Dannemuller et. al., 2000) suggest that the change in functional groups is an attempt to maintain proper membrane fluidity and permeability. If the cells internal chemistry is drastically different from the growth environment, cells would likely lyse or shrivel. In this experiment color changes were observed in both organisms, and were observed in both increased and decreased ionic strength cultures. The S-layer of some bacteria have been shown to shed in response to mineralization on the surface to prevent clogging of surface pores (Sara and Sleytr, 2000). Under stress the gene responsible for expression of the S-layer can be different, and could possibly explain a change in color. Here no investigation into the presence or absence of S-layers are presented, but should be further investigated in future studies.

When increasing the growth media ionic strength of *H. salinarum*, surface carboxyl group density increased by approximately 30% and total functional groups increased by approximately 10%. The increase in functional group density was less rapid than the decrease in functional groups. In decreased ionic strength the change in functional groups was observed after 3 generations of growth, but not until 6 generations of growth in increased ionic strength media.

Initially a decrease of functional groups was observed in increased ionic strength media. After a period of several generations the cells are able to recover and increase functional groups.

Similar results were observed in *R. litoralis*. When decreasing the ionic strength of growth media carboxyl group densities decreased by as much as 63%, and when increasing growth media ionic strength carboxyl group densities increased by as much as 30%. This microorganism also initially showed a reduction in carboxyl group density in generation 3 of growth in increased ionic strength growth media, but had recovered and increased by generation 6. Again, it is inferred that this initial drop in functional group densities is a result of the organisms being overstressed, followed by recovery and adaptation. The initial decrease in 1.0M ionic strength growth media was 15%, and the decrease in 1.5M ionic strength growth media was 65%. The greater decrease in the higher ionic strength media, and therefore more stressful environment, lends support to the conclusion that the observed initial decrease and increased ionic strength are related.

In comparison between *H. salinarum* and *R. litoralis* the magnitude of change in functional groups varied dependent upon the optimal ionic strength of the organism. The variance in the magnitude of change in functional group densities is likely a result of where the optimal ionic strength of the organism falls along the observed trend (Figure 17). For example, the decrease in average carboxyl group density in *R. litoralis* cultured in decreased ionic strength media was 63% while the decrease in carboxyl group density for *H. salinarum* was only 10%. Near the portion of the curve where an organism has lower optimal ionic strength, the change in functional group density in increased or decreased ionic strength is likely to have greater magnitude. Halophilic microorganisms, such as *H. salinarum*, are highly adapted to high salt environments, therefore in halophilic archaea with high optimal growth salinities adaptations,

such as unsaturation of membrane lipids, are found in higher proportions versus those with lower salt optimums (Dawson et. al., 2012). This perhaps supports the result that the organism with a lower optimal ionic strength, *R. litoralis*, would experience greater magnitudes of change for the same amount of change in ionic strength due to a lack of adaptations already present.

From the results of this experiment the rate at which surface functional group density changes occur is again dependent upon the optimal ionic strength of the organism. For example in *R. litoralis*, after 1 generation of growth, carboxyl group densities had increased or decreased from the density at optimal ionic strength by 30% and 12% respectively. After 1 generation of growth in *H. salinarum* cultures, analysis resulted in nearly identical values for carboxyl group densities. In both organisms the rate at which carboxyl group densities decreased was more rapid, and continued in future generations. Increases in generation 1 were followed by a decrease in carboxyl groups followed by an increase in generation 6 for both organisms. In non-laboratory settings, where *H. salinarum* is found, the change in fluid chemistry is likely to be a gradual change rather than the immediate jump in ionic strength found in this experiment. In environments such as sabkhas, or coastal lagoons, changes in ionic strength occur seasonally. The strain of *H. salinarum* used in this experiment was isolated from fluid inclusions from a salt pan. Evaporitic environments like deserts, and coastal lagoons are not subjected to large increases or decreases in ionic strength but rather gradual fluctuations. Considering gradual changes in ionic strength, the rate of change in the environment is likely to occur rapidly. It is important to note that increases in growth medium ionic strength were gradual increases (0.5M), and the tolerance of each organism to changing ionic strength was not studied in depth.

4.2 Native Marine Consortium

In a previous study investigating surface functional group densities of freshwater microorganisms a consortium of organisms was investigated and found to fit with data trends for other organisms (Johnson et. al., 2006). For this study a similar methodology was used to evaluate the relevance of lab experiments to real world examples. Evaluation of a marine consortium collected in Puerto Rico yielded similar results to that of Johnson et. al. (2006) in relation to the data set described in Figure 17. The ionic strength of the seawater collected was found to be 0.76M. The datapoint produced from analysis of the Puerto Rico seawater plots above the rest of the data. It is unclear if this result is due to lysis of the cells during transport from the field to lab or if this is an accurate result. Additional characterization of native marine consortia is needed to establish variations between pure cultures and environmental communities if any exist.

4.3 Mineral Nucleation

4.3.1 Carbonate Minerals

Microorganisms have, in recent years, become a large focus in the investigation into carbonate formation kinetics. Exopolymeric substances (EPS) have often been the focus of carbonate mineral formation in microorganisms. EPS can be found in biofilms and microbial mats. Bacteria isolated from microbial mats have been shown capable of producing large amounts of EPS, and are believed to enhance the process of carbonate precipitation (Braissant et. al., 2007). In the 2007 study by Braissant et. al. EPS were primarily comprised of sulfates, carboxylic acids, sulfonic acids, thiols, and amino acids some of which are relevant to this study. Increases in surface functional groups and EPS can in turn have a profound effect on rates of precipitation of carbonate minerals.

In this study it has been shown that organisms are capable of increasing or decreasing surface functional groups dependent upon the environment of growth. The primary functional group of interest in this study is a comprising component of EPS on microorganism's surfaces. The results of this study implies that it is possible for the concentration of EPS in a given environment to increase with changes in fluid chemistry. Furthermore, when comparing data from this study to previous studies investigating freshwater organisms and halophilic organisms, a general trend was observed to be the same for both data sets (Figure 17), independent of individual organism characteristics. This result suggests that rather than being organism specific, it appears to be a broad epigenetic response by microorganisms that leads to a phenotypical response in all organisms to the challenges posed by high salt environments. To further illustrate this, an evaluation of a native seawater consortium was also compared to the trend, and was found to plot closely with data obtained from lab experiments. To confirm that this result is indeed valid, many more consortia should be evaluated and compared to the data trend from this and previous studies.

This study has produced data that fills a gap in seawater salinity environments, however much more work is required to understand the kinetics of carbonate mineral formation, specifically dolomite, by microorganisms. Carbonates produced from different EPS have been shown to have varying concentrations of Mg ions due to preferential binding of Ca, increasing the Mg/Ca ratio in fluids (Bontognali et. al., 2014). However, increased Mg/Ca ratios were found to not be sufficient to induce dolomite precipitation alone (Morse et. al. 1997). It has also been shown that microorganisms are capable of mediating precipitation even when they are not living. In considering results from this and previous studies the ability of various organisms to facilitate carbonate mineral production should be further investigated.

4.3.2 Mg-Rich Clays

In many carbonate reservoirs, Mg-rich clay minerals are transformed into dolomite through diagenesis. Mg-silicates are highly active geochemically when compared with other clay minerals (Tosca and Wright, 2015). Depositionally, such clays are well understood, however, their crystallization process is not. Commonly lacustrine clays are almost exclusively Mg and Si in composition, and are often found in saline alkaline environments (Tosca and Wright, 2015). Although Mg-rich clays can readily nucleate directly from fluid, substrate surfaces such as EPS are commonly required to catalyze the reaction.

Results of this study suggest that microorganisms inhabiting a saline, alkaline, environment will have an increased surface functional group density, and possibly EPS, that can serve as a nucleation point for such clays. Deposits of clays can then theoretically, through diagenesis, dolomitize to produce potential hydrocarbon reservoirs. The processes involved in clay mineral precipitation and diagenesis is scarcely investigated and may fill a large knowledge gap in the origin of dolomitic hydrocarbon reservoirs. Given their ability to quickly adapt to saline environments, microorganisms can potentially play a large role in understanding these processes.

4.4 Ancient and Modern Dolomite Deposits

Dolomite deposits are common in the rock record, some of them being identified as being associated with microbial activity. The Mississippian Debolt Formation in the Dunvegan gas field of northwestern Alberta, Canada is a carbonate gas reservoir. Facies of this formation contain anhydrite, subaerial exposure features, bioturbation structures, and structures common to shallow marine environments (Baniak et. al., 2014). Features of these facies suggest that deposits formed in high salinity environments. There are extensive bioturbation features in some facies in

the Debolt Formation that are ideal for microorganisms to inhabit. Results from this study and previous studies suggest that the microorganisms inhabiting such an environment would have a high density of surface functional groups capable of promoting dolomite formation. Dolomite filled burrows in this formation make sharp contacts with surrounding the surrounding calcite matrix (Baniak et. al., 2014). From this study, one possible explanation for the sharp contact between dolomite and calcite can be found in the microorganism inhabiting mucous linings of the burrows in a high salinity environment. Nucleation of small dolomite crystals along the sides of burrows can lead to more rapid growth once nucleation has occurred.

Modern deposits of dolomite are much less common. One location of modern dolomite formation is found in Lagoa Vermelha, a hypersaline coastal lake in Brazil. A sulfate-reducing microorganism, *Desulfovibrio brasiliensis*, isolated from Lagoa Vermelha has been shown to precipitate low temperature dolomite in laboratory cultures, and is thought to play a major role in dolomite formation in Lagoa Vermehla. Variation in precipitation and evaporation in the region of Lagoa Vermelha by season causes fluctuations in the ionic strength of the lake (Figure 14) (Vasconcelos and McKenzie, 1997). Microorganisms inhabiting the lake are capable of adapting to fluctuating ionic strength conditions, and in doing so adapt their cell surface chemistry. During high ionic strength conditions, surface functional group densities will be higher, and this study shows that this is a function of salinity (Figure 17).

