Abstract

INVESTIGATING THE MICROBIAL COMMUNITIES ASSOCIATED

WITH ALUMINUM ALLOYS 2024 AND 7075

By

Kyra A. Price

December, 2020

Director of Thesis: Erin K. Field

Major Department: Biology

Aluminum (Al), one of the most versatile, cost-effective, and appealing metals for use in construction, has no known biological function. Al alloys in aquatic environments provide a surface for attachment and can be advantageous for nutrient acquisition. Due to Al's toxicity, there is little competition for colonizing the surface of Al alloys; therefore, organisms that survive in these conditions have an advantage over others that cannot. Previous research on the microbial communities that attach to a variety of surfaces like copper, Al, steel alloys, and plastics has shown that they are unique in composition and that certain community members may be preferentially selecting the surfaces they attach to. Because of this, I hypothesized that the microbial communities attached to Al alloys will be different than those attached to other metal alloys and surface materials. This will then be evident by variation in the microbial community composition between the

substrates. To test this hypothesis, a field-based environmental study was conducted at two locations in the Pamlico River in North Carolina for 8 months investigating the microbial communities that colonize different substrates of interest—Al alloys 2024 & 7075, stainless steel alloys 304 & 316, a non-metal biofouling plate, and sediment. After 6-8 weeks, DNA was extracted from the material attached to the metal coupons and the microbial community was sequenced via 16S rRNA gene amplicon Illumina sequencing. Results suggest the microbial communities attached to all substrates were more similar in July than December. Salinity and water temperature were found to drive the variation in community composition. *Gammaproteobacteria* were found to primarily contribute to the dissimilarity between Al 2024 and Al 7075, with a higher abundance on Al 2024. Using the biomass attached to the Al alloys, *Bacillus* and *Pseudoalteromonas* sp. were isolated from Al 2024 in the presence of aluminum. The isolate's growth limits were characterized to further understand the microorganisms that attach to Al surfaces and how they are able to withstand changes in the environment in the presence of Al. I hypothesized that decreasing the temperature below the isolate's optimal growth temperature would negatively affect its tolerance to aluminum. Results suggest that isolates from Al 2024 in an estuary environment are negatively affected by a 5°C drop in temperature at 1 mM $AlCl_3$, as their maximum optical density at 600 nm decreased. These findings can be used to understand the variation in microbial communities attached to Al 2024 in estuaries globally, and ultimately to develop specialized management strategies to preserve Al alloy infrastructure in aquatic systems.

INVESTIGATING THE MICROBIAL COMMUNITIES ASSOCIATED WITH ALUMINUM ALLOYS 2024 AND 7075

A Thesis

Presented to the Faculty of Biology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Molecular Biology & Biotechnology

By

Kyra A. Price

December, 2020

INVESTIGATING THE MICROBIAL COMMUNITIES ASSOCIATED

WITH ALUMINUM ALLOYS 2024 AND 7075

By

Kyra A. Price

APPROVED BY:

DIRECTOR OF THESIS: _____

Erin K. Field, PhD

COMMITTEE MEMBER:_____

Eric S. Anderson, PhD

COMMITTEE MEMBER:_____

April M. Blakeslee, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY:_____

David Chalcraft, PhD

DEAN OF THE GRADUATE SCHOOL:_____

Paul J. Gemperline, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

ENVIRONMENTAL STUDY OF ALUMINUM ALLOYS 2024 & 7075 IN PAMLICO RIVER

INTRODUCTION

The toxic and inert nature of aluminum (Al), one of the most abundant metals in Earth's crust, is an increasingly interesting field of research. Not only is it the most abundant metal in Earth's crust, but it is one of the most versatile, cost-effective, visually appealing metals and is completely recyclable (Emsley, 2011). Al has been used in everyday materials from wrapping foil to components of airplanes since 1854 (Emsley, 2011, Hatch, 1984). Due to its density and overall light weight its use in structural applications, when alloyed with other metals, comes second to steel in its industrial use and is a third of the weight of steel (Emsley, 2011). Once exposed to air, a passive Al oxide film forms to protect the surface of Al alloys from further oxidation which could eventually result in corrosion of the metal surface (McNamara et al., 2005, Rajasekar & Ting, 2010). Unlike iron-based metals like carbon and stainless-steel alloys, Al alloys exhibit higher corrosion resistance competitive with other corrosion resistant metals alloyed with titanium and copper (Emsley, 2011, Hatch, 1984). Like all metal alloys, compared to the atmosphere, Al alloys corrode faster when submerged in an aquatic environment due to the pH and surrounding ions which has sparked the interest of researchers to identify causes of accelerated deterioration (McNamara et al., 2005, Rajasekar & Ting, 2010, Total Materia*,* 2008). When Al infrastructure does corrode, it displays "pitting" which is a non-uniform localized area of corrosion across the metal surface (Hatch, 1984, Total Materia*,* 2008). Previous research on the surface of submerged Al alloys, as well as inside jet fuel tanks constructed with Al alloys, has revealed microbial communities that attach to the metal surface, penetrate the passive Al

oxide layer, and either exacerbate these conditions or protect them in a biofilm state (McNamara et al., 2005, Rajasekar & Ting, 2010).

Due to its reactivity, Al is always found complexed in nature primarily in the form of Al silicate minerals such as bauxite or cryolite ores (Bruckard et al., 2010, Total Materia*,* 2008). To be used, it needs to be extracted using an energetically expensive process called the Hall–Héroult process—this is where Al oxide in the molten ore is dissolved and electrolytically reduced to pure Al giving it qualities characteristic of the Al we use day-to-day, difficult to corrode and easy to recycle (Bruckard et al., 2010, Emsley, 2011, Hatch, 1984). Al is becoming an increasing environmental concern. The use of Al in food products, pharmaceuticals, and water treatment along with acid rain and other anthropogenic activities, have led to an increase in the solubilization of Al, overall increasing the concentration of toxic Al in the environment (Bruckard et al., 2010, Emsley, 2011, Hatch, 1984).

In the 1930's, the booming innovation of aircraft industries resulted in the development of new alloys displaying better strength while maintaining a light weight (Öksüz et al., 2013, Total Materia*,* 2004). This study used two of the three most commonly used Al alloys used in the construction of aircrafts during World War II (WWII): Al 2024 and Al 7075. Al alloy 2024 has been in use since 1931, with copper as the primary alloying element and trace elements like magnesium and manganese, it showed increased strength from any other Al alloy used before (Öksüz et al., 2013, Total Materia*,* 2004). It was also favored over other alloys due to its high strength:weight ratio and primarily used when high cyclic stress resistance is required like in the aerospace industry, more specifically in aircraft structures, the transportation industry, like in hydraulic systems, and in marine applications where fatigue resistance is needed (Öksüz et al., 2013, Total Materia*,* 2004). Al alloy 7075 was put in use in the US in 1943; with zinc as the

primary alloying element it has a high strength:weight ratio and is one of the highest strength Al alloys available. The high strength and light weight of Al 7075 allow its use in a variety of fields like aerospace, marine, transportation, and military applications; even some high-end bicycles contain Al 7075 (Öksüz et al., 2013, Total Materia*,* 2004).

Differences in the chemical composition of Al alloy 2024 and 7075 can be seen in Table 1. The main difference is in the primary alloying element—copper for Al 2024 and zinc for Al 7075 (Öksüz et al., 2013, Total Materia*,* 2004). Al 2024 does contain some zinc and Al 7075 contains some copper, but they are at trace amounts compared to their primary alloying metal counterpart. Al 2024 contains more manganese while Al 7075 contains more magnesium and chromium. Even though Al 2024 is one of the most durable Al alloys, having copper as the main alloy element makes 2024 more susceptible to corrosion and less ductile (Öksüz et al., 2013, Total Materia*,* 2004, Total Materia, 2008). The chemical composition of Al 7075 makes it less ductile than Al 2024 but provides significantly better stress and corrosion resistance, making it more suitable for applications that require high constant stress resistance (Öksüz et al., 2013, Total Materia*,* 2004, Total Materia, 2008).

Table 1: Variations in metal composition of the two Al alloys of interest: Al 2024 and Al 7075. The concentrations of each metal are in % wt. and were obtained from the documentation received from the manufacturer, Metal Samples Company (ALSPI). The different amounts of metals are bolded to emphasize them from the other metal species.

Al 2024	**Al**	**Si**	**Fe**	**Cu**	**Mn**	**Mg**	**Cr**	**Zn**	**Ti**	**V**	**Zr**	**Other**
Min (wt. %)	90.65	0.00	0.00	3.8	0.30	1.2	0.00	0.00	0.00	0.00	0.00	Each, 0.05
Max (wt. %)	94.55	0.50	0.50	4.9	0.9	1.8	0.10	0.25	0.15	0.05	0.05	Total, 0.15
Al 7075	**Al**	**Si**	**Fe**	**Cu**	**Mn**	**Mg**	**Cr**	**Zn**	**Ti**	**V**	**Zr**	**Other**
Min (wt. %)	87.07	0.00	0.00	1.2	0.00	2.1	0.18	5.1	0.00	0.00	0.00	Each, 0.05
Max (wt. %)	91.45	0.40	0.50	2.0	0.30	2.9	0.28	6.1	0.20	0.05	0.05	Total, 0.15

Aquatic environments are limited in nutrients and hard substrata; thus, attachment to surfaces such as metal infrastructure can be advantageous for nutrient acquisition (N-Uptake, 1999). Due to Al's toxicity, there is little competition for colonizing the surface of Al alloys; therefore, organisms that can survive in these conditions have an advantage over others that cannot. Previous research on the microbial communities that attach to a variety of surfaces like copper, Al, steel alloys, and plastics has shown that they are unique in composition and that certain microbial community members may be preferentially selecting the surfaces they attach to (Zhang et al., 2019). In a study conducted by Zhang et al., 2019, the dominant genera found on different metal alloys were found to be the copper-tolerant, acid-producing *Lactobacillus* on the copper alloys, common aerobic surface colonizers *Bacillus* and *Ruegeria* for Al alloys, and aerobic biofilm-forming *Pseudomonas* on carbon steel alloys. For surfaces that have the same composition, the microbial communities demonstrate consistent patterns of taxonomic distributions between replicates (McBeth & Emerson, 2016). This supports the hypothesis that the metal type present in an environment influences the microbes that attach and subsequently, the microbial interactions with each other and the metal surface.