5. Conclusions

Two microorganisms were analyzed after culture in varied ionic strength media to assess their surface characteristics. Several key conclusions were made from results of this experiment, and are as follows:

1. Increasing growth media ionic strength of microorganisms results in an increase in surface functional groups.
2. Decreasing growth media ionic strength of microorganisms results in a decrease in surface functional groups.
3. The magnitude of change in surface functional groups follows a predictable trend.
4. Changes in surface functional groups is rapid and is likely to occur in any environment with fluctuating fluid chemistry either by mixing or evaporative processes.

Carboxyl group densities were found to have increased by as much as 69% in increased ionic strength media, and decreased by as much as 30% in decreased ionic strength media. Changes were observed quickly in both organisms, after just 6 generations in both. The rate of change in these organisms falls well within seasonal cycles that are likely to produce changes in ionic strength in lacustrine or near shore marine environments, as well as sabkha environments.

Rapid adaptations seen in this study by microorganisms to changing growth environment ionic strength have several important geological implications relating to adsorption of metals, and facilitation of mineral formation. In considering the factors important in modeling the geochemistry of geological systems it is necessary to consider the biological interaction within the environment. Slight changes in a system can play a large role in determining the characteristics of microorganism-environment interactions.

This study addressed the ability of and rate at which microorganisms can adapt their surface chemistry as a function of growth media ionic strength. The ability of microorganisms to adapt to changing ionic strength environments, and the resulting data plotting along a trend suggests that perhaps above a threshold of surface carboxyl group density, any organism may be capable of facilitating mineral nucleation.

6. References

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Figure 1. Location of collection of marine consortium. The star represents the approximate sample collection location. Puerto Rico is shown in the upper left map with Crashboat Beach represented by the triangle.

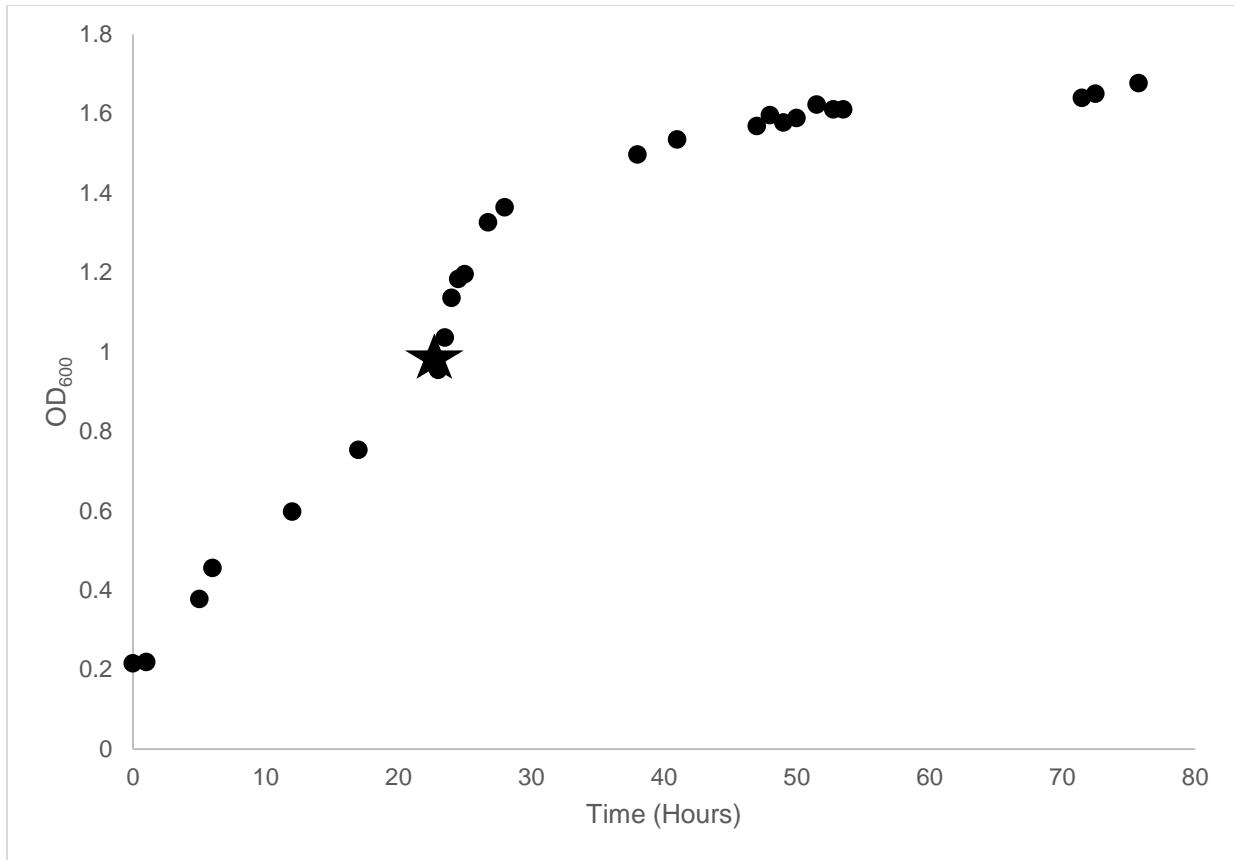


Figure 2. Growth curve for *R. litoralis*. The star represents mid-exponential phase at approximately 24 hours.

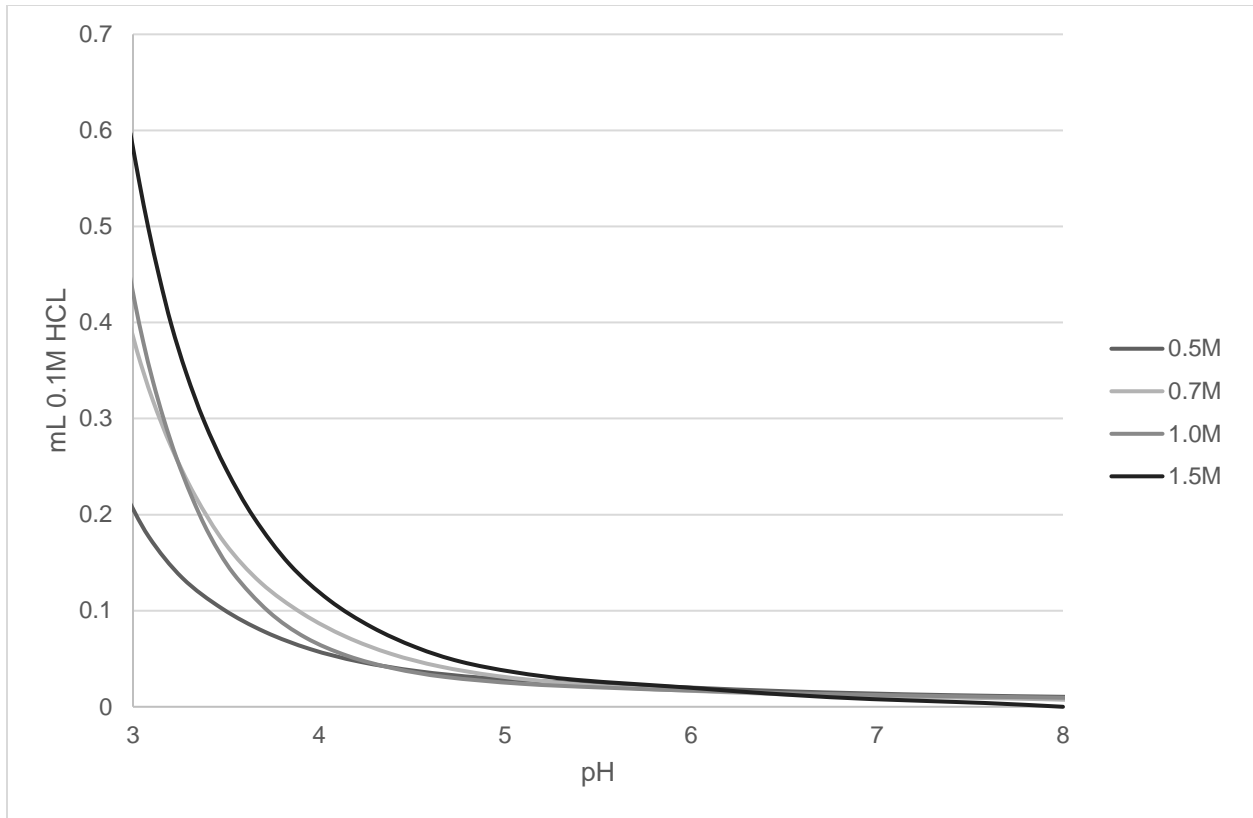


Figure 3. *R. litoralis* titration curves for varied ionic strength growth media. Samples were titrated at 1 g ml^{-1} bacteria to electrolyte.

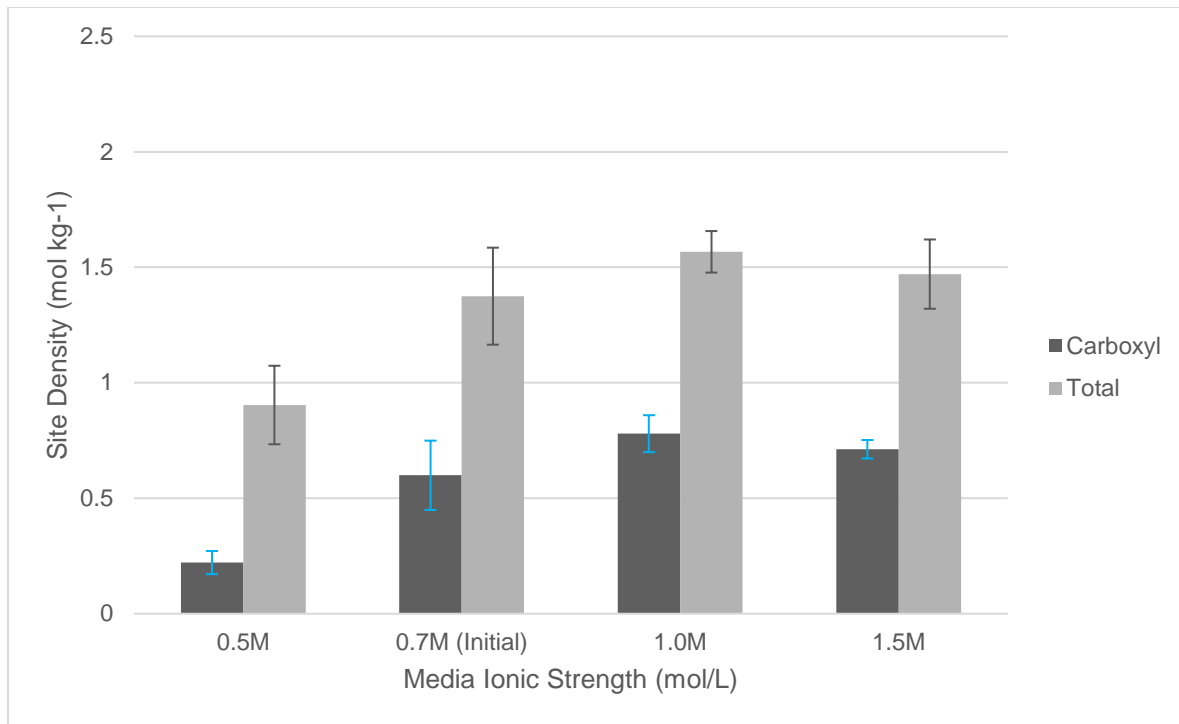


Figure 4. A plot of *Roseobacter litoralis* data summarizing averaged carboxyl and total site densities for varied ionic strength media. Error bars in this figure represent the combined error in the average values for carboxyl and total functional groups.

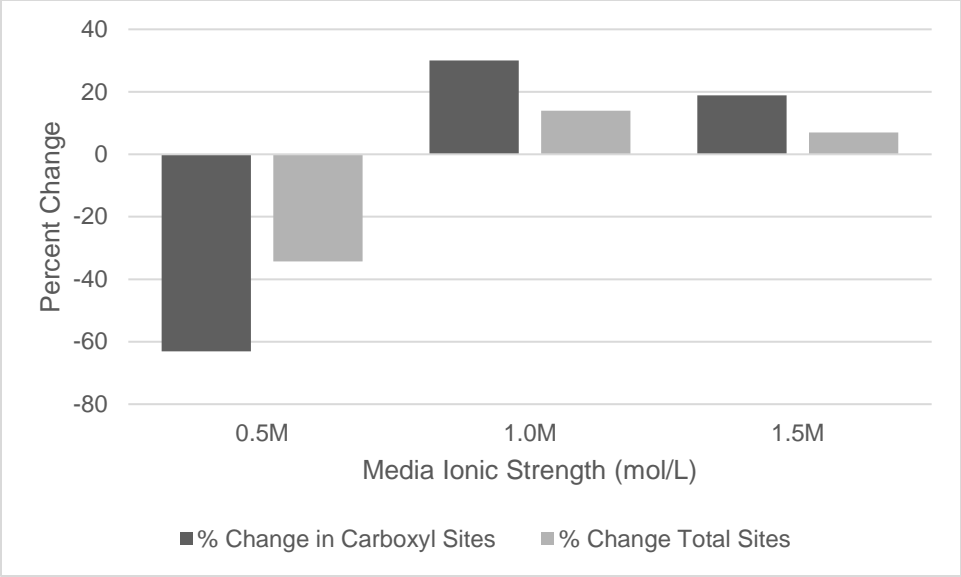


Figure 5. Percent change in carboxyl and total site density for *Roseobacter litoralis* for organisms grown in varied ionic strength growth media.

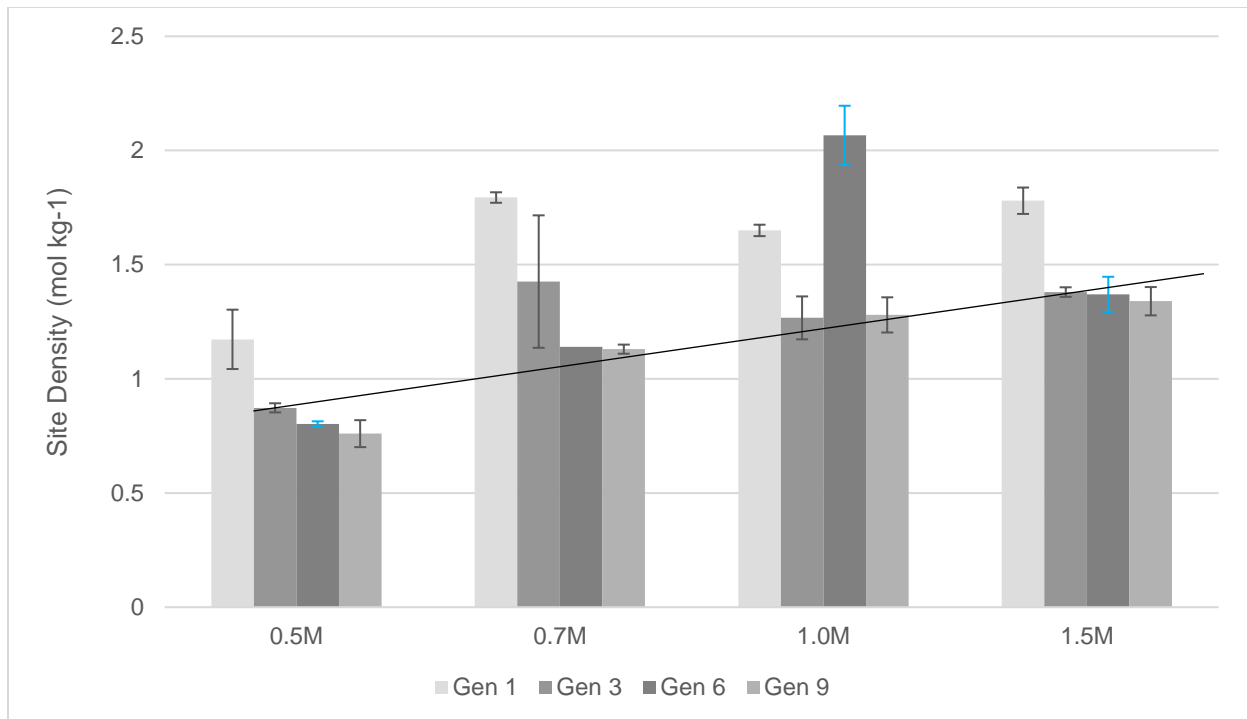


Figure 6. ProtoFit results for each generation of growth for *R. littoralis* for total functional group site density. The line in the figure represents the increasing trend in functional group density with increasing growth media ionic strength.

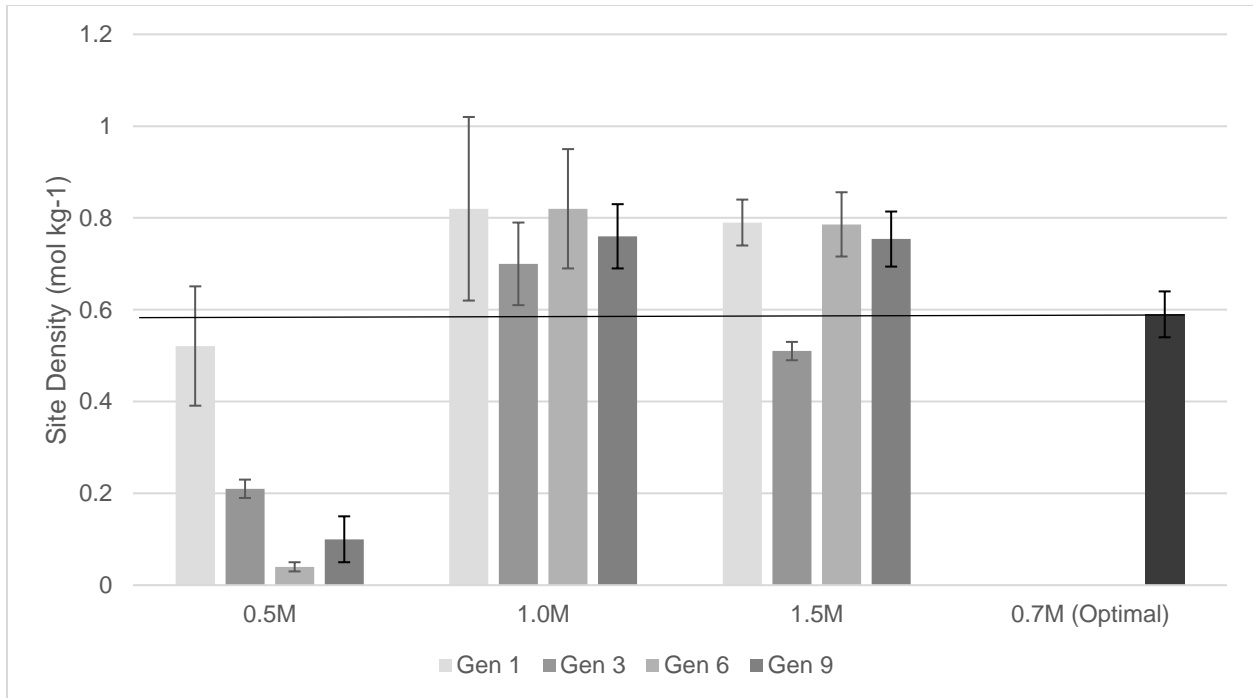


Figure 7. Carboxyl group density_results from ProtoFit for each generation of growth at varied ionic strengths for *R. littoralis*. The line in this figure represents the average carboxyl group density for organisms grown at the optimal ionic strength.