Due to Al's toxic and inert nature, the microbes that colonize the surface of Al alloys 2024 and 7075 may have adapted tolerance to this metal and equip themselves with biological defense systems to reduce or eliminate toxic effects (Little et al., 1991, Little et al., 1992, Zuo et al., 2005, Zuo, 2007). Al has no known biological function and is toxic to cells. Al, like other heavy metals, acts as a competitive inhibitor to disrupt enzyme structure and function (Sterritt & Lester, 1980). Al can bind with thiol and other groups on protein molecules, replace metals naturally occurring in enzyme prosthetic groups, and potentially bind to and affect DNA (Sterritt & Lester, 1980). If Al is toxic to biological cells, how can these microbes attach and survive on

Al surfaces? Known mechanisms that aid in microbial tolerance of such conditions include producing a biofilm, export, chelation, and metabolism (Mansfeld, 2007, Sterritt & Lester, 1980, Videla & Herrera, 2009, Zarasvand & Rai, 2013, Zuo, 2007, Zuo et al., 2005).

Some bacteria have the ability to organize themselves with others in a biofilm matrix, composed of extracellular polymeric substances, DNA and proteins (Davey & O'toole, 2000, Zuo, 2007). Biofilm formation is considered a selective advantage in aquatic systems where hard surfaces are uncommon; attachment to surfaces in aquatic systems provides microorganisms with more available resources for nutrient acquisition (Davey & O'toole, 2000, Zuo, 2007). Besides providing an attachment advantage, microbial cells act differently in a biofilm than their planktonic counterparts. They can display unique morphological as well as physiological traits like enhanced antibiotic and heavy metal resistance (Davey & O'toole, 2000, Zuo, 2007). These traits allow biofilms to survive on surfaces for long periods of time, and influence the metabolic functions carried out by members of the biofilm community. To exploit metal surfaces in aquatic environments that most organisms cannot tolerate, microbes can change the conditions of the metal to make it more suitable for colonization and then finish by ridding their cells of these toxic metal species (Juzeliūnas et al., 2006, Mansfeld, 2007). When in a biofilm, they have the ability to change the electrochemical conditions of metal surfaces by producing substances like metal-binding proteins also known as chelating-agents. Previous research on organism *Rhizobium viscosum* by Jo et al., 1997 found a protein related to Al tolerance called ALU1-P. Homologs of this protein are found in other bacteria like *Pseudomonas aeruginosa* (ie. ExsB) which function as transcriptional regulators that control exoenzyme levels. When this fragment of DNA was transformed into *E. coli,* which is an Al susceptible organism, they noticed the organism began to tolerate Al more than normal (Jo et al., 1997). Zuo et al. (2005) looked for

novel Al and mild steel-binding proteins that have the potential to protect metals against corrosion. By forming a biofilm, bacteria can stably attach to metal surfaces, and express these metal-binding proteins (Zuo et al., 2005). Microbes use chelating agents such as siderophores to break the passive layer that form on the metal surface. Siderophores bind to metal cations, like iron, which exist in the oxide film and promote iron oxide dissolution, which influences corrosion (Zuo, 2007, Zuo et al., 2005). Exley & Mold, 2015, discuss how siderophores involved in iron acquisition have the ability to form strong complexes with Al (Exley & Mold, 2015).

The production of metabolic substances are additional mechanisms through which microbes can tolerate aluminum. Some organisms can create sack-like structures made up of a variety of compounds to sequester heavy metals (Appanna et al., 1994, Appanna & St. Pierre, 1996, Appanna & St. Pierre, 1994). Appanna & St. Pierre, 1996 evaluated the growth of *Pseudomonas fluorescens* in the presence of millimolar concentrations of Al and saw the formation of inclusion bodies made up of gelatinous phosphatidylethanolamine (PE) residues where Al was sequestered (Appanna et al., 1994, Appanna & St. Pierre, 1996, Appanna & St. Pierre, 1994). A study by Appanna & Hamel, 1996 looking at *Pseudomonas fluorescens* and how iron affects its ability to detoxify Al from its cell, showed citrate is rapidly used and the two trivalent metals are immobilized in a gelatinous lipid-rich residue (Appanna & Hamel, 1996). A combination of these mechanisms may be used by the microbial communities present and determining which taxa are present aids in our ability to understand how microbes are interacting with Al surfaces in aquatic environments.

Environmental factors like water temperature and salinity are important drivers of which microbes can survive in a particular ecosystem (Faust et al., 1975, Fu et al., 1991, Gikas et al., 2009, Li & Torres, 1993, McMeekin et al., 1987, Price & Sowers, 2004). Estuaries are dynamic

ecosystems that contain strong gradients due to the mixing freshwater and marine water, where temperature and salinity play a major role in organismal interactions. Depending on the proximity of the estuary to its outlet, the salinity fluctuates largely but it is always temporary and recoverable (Crump et al., 2004). With the climate warming over time, and evaporation of water sources increasing, the temperature and salinity of water are steadily increasing which may have long-term impacts on the structure of estuary ecosystems (Dahlman & Lindsey, 2020, Jenkins, 2014). Saltwater intrusion is also expected to increase in low-lying coastal areas like Eastern NC so studying its effects on overall functional diversity of ecosystems is important (Colombani et al., 2016). Water temperature and salinity also change in response to season (Sieburth, 1967). Investigating the microbial communities attached to Al structures over time can be used to understand how salinity, temperature, and other environmental factors affect community composition and biofilm attachment. These results can then be applied to other aquatic environments around the globe.

To date, there are no studies comparing the microbial communities attached to Al alloys 2024 & 7075, stainless steel alloys 304 & 316, and a non-metal substrate over different seasons, in the same estuarine system, and at two different sites. Understanding how the microbial communities are different between substrate type, season and site and overall identifying who is present on them will help to understand how the interactions between the microbes themselves, and the metals surface influence the quality and integrity of the metal alloy. These metal substrates provide microorganisms with an opportunity for attachment, and because Al is toxic, having the ability to colonize it is more favorable than not. I hypothesize that the microbial communities attached to Al alloys will be different than those attached to other substrates (eg. Stainless steel, non-metal biofouling plate, sediment) and the communities attached to the Al

alloys will be influenced by seasonal and environmental changes. If the communities differ between the Al alloys and the stainless-steel alloys, then the microbes present on the Al and not the steel may suggest a preference for Al over iron or were outcompeted from attachment. If the community composition changes between the months and sites, then the microbes present on the Al alloys are sensitive to environmental changes.

METHODS

Environmental Study

Metal alloy coupons (Al 2024 & 7075, 304 & 316L stainless steel, and non-metal biofouling plate) were deployed in duplicate at two sites (P3-Mallard Creek & P7-North Creek Landing) that have been previously used for an environmental study on stainless steel along the Pamlico River in North Carolina (Garrison et al., 2019). The communities that are found in or attached to the stainless-steel metals, non-metal biofouling plate and sediment that was collected from the site, serve as controls when comparing the microbial communities found on the Al alloys. The metal coupons were protected inside a 50 mL conical with the bottom sawed off and were zip-tied to create a “coupon tube”. The deployment apparatus was a milk crate filled with oyster shell and the aforementioned coupon tubes were zip-tied to the outsides of the crate (Figure 1).

Figure 1: Deployment crates used to attach protected metal coupons (A) and biofouling plate (B).

This crate was attached via rope to a dock and submerged approximately 1 m into the water column, sitting on top of the sediment.

The two sampling sites were chosen because they are brackish, well-protected from natural disturbances, and experience similar fluctuations in water temperature and salinity but are consistently different from each other on the same sampling day. Air and water temperature, salinity, trap depth, and dissolved oxygen were recorded for each sampling trip (Table 2), except for the month of July which is missing data for dissolved oxygen due to instrumental error in the field. Although these end point measurements were taken for each sampling trip, it should be noted they are not representative of the fluctuations in environmental parameters experienced by these communities *in situ*.

Table 2: Sampling details from Environmental study conducted July-December 2019. Parameters annotated with NA were not able to be determined due to instrumental error while out in the field.