<i>R. littoralis</i> ProtoFit output								
0.5M		0.7M		1.0M		1.5M		
Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	
Generation 1								
Trial 1	0.37	0.87	0.77	1.45	0.85	1.54	0.84	2.29
Trial 2	0.57	0.99	0.82	1.53	0.81	1.60	0.73	1.45
Trial 3	0.61	1.64	0.79	2.39	0.80	1.81	0.82	1.60
Average	0.52	1.17	0.79	1.79	0.82	1.65	0.80	1.78
Generation 3								
Trial 1	0.19	1.08	0.80	1.41	0.75	1.29	0.49	1.59
Trial 2	0.23	0.85	0.72	1.78	0.59	1.13	0.51	1.19
Trial 3	0.21	0.68	0.25	1.08	0.76	1.39	0.53	1.35
Average	0.21	0.87	0.59	1.42	0.70	1.27	0.51	1.38
Generation 6								
Trial 1	0.06	1.18	0.54	1.14	0.68	1.86	0.73	1.28
Trial 2	0.03	0.70	0.53	1.13	0.96	2.35	0.52	1.30
Trial 3	0.04	0.52	0.52	1.07	0.83	1.98	0.84	1.46
Average	0.04	0.80	0.53	1.12	0.82	2.06	0.7	1.35
Generation 9								
Trial 1	0.17	1.06	0.46	1.09	0.78	1.35	0.69	1.29
Trial 2	0.08	0.62	0.49	1.33	0.68	1.19	0.82	1.40
Trial 3	0.06	0.61	0.45	0.99	0.83	1.31	0.75	1.34
Average	0.10	0.76	0.47	1.14	0.76	1.28	0.75	1.34

Table 4. All carboxyl group and total group density ProtoFit results for *R. littoralis* including replicates at all generation.

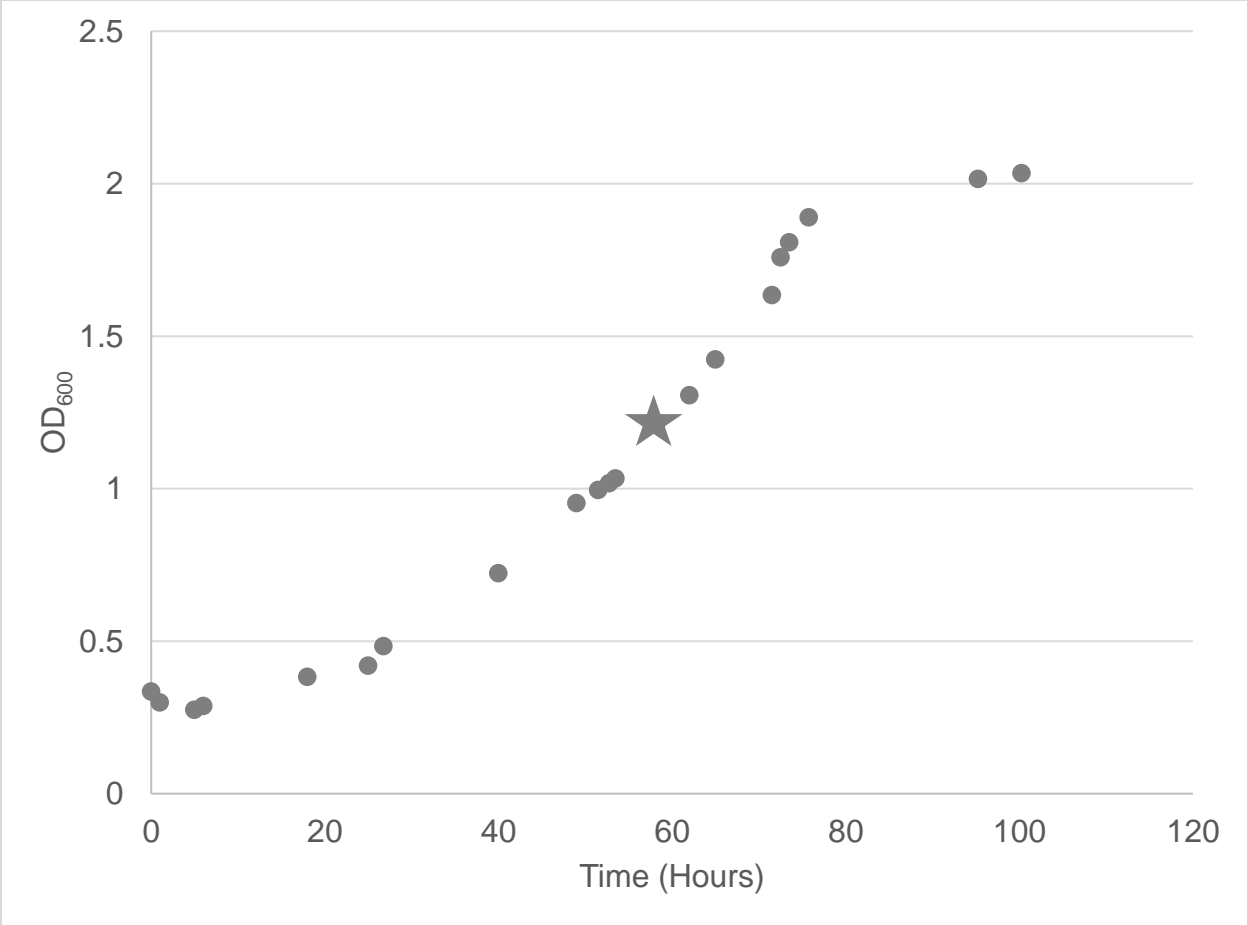


Figure 8. Growth curve for *H. salinarum*. The star represents mid-exponential phase at approximately 60 hours.

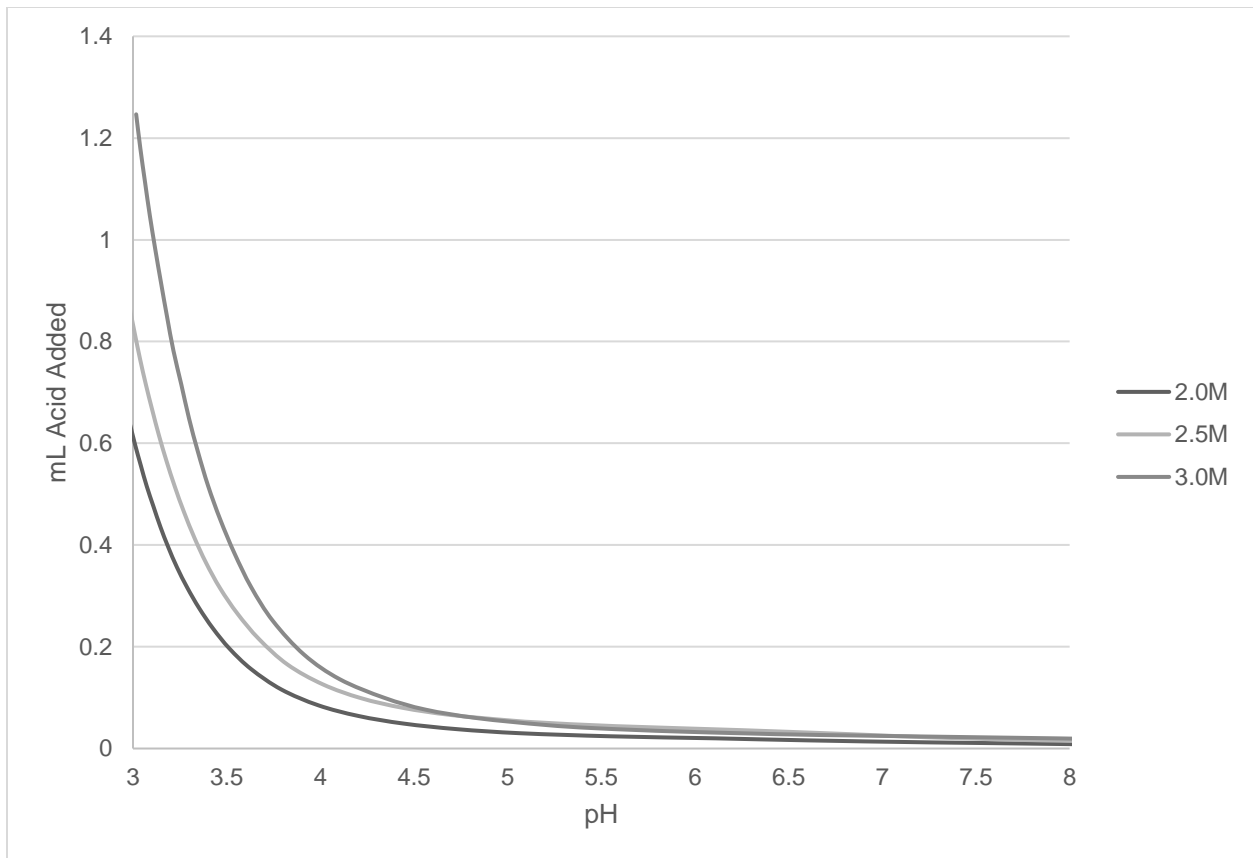


Figure 9. *H. salinarum* titration curves for varied ionic strength media. Samples were titrated at 1 g mL^{-1} cells to electrolyte solution.

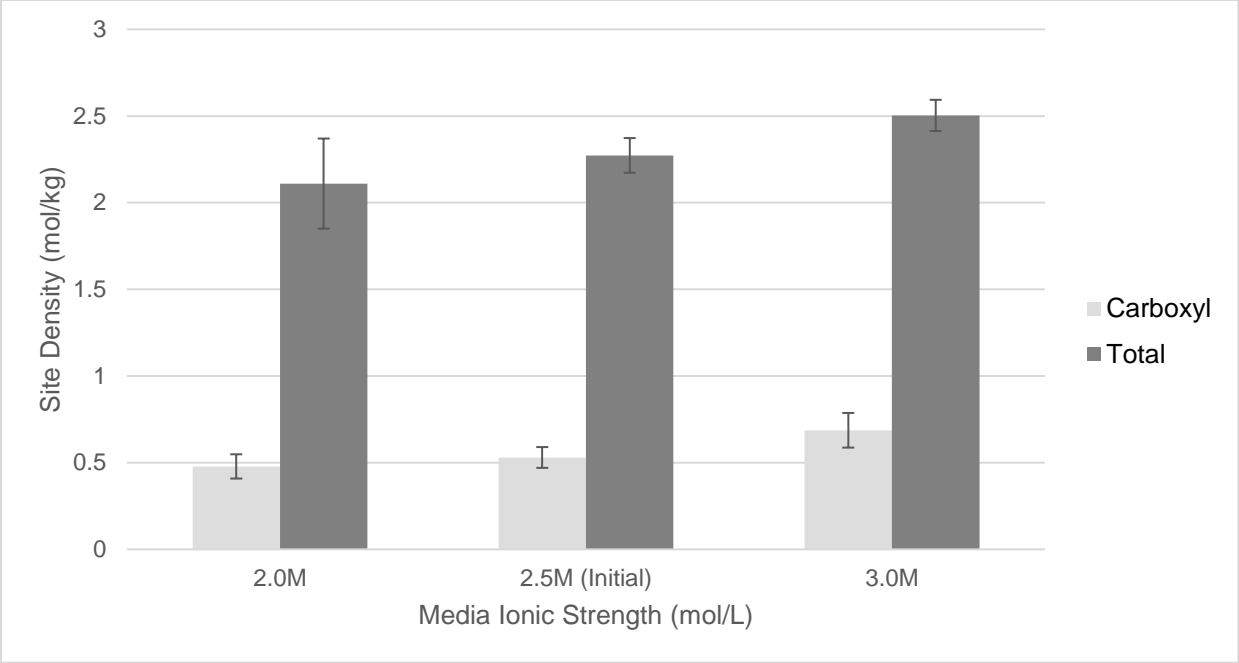


Figure 10. A plot of *Halobacterium salinarum* data summarizing averaged carboxyl and total site densities for optimal (2.5M) and varied ionic strength media.

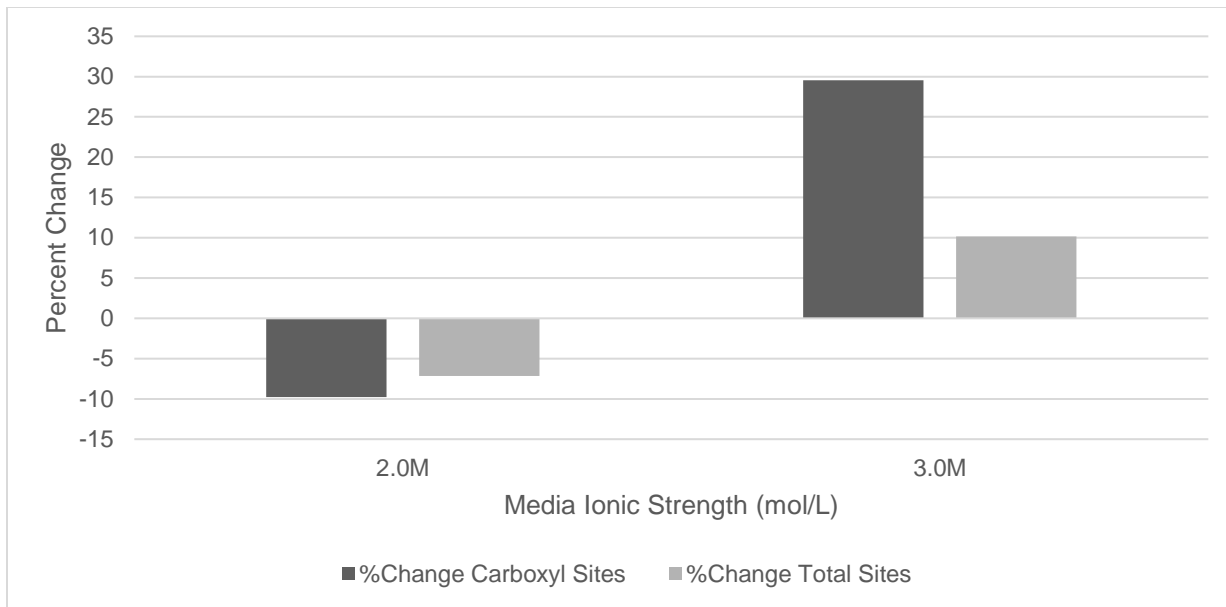


Figure 11. Percent change in carboxyl and total site density for *Halobacterium salinarum* for organisms cultured in varied ionic strength growth media.

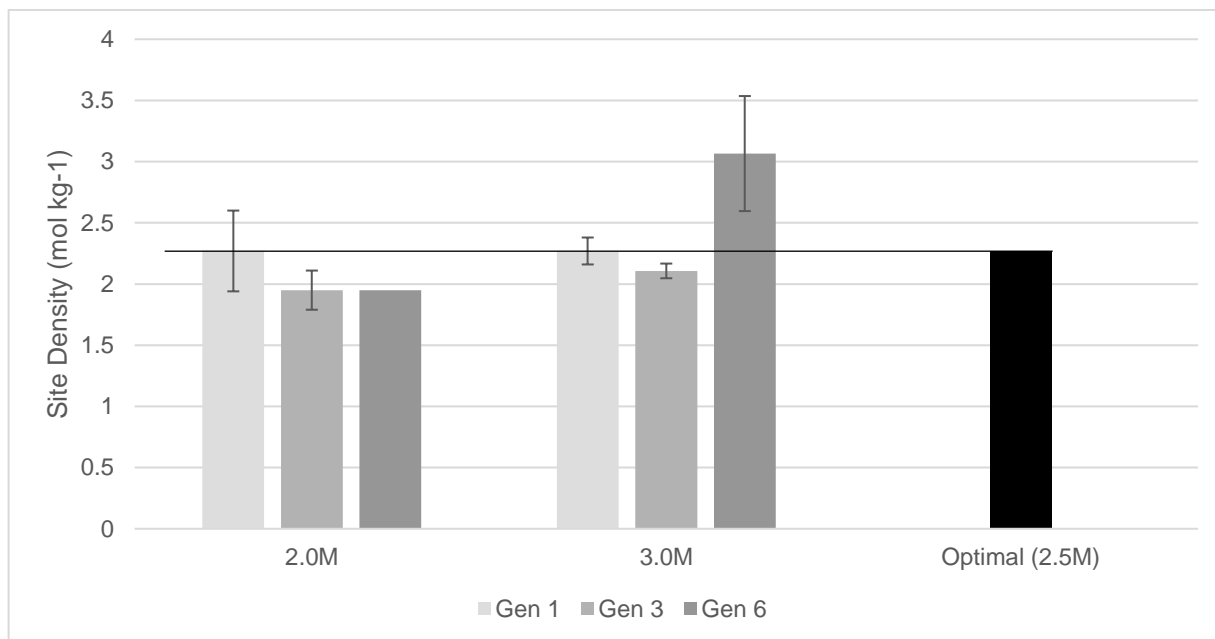


Figure 12. ProtoFit results for each generation of growth in *H. salinarum* for total functional group site density. The line in this figure represents the average functional group density for organisms grown at the optimal ionic strength.

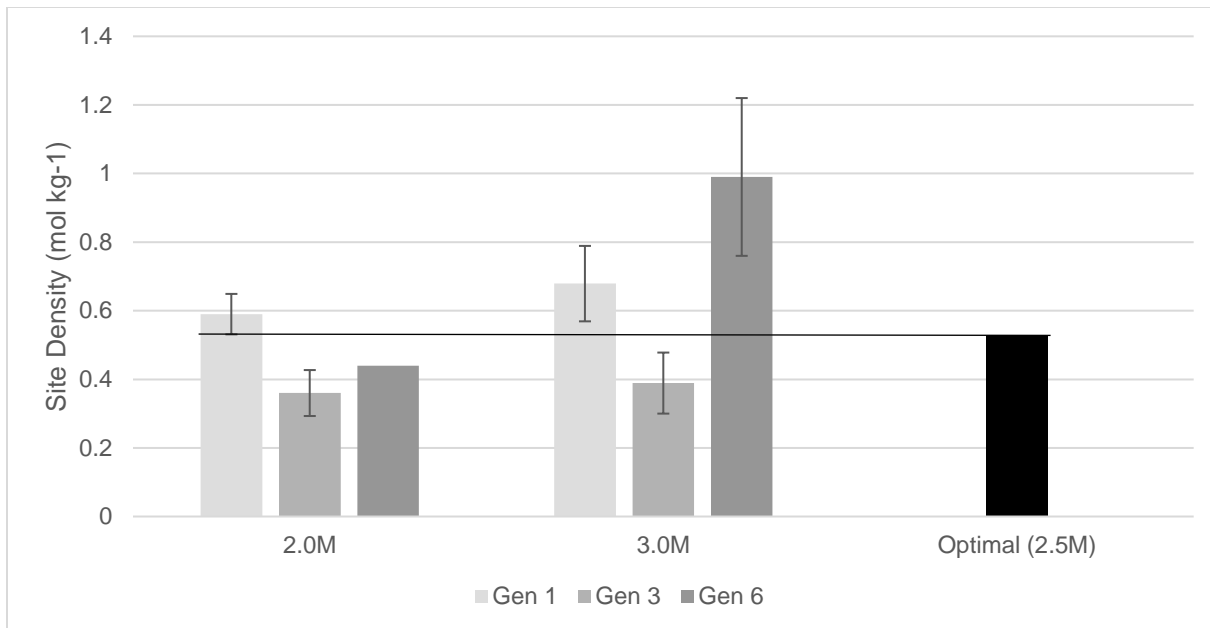


Figure 13. ProtoFit results for each generation of growth in *H. salinarum* for carboxyl group site density. The line in this figure represents the average carboxyl group density for organisms grown at the optimal ionic strength.