Month	Site	Salinity (ppt)	Water temp. (ºC)	Air temp. (ºC)	Dissolved O_2 (mg/L)	Trap Depth (m)	Samples Collected (duplicate)
July	P3	5.0	34.6	34.0	NA	0.53	Al 2024
							Al 7075
							304 SS
	P7	7.6	31.5	32.0	NA	0.92	316 SS
							Biofouling Plate
							Sediment
September	P3	11.8	27.0	30.0	4.87	0.6	Al 2024
							Al 7075
							304 SS
	P7	14.0	28.3	27.0	7.44	1.2	316 SS
							Biofouling Plate
							Sediment
November	P3	11.0	16.5	16.0	7.04	0.3	Al 2024
							Al 7075
							304 SS
	P7	14.6	18.0	16.0	8.97	0.96	316 SS
							Biofouling Plate
							Sediment
December	P3	10.5	13.5	19.4	8.93	0.38	Al 2024
							Al 7075
							304 SS
	P7	15.5	13.5	18.3	8.13	0.96	316 SS
							Biofouling Plate
							Sediment

The Mallard Creek site (referred to as P3) is located at 35.4754 °N, -76.926 °E and over the span of 8 months in the year 2019 (May-December) the water temperature and salinity varied from 13.5-34.6 °C and 1.5-11.8 ppt respectively, while the North Creek Landing site (referred to as P7) located at 35.4286 °N, -76.7088 °E experienced water temperature and salinity variability from 13.5-31.5°C and 2.8-15.5 ppt respectively (Figure 2). At P3, the trap was deployed just off the dock which is next to the shoreline and on average was half of a meter down into the water. At P7, the trap was deployed off the dock but further from the shore by approximately 10 m and on average was one meter down into the water. Coupons were submerged for a 6-8-week period

over an 8-month period to span multiple seasons for comparative purposes. To collect them, the zip-tie that secured the coupon was clipped off and a 50 mL conical was used to catch the coupon; the tube was subsequently filled to the top with *in situ* water and stored in a cooler until taken back to the lab for processing.

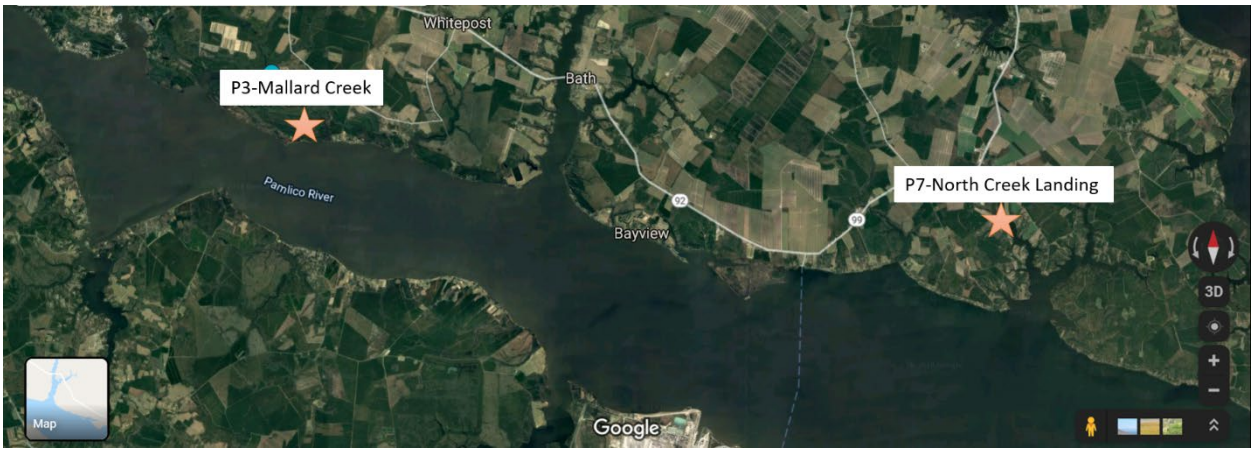


Figure 2: The two sites used for the environmental study are located along the Pamlico River. P3-Mallard Creek is located 35.4754 °N, -76.926 °E and P7-North Creek Landing is located 35.4286 °N, -76.7088 °E. Photo modified from Google Earth.

Processing Coupon Samples

In the lab, the outer surface of the coupons was aseptically scraped with a pre-autoclaved spatula and captured in the 50 mL conical the coupon was originally collected in. The tubes were centrifuged at 4,000 x g for 15 minutes and then the supernatant was removed until ~5 mL was left so that the pellet could be stored at -80°C for later use in nucleic acid extractions. This material was also used to inoculate enrichment cultures (see Chapter 2).

Microbial Community Analysis

DNA extractions of the material collected (250 µL or 0.25 g) from the metal coupons, biofouling material, and nearby sediment was performed with the DNeasy Powersoil kit (Qiagen, Inc., Carlsbad, Ca). The resulting DNA was sent off for microbial community sequencing of the

V4-V5 region of the 16S rRNA gene at the Integrated Microbiome Resource (IMR) at Dalhousie University to identify the microbial community members associated with colonizing the different metal alloys (Lane, 1991, Turner et al., 1999). Only one sample came back having failed to sequence, P7-Al 7075 #2 from November, and was therefore removed from the data set since it could not be used for analysis. Sequences were analyzed using MOTHUR v1.43.0 to identify the microbial community structure. Diversity indices were calculated to investigate the variation in microbial community composition between substrates and seasonality/location: richness was measured using Chao1, evenness was measured using Pielou's evenness, and alpha diversity was measured using Shannon's diversity index. The Krustal-Wallis test was used to determine statistical significance. Variation in community composition was analyzed between the substrate types (e.g. Al alloys, stainless steel alloys, non-metal biofouling plate, sediment), geographical sampling sites, environmental conditions of these sites (salinity, water temperature, dissolved oxygen, and trap depth), and seasons (over 8-month period, with 4 collection dates). The aforementioned factors were analyzed together using both CCA (canonical correspondence analysis) plots and NMDS (non-metric multidimensional scaling) plots, to order the variables in terms of which influences dissimilarities between the communities. For the CCA plot, ANOVA (analysis of variance) was used to evaluate statistical significance of each of the environmental parameters and stress values were used to estimate whether the NMDS plots were of a good fit. The communities found on the two Al alloys (Al 2024 and Al 7075), summer and winter seasons (July and December), and sites were compared using SIMPER analysis which makes species comparisons between two groups using Bray-Curtis similarities. This analysis helped to determine which species, if any, significantly contribute to dissimilarities in community composition. Relative abundance box plots of specific members in the microbial communities

were created along with bar charts of phyla present in the communities to help visualize and support the SIMPER results as well as isolation results from Chapter 2.

RESULTS

Microbial Community Diversity between Substrates

Of the substrates used in the environmental study, sediment exhibited the highest species richness, evenness, and alpha diversity (4923.87 ± 700.95, 0.91 ± 0.02, and 7.02 ± 0.23, respectively), while Al 2024 exhibited the most variation (2185.24 ± 714.94, 0.78 ± 0.09, and 5.45 ± 0.86, respectively) in diversity indices compared to all other substrates used in the environmental study (Al 7075, 304 & 316 SS, and non-metal biofouling plate; all $p<0.01$) (Figure 3).

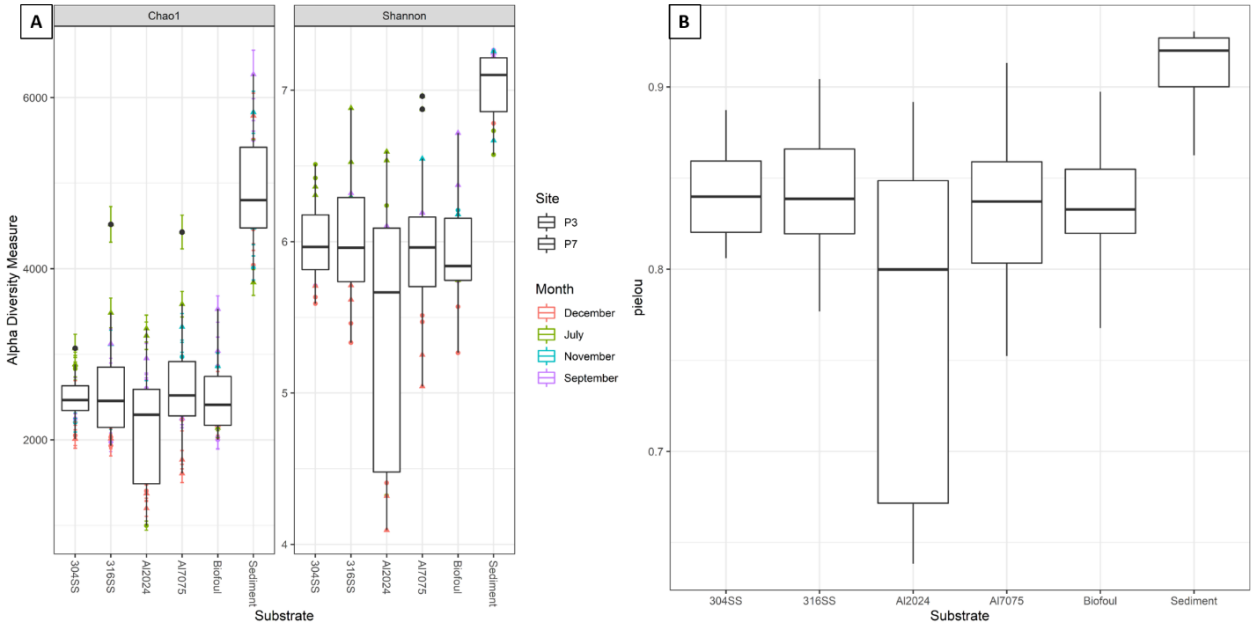


Figure 3: Diversity indices used to determine the differences in (A) richness, alpha diversity and (B) evenness between the communities attached to the substrates from all samples collected ($p<0.01$).