<i>H. salinarum</i> ProtoFit output						
	2.0M		2.5M		3.0M	
	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)	Carboxyl Groups (mol/kg)	Total Groups (mol/kg)
	Generation 1					
Trial 1	0.53	2.79	0.66	2.29	0.57	2.05
Trial 2	0.59	1.49	0.67	2.65	0.79	2.61
Trial 3	0.65	2.54	0.48	1.98	0.67	2.13
Average	0.59	2.27	0.60	2.31	0.68	2.26
	Generation 3					
Trial 1	0.44	1.68	0.29	2.19	0.47	2.23
Trial 2	0.31	1.89	0.23	1.89	0.39	2.07
Trial 3	0.34	2.27	0.24	1.84	0.29	1.99
Average	0.36	1.95	0.25	1.97	0.38	2.10
	Generation 6					
Trial 1	0.44	2.26	0.73	2.60	0.99	2.99
Trial 2	0.53	2.64	0.73	2.46	0.74	2.47
Trial 3	0.30	1.20	0.41	1.89	1.19	3.37
Average	0.42	2.03	0.62	2.32	0.97	2.94

Table 5. All carboxyl group and total group density ProtoFit results for *H. salinarum* including replicates at all generation.

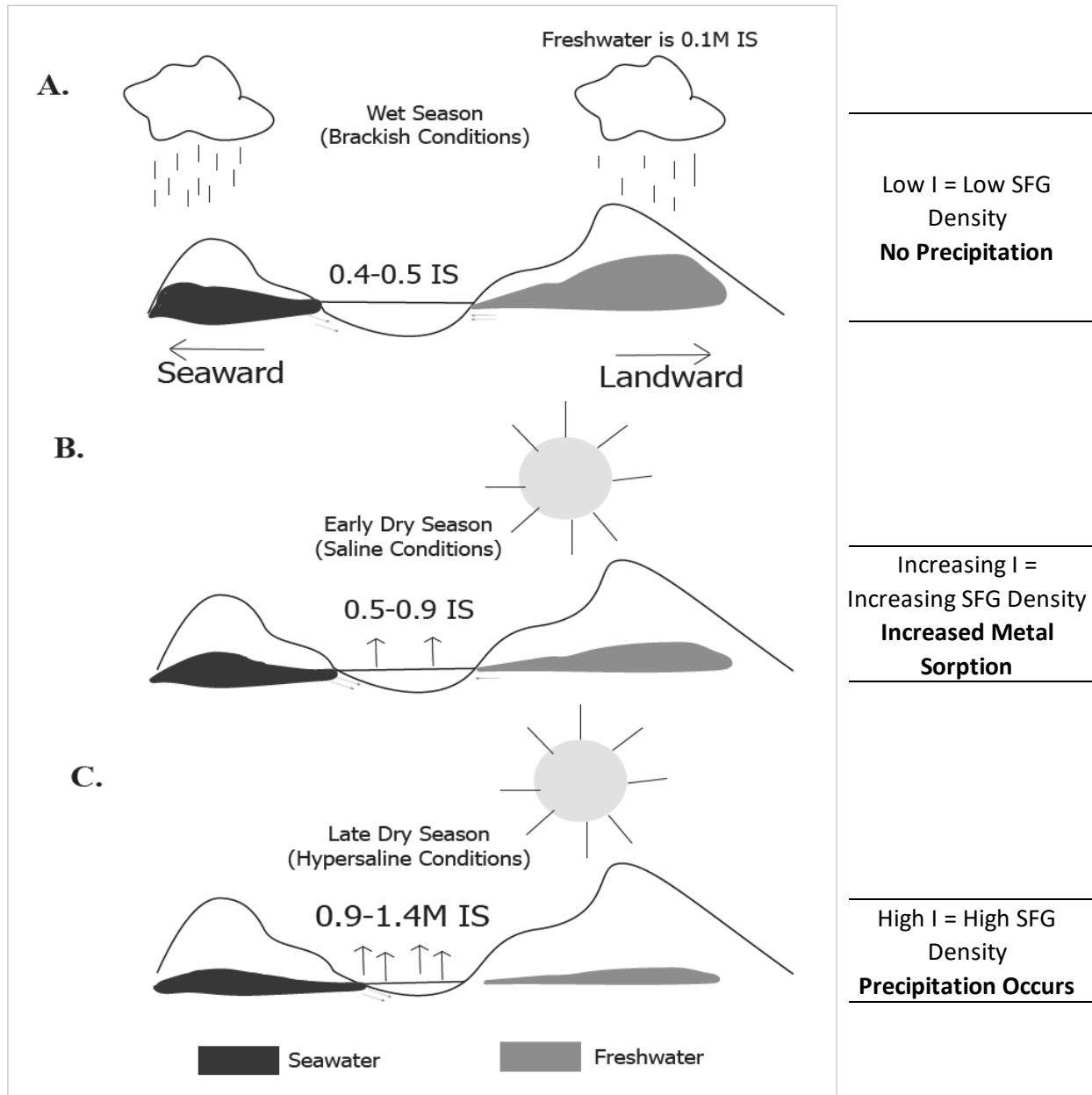


Figure 14. Conceptual model of Lagoa Vermelha representing the seasonality of salinity in Lagoa Vermelha. Evaporation and unbalanced inflow results in increased ionic strengths in the dry season. Microorganisms react to changes and increase functional groups allowing greater quantities of Mg^{2+} to be sorbed. Biomass in the fluid with increasing carboxyl group density will act as a nucleation point for Mg-rich clays and dolomite (Ex. C) along the lagoon floor. SFG = Surface Functional Groups. (modified from Vasconcelos and McKenzie, 1997).

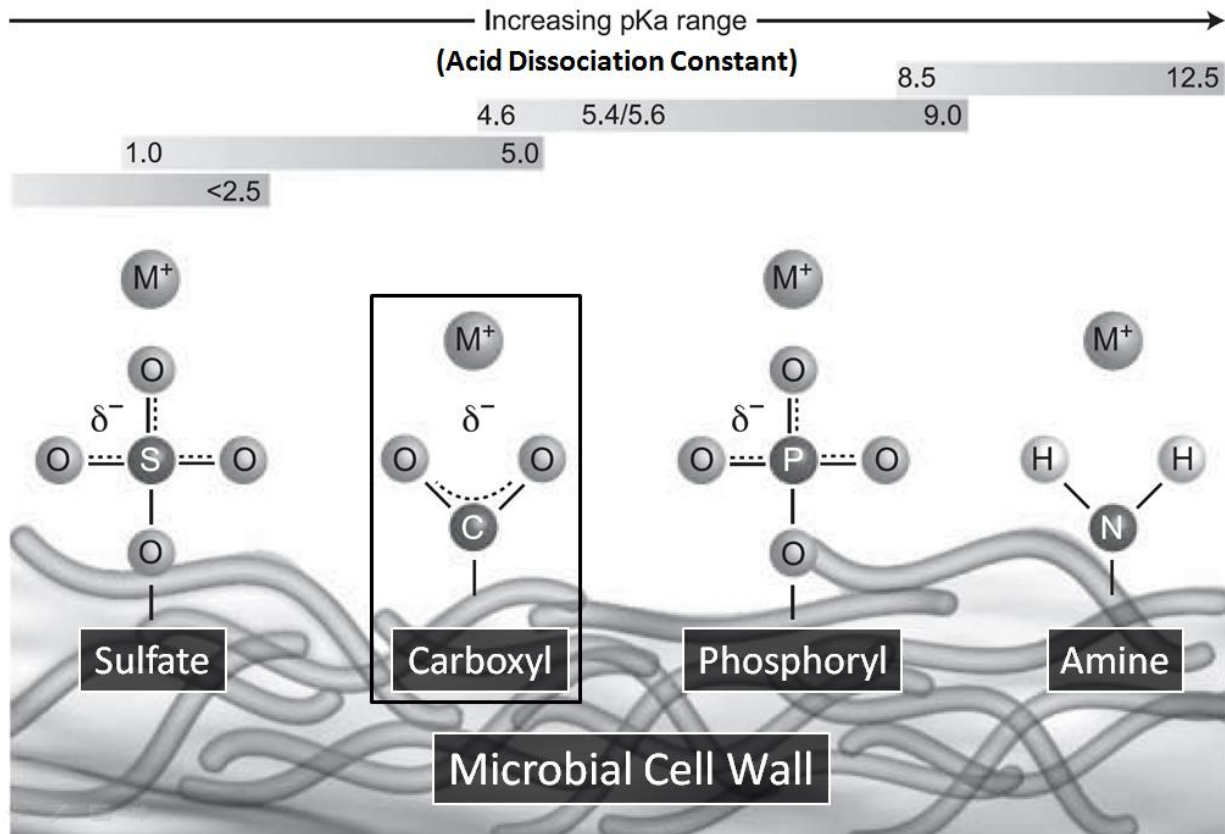


Figure 15. Diagram representing key surface functional groups of microbial surfaces and their related structures and pKas. (modified from Braissant et. al., 2007 and reproduced from Voegerl, 2014)