Richness, evenness, and diversity were more variable in communities associated with Al 2024 regardless of which site they were collected from (Figure 4; all $p<0.01$ for both site).

Microbial Community Diversity between Sites and Seasons

All of the diversity indices were higher for site P7 on all the substrates, especially Al 2024, supporting my hypothesis that environmental location influences who can attach to Al 2024. The variation in diversity indices on Al 2024 can be accounted for when the communities are separated by month (Figure 5 & 6).

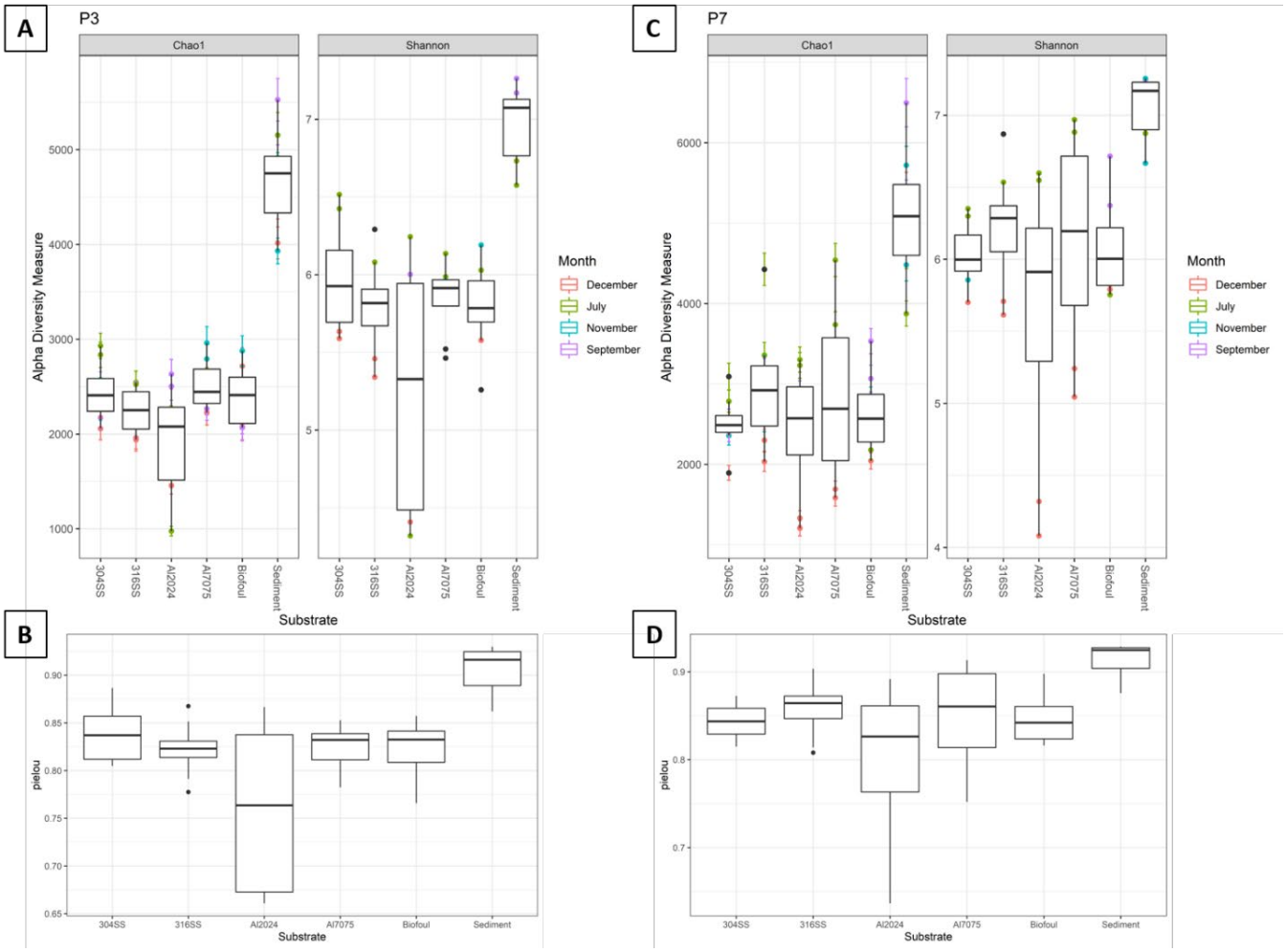


Figure 4: Diversity indices for samples collected at (A) P3 Mallard Creek and (C) P7 North Creek Landing as well as evenness at (B) P3 Mallard Creek and (D) P7 North Creek Landing (all $p<0.01$).

In July (Figure 5A & 5B), the richness, evenness, and diversity on Al 2024 was higher (2447.99 ± 1072.74; $p<0.05$, 0.83 ± 0.12; $p>0.05$, and 5.92 ± 1.08; $p<0.05$, respectively) than in December (1373.12 ± 130.30; $p<0.01$, 0.67 ± 0.01; $p<0.01$, and 4.33 ± 0.17; $p<0.01$) (Figure 6C

& 6D). The communities attached to the substrates tested were more similar between September (Figure 5C & 5D) and November (Figure 6A & 6B). The diversity results separated by month support my hypothesis that the communities attached to Al alloys, specifically Al 2024, are influenced by seasonal changes.

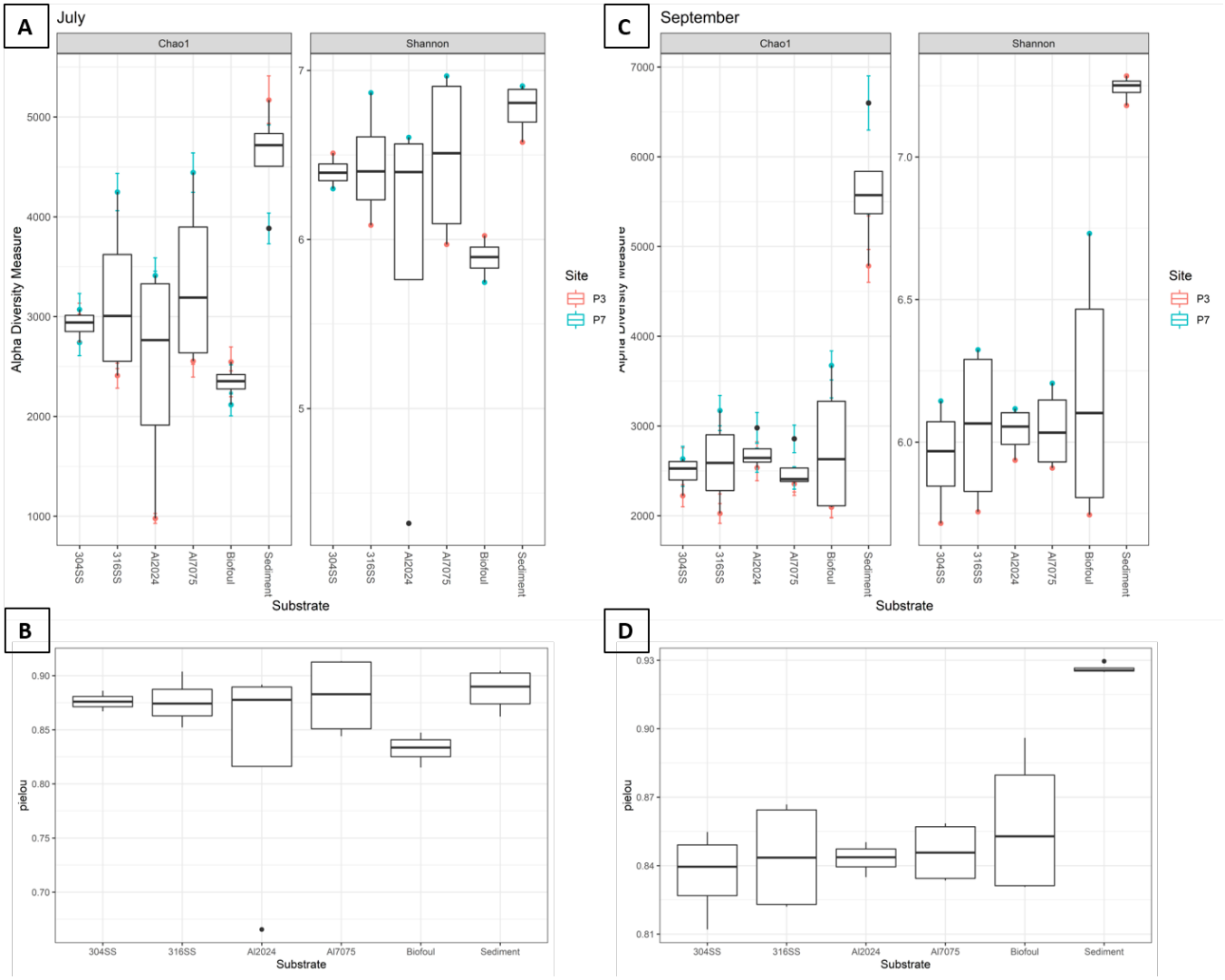


Figure 5: Diversity indices for samples collected in (A) July ($p<0.05$) and (C) September ($p>0.05$) as well as evenness in (B) July and (D) September (both $p>0.05$).

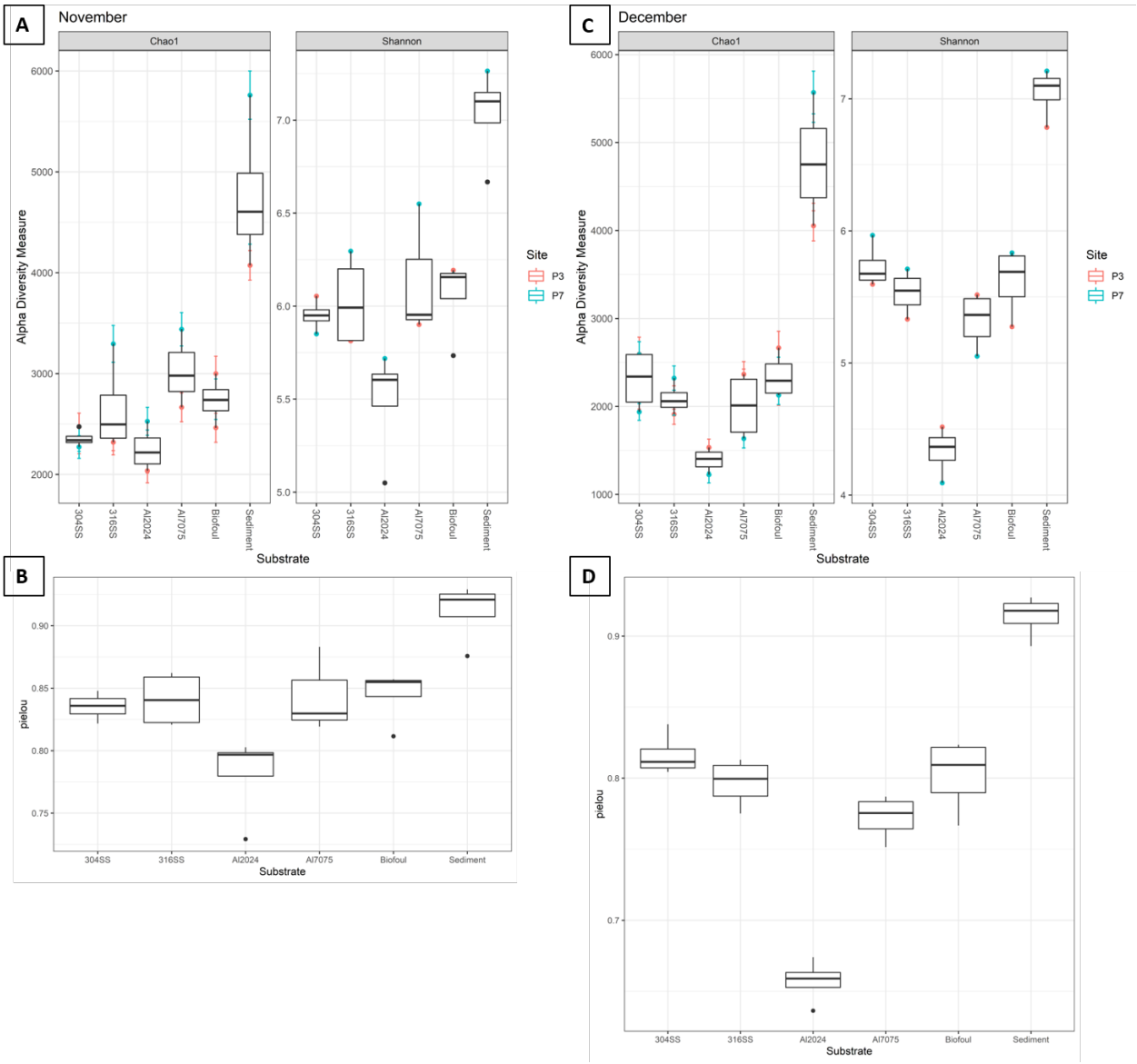


Figure 6: Diversity indices for samples collected in (A) November and (C) December as well as evenness in (B) November and (D) December (all $p<0.01$).

Microbial Community Composition between Sites and Seasons

In order to determine how similar or dissimilar communities were based on substrate, site, and month, NMDS analysis was performed. Results suggest the communities in the NMDS plots were influenced by environmental location and seasonal changes (Figure 7). When separated by site, the samples cluster together based on substrate type at P3 and by month at P7, suggesting the communities attached to the different substrates at P3 are more similar to each

other than at P7 (Figure 7). When plotting the months separately, the samples cluster together based on site rather than substrate, and the stress values decreased to 0.05-0.08 which indicates the months explain a portion of the variation seen in the composition of the communities attached to the substrate types (Figure 8A-D). Based on the NMDS plots separated by month, the communities that attach to the different substrates appear to be more similar in July than in December, suggesting seasonal variation in attachment.

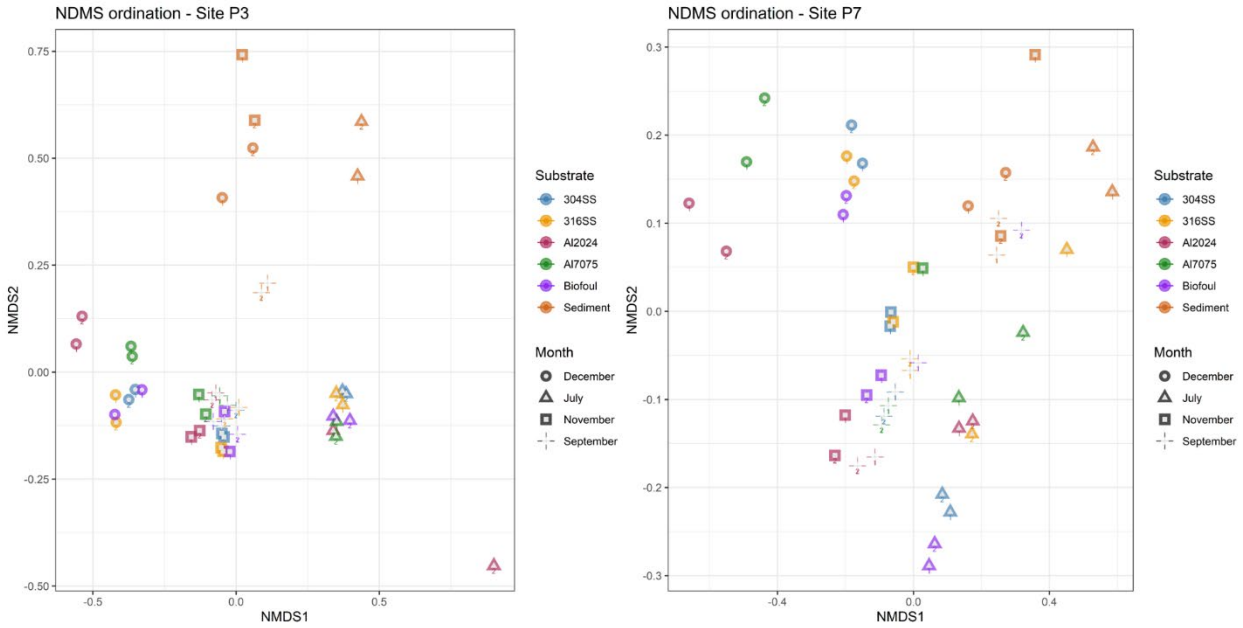


Figure 7: Non-metric multidimensional scaling (NMDS) plots of 16S rRNA gene amplicon sequence data which demonstrate differences in microbial community composition between sites, P3 (left) and P7 (right). Microbial community composition more similar by month at P3 and by substrate at P7. Stress values for P3 and P7 plots are 0.09 and 0.08, respectively.