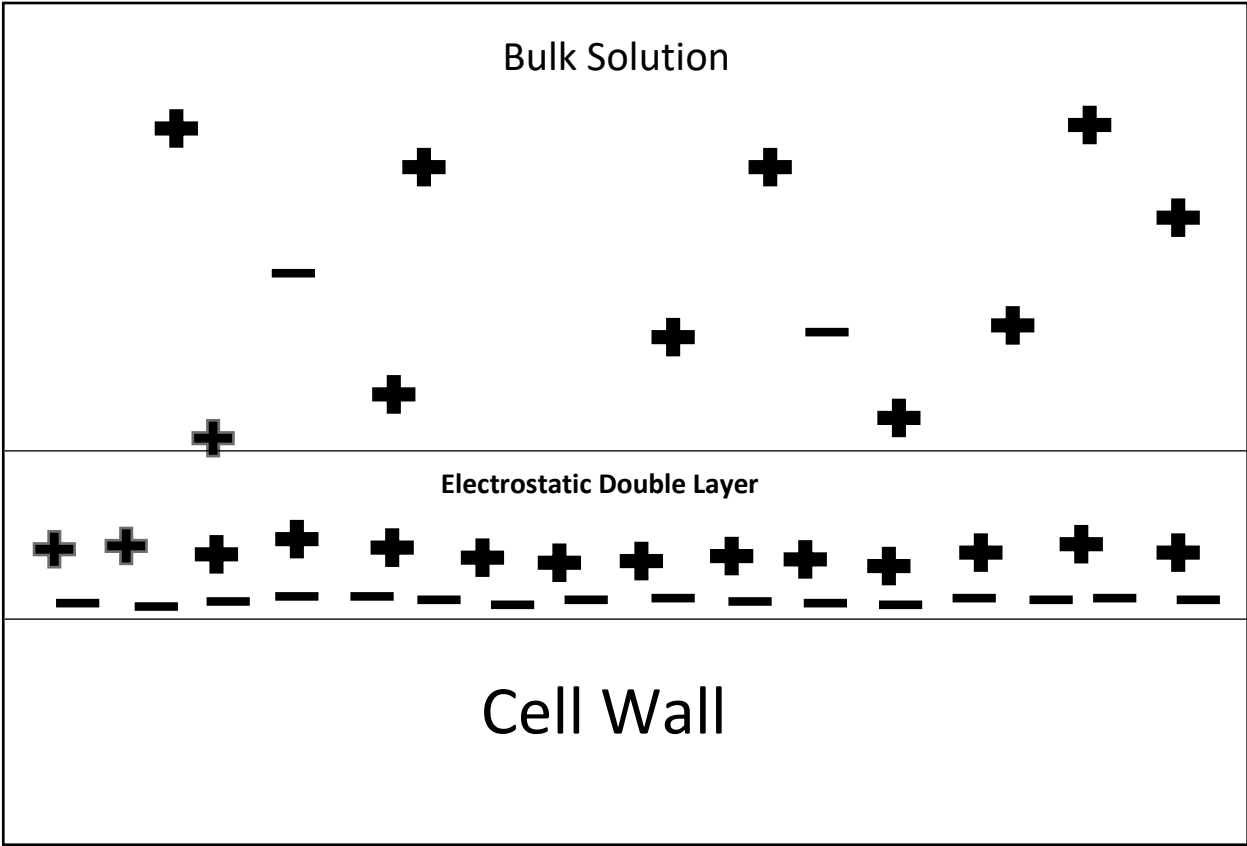


Figure 16. A model representation of the electrostatic double layer where metal-surface interactions take place.

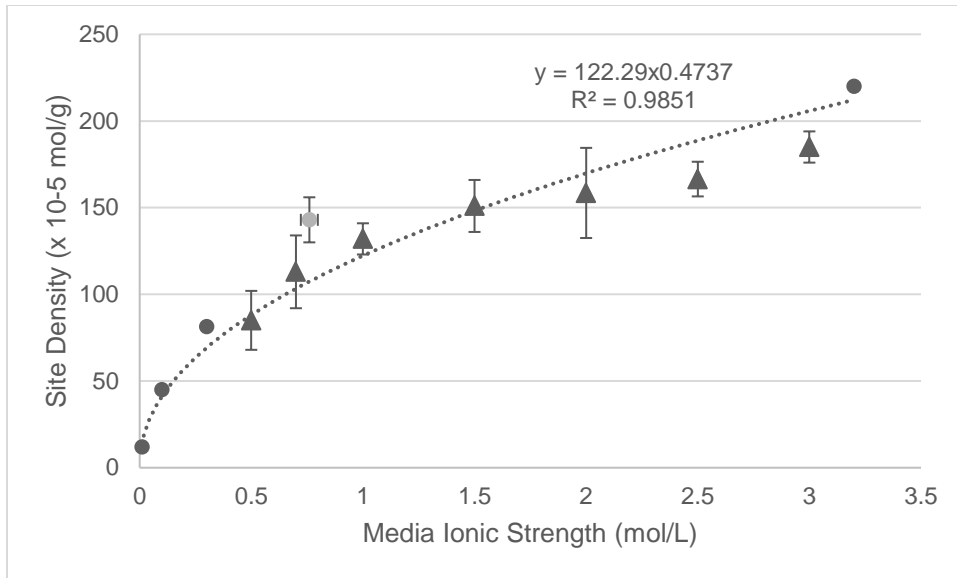


Figure 17. Plot of sum of the two largest functional site densities. Data from previous studies (Johnson et. al., 2006; Kenward, 2010; Kinnebrew, 2012) are represented as blue dots, data from this study are represented as orange triangles, and the native marine consortium is represented as the green square. Fitting a power function to the data shows the predictable relationship between functional group density and growth media ionic strength.

Appendix

<i>Halobacterium Salinarum</i> ATCC Medium 2185	
Basal Medium	
NaCl	250.0g
MgSO ₄ · 7H ₂ O	20.0g
Trisodium Citrate	3.0g
KCl	2.0g
Tryptone	5.0g
Yeast Extract	3.0g
Distilled H ₂ O	1000 mL
Autoclave at 121C for 15 minutes.	
Trace Metal Solution	
ZnSO ₄ · 7H ₂ O	1.32g
MnSO ₄ · H ₂ O	0.34g
Fe(NH ₄) ₂ SO ₄ · 6H ₂ O	0.82g
CuSO ₄ · 5H ₂ O	0.14g
Distilled Water	200 mL
Add 0.1 mL of trace metal solution to autoclaved basal medium. Filter Sterilize. Yields a 2.5M ionic strength medium.	

Table 1. Recipe for *Halobacterium salinarum* to obtain a 2.5M medium

<i>Roseobacter litoralis</i> ATCC Medium 2	
Media prepared using Difco Marine Broth 2216. For simplicity and repeatability 37.4g of Difco Marine Broth 2216 was added to 1000 mL of distilled H ₂ O. Contents are listed below. Boil solution for 1 minute to dissolve powder and autoclave at 121C for 15 minutes. Yields a 0.7M ionic strength medium.	
Peptone	5.00g
Yeast Extract	1.00g
Ferric Citrate	0.10g
NaCl	19.45g
MgCl	8.8g
Na ₂ SO ₄	3.24g
CaCl ₂	1.8g
KCl	0.55g
NaHCO ₃	0.16g
KBr	0.08g
SrCl ₂	0.034g
H ₃ BO ₃	0.022g
Na ₂ SiO ₃	0.004g
NaF	0.0024g
NH ₄ NO ₃	0.0016g
Na ₂ HPO ₄	0.008g
Distilled H ₂ O	1000 mL

Table 2. Recipe for *Roseobacter litoralis* to obtain a 0.7M ionic strength medium. Recipe for *R. litoralis* medium taken from ATCC.

Media Manipulations					
<i>R. litoralis</i>			<i>H. salinarum</i>		
Ionic Strength	Manipulation to Original Medium	# of Generations	Ionic Strength	Manipulation to Original Medium	# of Generations
0.5M	18.7g Marine Broth in 730 mL distilled H ₂ O	9	2.0M	195g NaCl	6
0.7M	37.4g Marine Broth in 1000 mL distilled H ₂ O	9	2.5M	250g NaCl	6
1.0M	37.4g Marine Broth + 16g NaCl in 1000 mL distilled H ₂ O	9	3.0M	312g NaCl	6
1.5M	37.4g Marine Broth + 45g NaCl in 1000 mL distilled H ₂ O	9			

Table 3. Media manipulations for *R. litoralis* and *H. salinarum* at each ionic strength including the optimal ionic strength medium.

	Site 1	Site 2	Site 3
0.5M	4.18	6.12	9.53
0.7M	3.88	6.03	8.80
1.0M	3.60	6.19	8.70
1.5M	3.84	6.14	9.24

Table 4. ProtoFit output of pKa values for *Roseobacter litoralis* for all ionic strength media.

	Site 1	Site 2	Site 3	Site 4
2.0M	3.96	5.97	8.76	11.61
2.5M	4.06	5.92	8.01	11.18
3.0M	3.80	5.94	8.29	11.14

Table 5. ProtoFit output of pKa values for *Halobacterium Salinarum* for all ionic strength media.