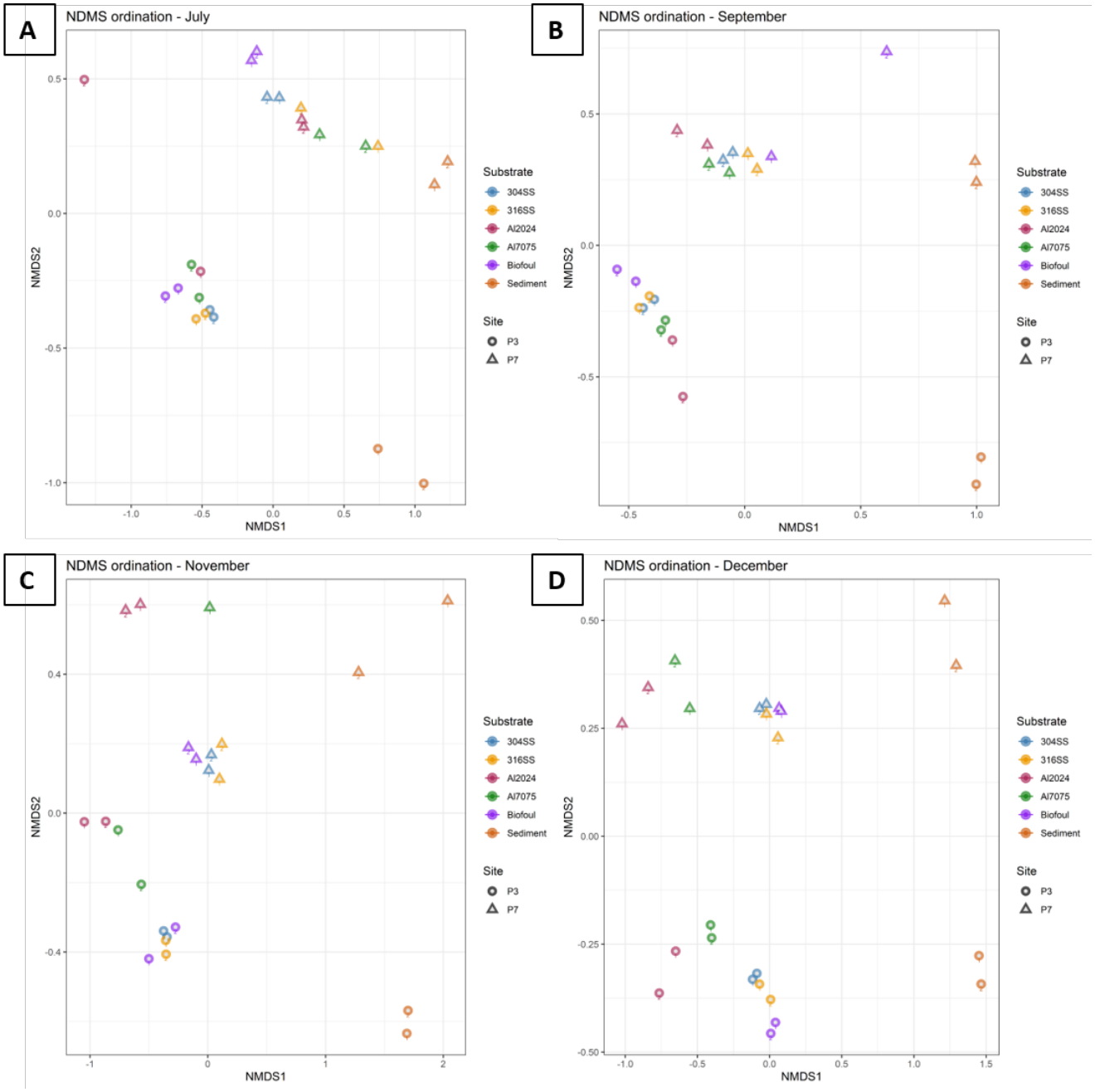


Figure 8: NMDS plots of 16S rRNA gene amplicon sequence data which demonstrate differences in microbial community composition based on the 4 months used in this study: (A) July, (B) September, (C) November and (D) December. Microbial community composition was distinct between the sites for all months. Stress values were 0.05, 0.08, 0.08, and 0.07, respectively.

Environmental Factors Influence Variation in Community Composition

A CCA analysis was performed in order to determine what environmental factors influences the dissimilarities seen in community composition. Based on the CCA plot, the communities separate from each other by month, site, and substrate supporting my hypothesis. Salinity and water temperature were determined to be the largest drivers of community composition on the substrate types ($p<0.001$) (Figure 9).

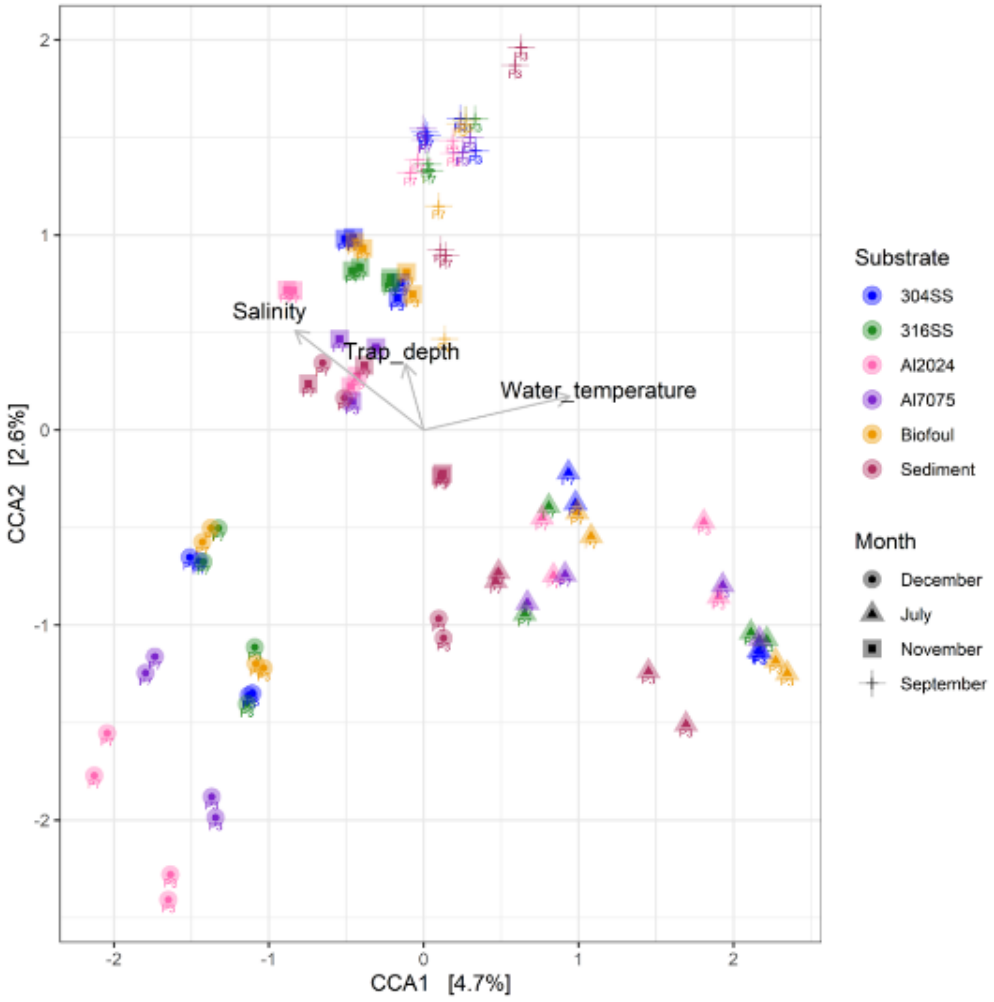


Figure 9: Canonical correspondence analysis (CCA) plot shows salinity and water temperature largely influence who can attach to the substrates by the length of their respective arrows. Samples also diverged by month which support the finding that salinity and water temperature drive community composition.

Contribution of Taxa to Dissimilarities in Composition between Al 2024 and Al 7075

When investigating the composition of the microbial communities, there was a distinction in the taxa present in all samples, and more interestingly between Al 2024 and Al 7075. The taxa present in the communities were also influenced by site and month. The taxa that contributed to the most differences seen in community composition between Al 2024 and Al 7075 at the phyla level were *Bacteroidetes* (SIMPER analysis: 5.7%) and *Proteobacteria* (32.9%), both found more abundant on Al 2024 with *Proteobacteria* showing the most variation. At the class level, *Alphaproteobacteria* (4.8%), *Betaproteobacteria* (1.2%), *Deltaproteobacteria* (1.9%), and *Gammaproteobacteria* (25.1%) contributed to the 32.9% dissimilarity seen in *Proteobacteria,* with only *Gammaproteobacteria* being most abundant on Al 2024, while *Flavobacteria* (4.7%) and *Sphingobacteria* (1.0%) contributed to the 5.7% dissimilarity in *Bacteroidetes, Flavobacteria* being more abundant on Al 7075, and *Sphingobacteria* being of similar abundance to the other substrates (Figure 10A). The taxa that contributed to the most variation seen in community composition between the two sites at the class level were unclassified bacteria (1.2%), *Flavobacteria* (1.0%) and *Gammaproteobacteria* (1.4%) (Figure 10B). The contrasting community composition between the summer and winter seasons (months July and December) at the class level was influenced by *Alphaproteobacteria* (2.8%), *Gammaproteobacteria* (2.5%), and *Flavobacteria* (2.5%). Based on previous literature and the isolation of bacteria in Chapter 2, abundances of *Bacillus* and *Pseudoalteromonas* sp. and their respective higher order classifications were visualized to determine how representative these taxa are of the communities that attached to the Al 2024 in the environmental study (Figure 10C & 10D).