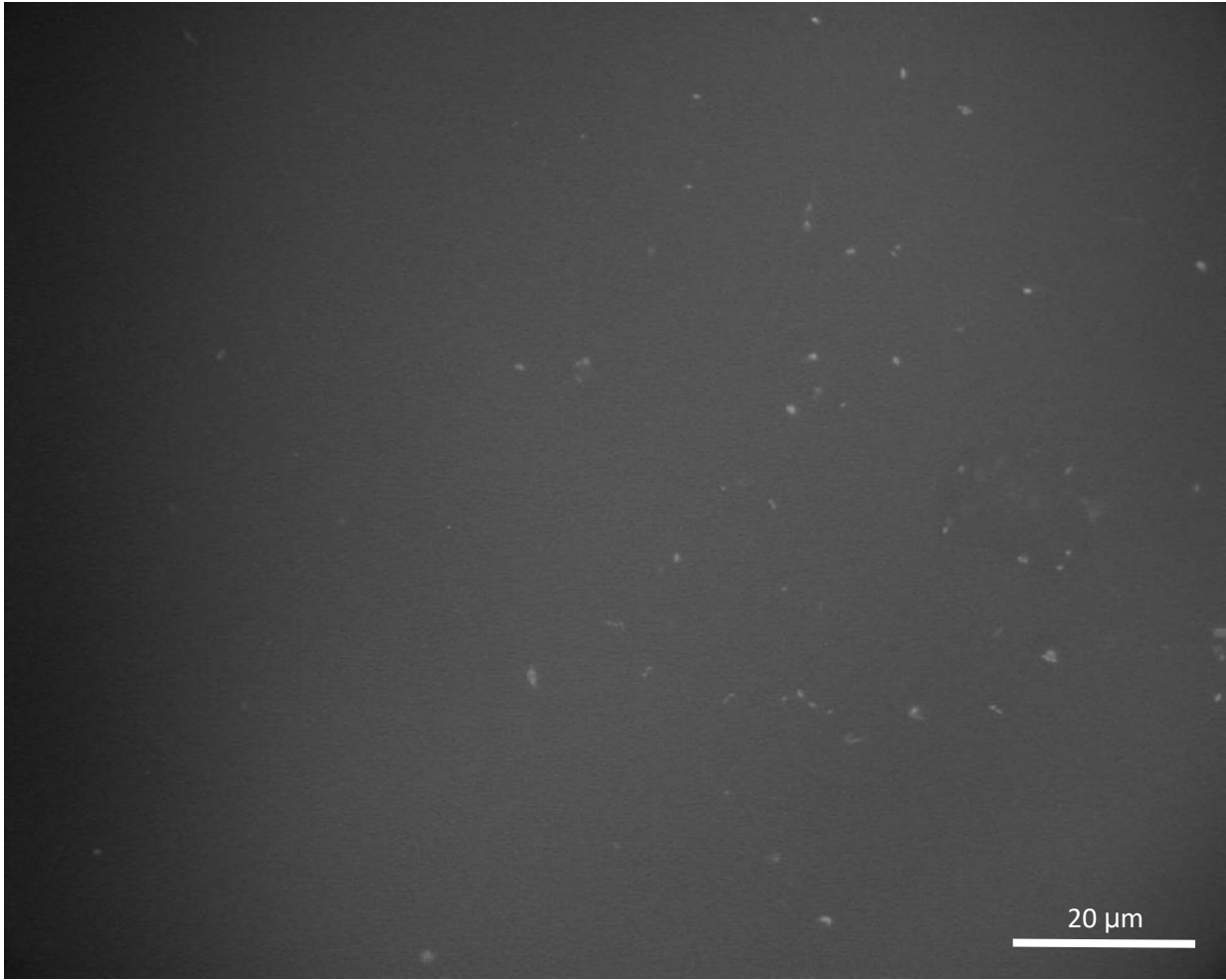


Figure 18. Oil immersion microscope image of *H. salinarum* at 100x magnification.

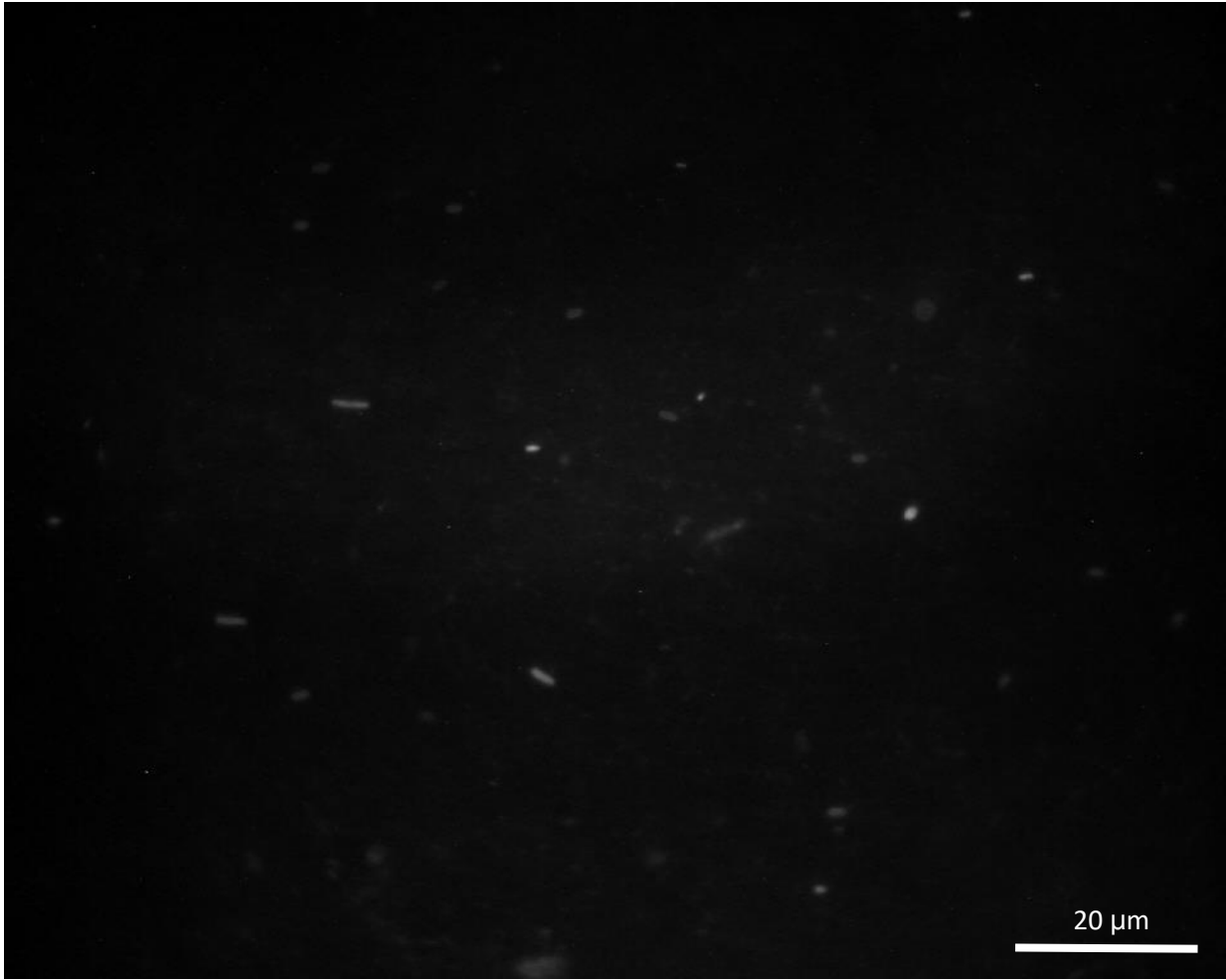


Figure 19. Oil immersion microscope image of *R. litoralis* at 100x magnification.

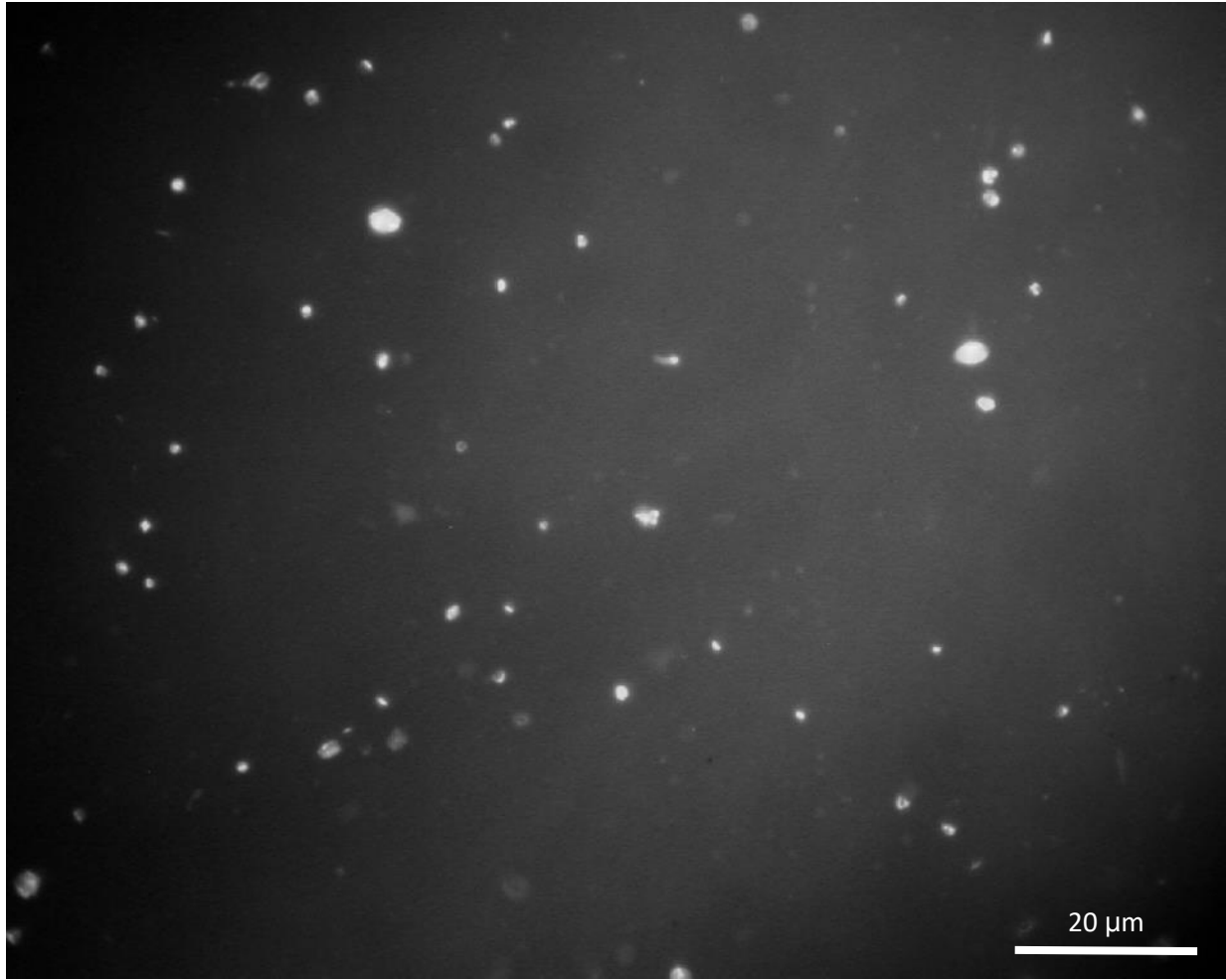


Figure 20. Oil immersion microscope image of the Puerto Rico seawater consortium at 100x magnification.