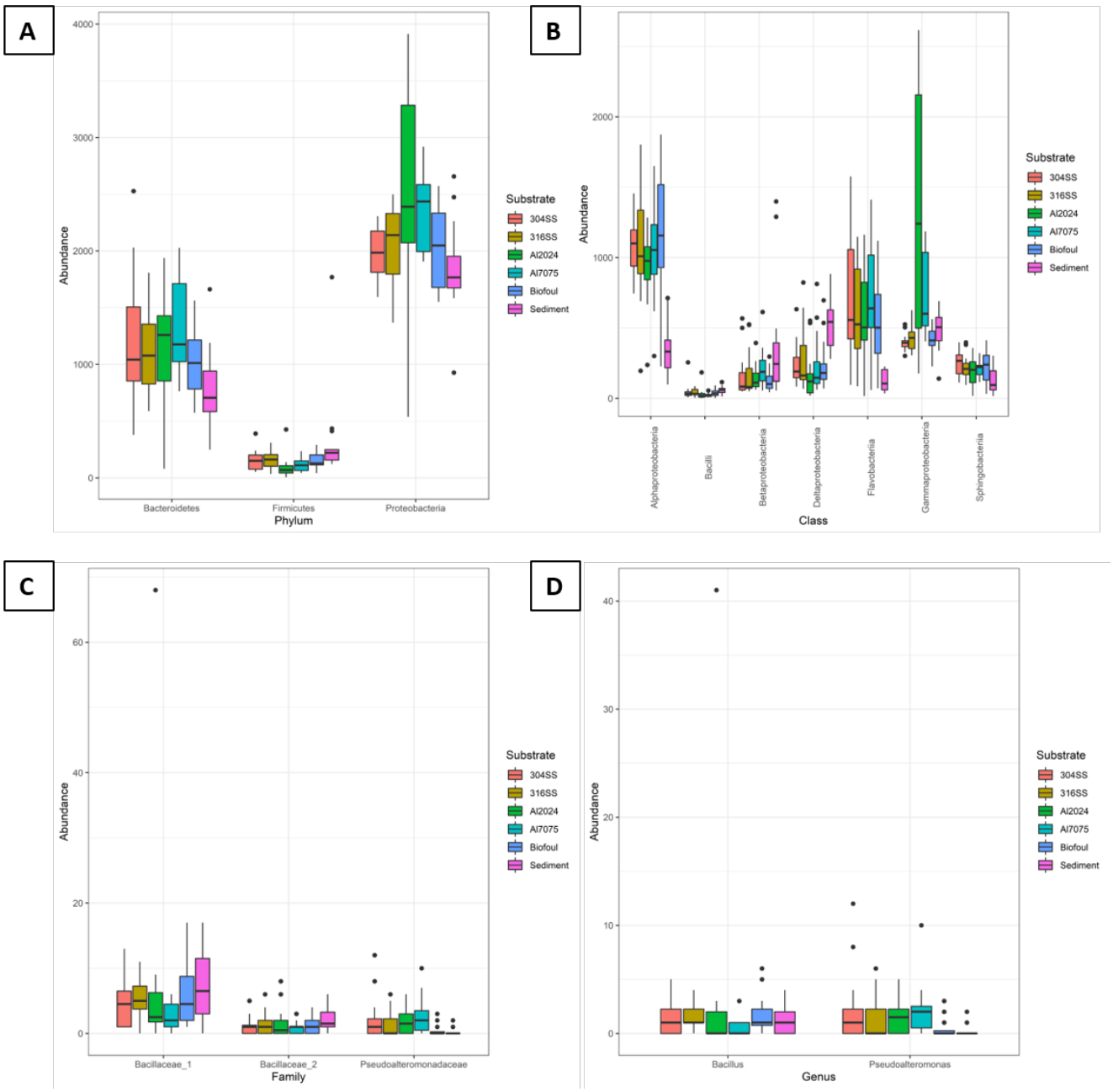


Figure 10: Taxa plots show abundance of significant taxa either attributing to dissimilarities between Al 2024 and Al 7075 or of particular interest for investigation in Chapter 2. (A) *Proteobacteria,* the most abundant and varied phylum, varied the most on Al 2024, and made up of mostly (B) *Gammaproteobacteria. Bacillus* and *Pseudoalteromonas* and their respective family classifications (C & D) were included in this investigation based on isolation results from Chapter 2 and previous literature. *Bacillus* sp. were not as abundant as expected, while *Pseudoalteromonas* sp. were more abundant on the Al alloys.

Bar graphs of the phyla present in the microbial communities on each substrate were used to visualize the SIMPER results (Figure11A). The community composition varies slightly at the phyla level between sites, with the presence of *Actinobacteria* at P3 (Figure 11B) and *Chloroflexi* at P7 (Figure 11C) on Al 2024, and the abundance of *Planctomycetes* decreasing from P3 to P7. On the SS alloys, *Acidobacteria* were only present at P3 (Figure 11B). The results separated by month support the variation seen in *Proteobacteria* on Al 2024 between months from the SIMPER analysis and taxa plots specifically made for that phylum (Figure 12). When looking further at the community composition between the months, the communities on all of the substrates tested were more similar in September (Figure 12B), and distinct from each other in July, November, and December (Figure 12A, 12C, & 12D), supporting the previous results seen in the diversity indices and NMDS plots. There was a lot of variation in the presence of *Bacteroidetes* and *Proteobacteria* between the months tested on all metal alloys, with an increase in abundance from July to December in *Proteobacteria* on Al 2024.

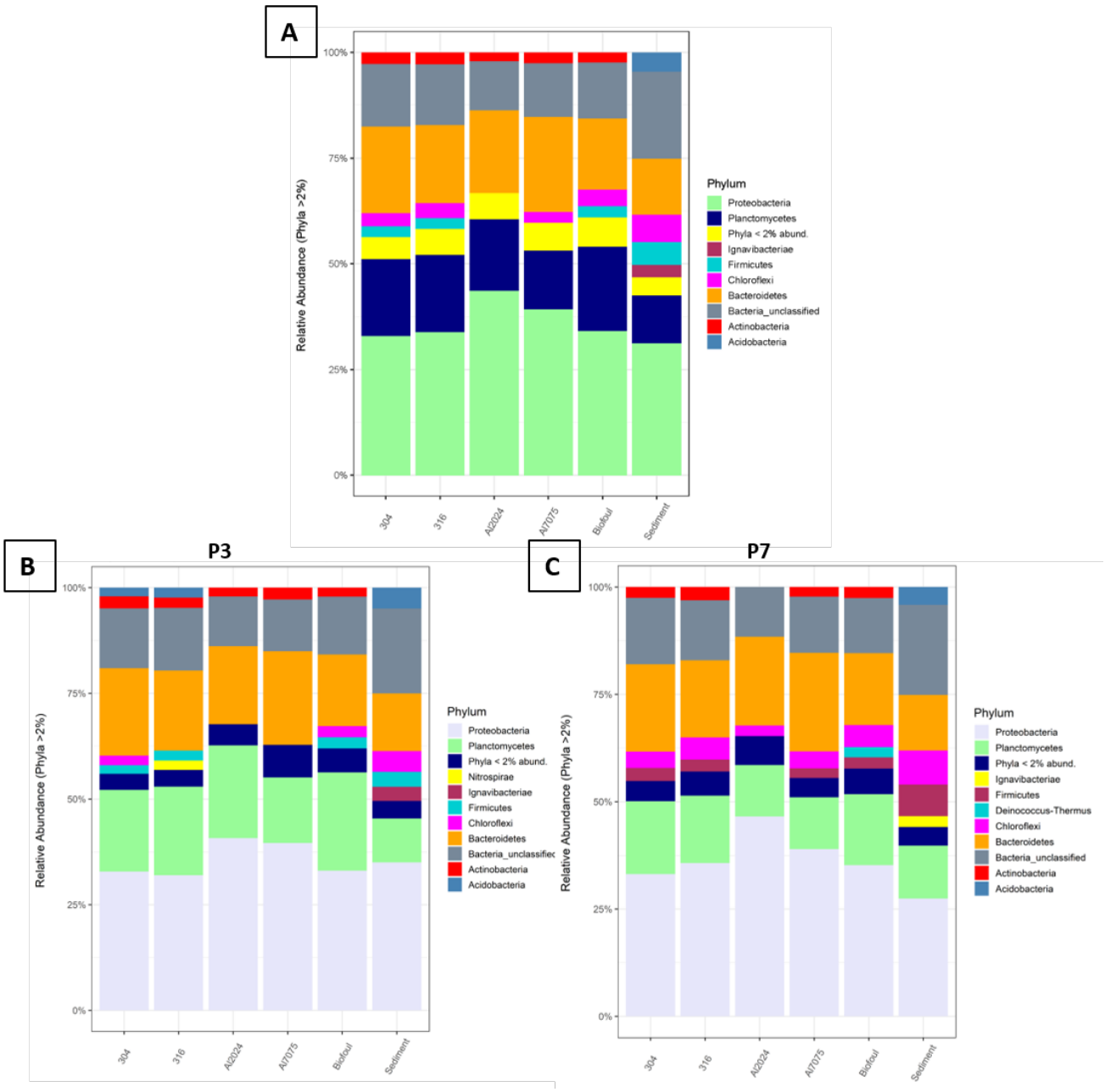


Figure 11: (A) Bar charts of taxa that make up the communities on all the substrates at the phyla level. The community composition varies slightly at the phyla level between (B) P3 and (C) P7, with the presence of *Actinobacteria* at P3 and *Chloroflexi* at P7 on Al 2024, and the abundance of *Planctomycetes* decreasing from P3 to P7.

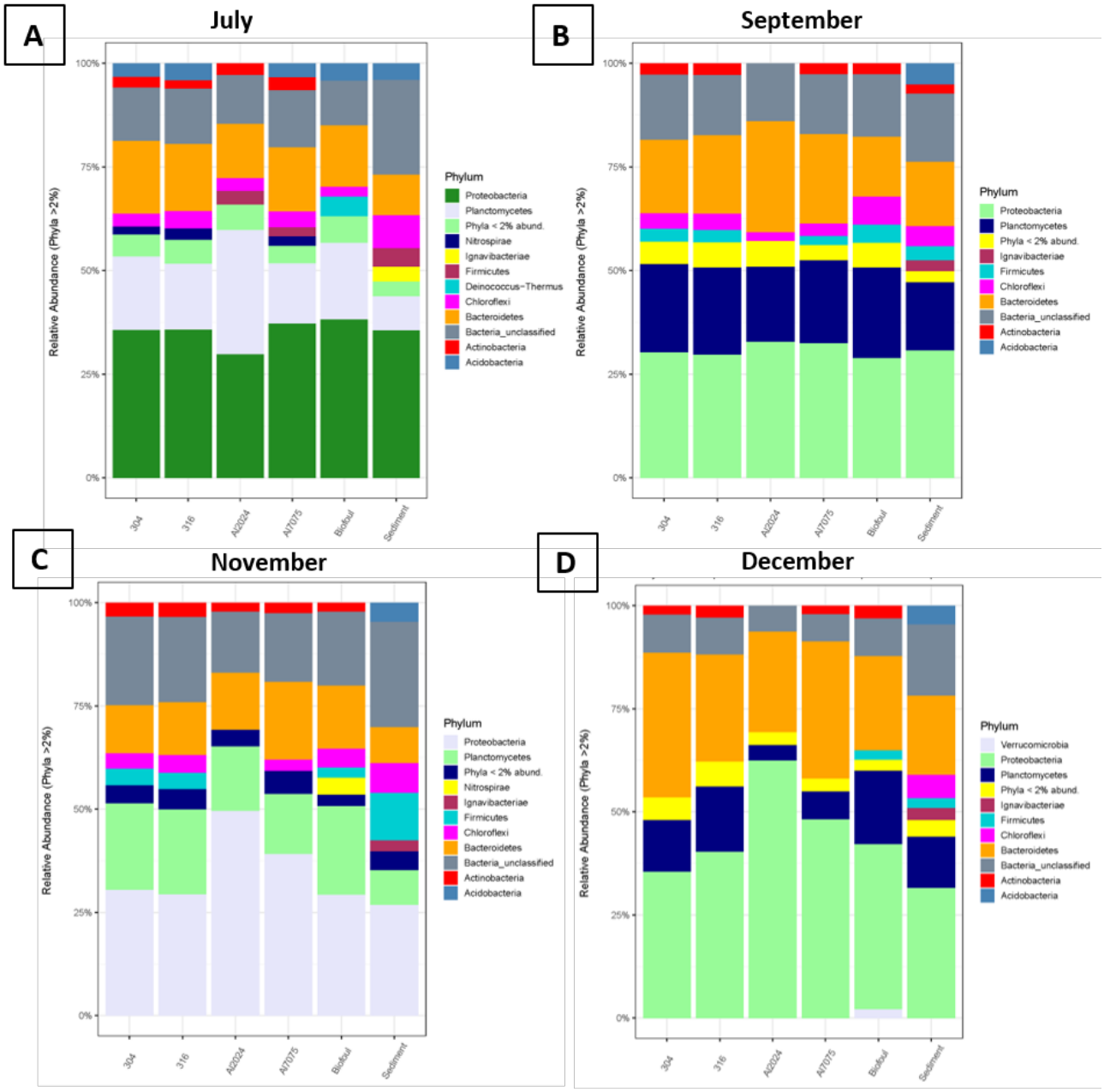


Figure 12: Bar charts of taxa that make up the communities on all the substrates at the phyla level between months. There was a lot of variation in the presence of *Bacteroidetes* and *Proteobacteria* between the months, with an increase in abundance from (A) July to (D) December in *Proteobacteria.* The composition in (B) September and (C) November were notably more similar than between (A) July and (D) December.

DISCUSSION

This study focused on investigating the microbial communities that attach to Al 2024 and Al 7075, commonly used in the aerospace industry and hydraulic systems, at two sites along the

Pamlico River over an 8-month period with 4 collection dates. The results from this study aim to provide fundamental knowledge of the microbial communities associated with Al alloys 2024 and 7075, in comparison to other metal and non-metal substrates, so that future research can be conducted to understand who attaches to these surfaces in other environments and how these particular microorganisms are able to attach to toxic Al surfaces.

Sediment exhibited the highest alpha diversity compared to all other substrates, suggesting only a subset of microorganisms are capable of attaching to hard substrates in aquatic environments. Between the two Al alloys, Al 2024 generally exhibited lower diversity than Al 7075, with a mix of high and low diversity in the summer months (July) and the lowest diversity of all substrates, sites, and seasons, in the winter months (December). The decrease in diversity for Al 2024 may be due to seasonal changes in the environment like an increase in salinity and decrease in temperature during winter months, which adds additional stress to the organisms that can attach. The differences in diversity observed between the microbial communities attached to Al 2024 and Al 7075 could be due to their chemical composition. Al 2024 primarily contains copper with a small amount of zinc while Al 7075 primarily contains zinc with less copper. Microorganisms have been found to be able to tolerate high concentrations of these metals, especially in estuaries and coastal systems contaminated with industrial runoff (Rajaram et al., 2013). Since diversity is lowest in December for Al 2024, further hypotheses could be made to test whether a decrease in temperature affects organismal attachment to Al alloys made with copper. Determining who in the community contributes to the variation in diversity for Al 2024 between months could also be used to understand which organisms are better suited to deal with high concentrations of copper instead of zinc between the different seasons.

The microbial communities were found distinct in their composition between substrates in December only suggesting seasonal environmental changes, like a decrease in temperature, influences who can attach to all substrates. When looking at the variation in composition between sites (P3 and P7) and months, there was more variation seen on the Al alloys compared to the other substrate types, especially between the summer and winter seasons (July and December), supporting my hypothesis that environmental changes influence attachment. Since the main distinguishing factor between the substrates is the presence of toxic Al, organisms that can attach to and tolerate these surfaces are impacted by their surrounding environment.

Seasonality was the biggest driver of microbial attachment to Al 2024 and was observed in the microbial community analyses as variation in diversity and composition was seen between months. The environmental parameters most notably affected by changes in season are water temperature and salinity, which were found to heavily influence microbial community composition on Al 2024. The two seasonal extremes, represented by samples collected in July and December, contrasted the most in community composition and diversity. September and November were more alike due to the subtle variability in environmental conditions between these months. The September samples were collected after a hurricane, so the lack of diversity and variation in community composition between the substrate types and sites may have been due to this weather event. These findings suggest that the environmental factors salinity and water temperature drive variation in the microbial communities attached to all the metal alloys, most prominently Al 2024 due to the presence of Al and copper in the alloy which is not found in the other metals, allowing assumptions to be made for Al 2024 in other similar aquatic systems globally. These findings also support the idea that succession in the microbial communities attached to metal substrates is variable and dependent on the environment.

Identifying how the communities that attach to Al 2024 and Al 7075 contrast to other substrates in this estuary system allows predictions to be made about what they are doing at the metal surface and aids in further hypothesis development.

The microorganisms that were found to contribute to the dissimilarities between Al 2024 and Al 7075 were found to be *Alphaproteobacteria, Gammaproteobacteria,* and *Flavobacteria*, the same classes that contributed most to the dissimilarities between the sites and seasons. Both *Alphaproteobacteria* and *Flavobacteria* were consistently more abundant on Al 7075, while *Gammaproteobacteria* was more abundant on Al 7075 in July and September, and then more abundant on Al 2024 in November and December. Members of both classes, *Alphaproteobacteria* and *Gammaproteobacteria*, have been previously isolated from Al surfaces (Mansfeld, 2007, McNamara et al., 2005, Rajasekar & Ting, 2010, Zhang et al., 2019), suggesting some taxa in this class are capable of attaching to a variety of Al surface types in different environmental locations. The SS alloys were found to be consistently more similar to each other and the non-metal biofouling plate than either Al alloy, but Al 7075 was found more similar to the other substrate types than Al 2024. Since there is no copper in the stainless steels and less in Al 7075 than Al 2024, the amount of copper present in the metal alloy may influence attachment.

For Chapter 2, microorganisms were isolated from Al 2024 in the presence of aluminum and used for further studies. These isolates were identified as *Bacillus* sp. and *Pseudoalteromonas sp; Bacillus* sp. are part of the phylum *Firmicutes* while *Pseuodalteromonas* sp. are part of the *Proteobacteria* phylum. After analyzing the taxa plot results, *Bacillus* sp. and *Firmicutes* were found to be at very low abundances in the communities that attached to Al 2024 in the environmental study. However, the opposite was found for the *Pseudoalteromonas* sp.

when looking at taxonomic levels higher than order. The *Gammaproteobacteria,* a large and diverse class, were the most abundant *Proteobacteria* on Al 2024 and contributed to the variation in composition seen between month, warranting further study of members in this class.

There are countless variables that may have affected the microbial communities that attach to the Al alloys investigated in this study, especially due to the dynamic nature of an estuary system adding to the novelty of this research, but the results suggest the variation seen in this study was influenced by the environmental location and season. Salinity was a main driver in the results of this study, but due to the fluctuating salinity in estuarine environments, it may be more of an influence than what the results of this study suggest. Obtaining continuous measurements of salinity over time would provide more insight to the role it plays in microbial attachment to Al alloys. In an environment with stable salinity concentrations, like marine systems, the microbial communities will likely be influenced more so by changes in temperature.

Temperature was also a main driver in the results of this study, suggesting it plays a major role in microbial attachment to Al alloys in the environment. Furthermore, microbial communities attached to Al alloys that are geographically separated will vary in both composition and diversity. In aquatic systems that experience higher temperatures the communities attached to different metal substrates are expected to be more similar to each other in composition, than that of communities in colder environments. Also, in these warmer environments, the communities attached to Al 2024 are expected to have both high and low diversity measures, whereas Al 2024 in colder environments should have only low diversity.

The presence of Al surfaces in aquatic environments provides a substrate for attachment, but also provides a selective pressure; if organisms are able to attach, they are at an advantage in this system. This ability to attach may be random or dependent on cellular physiology, but

regardless, will be influenced by the surrounding conditions of the aquatic system, especially for structures made with aluminum. Outside of a dynamic estuarine system, like in the ocean, these communities may be more stable in their succession due to the lack of environmental fluctuations. Regardless of the aquatic environment, results from this study can aid researchers interested in microbial attachment to Al alloys by providing knowledge of how these communities are influenced by their surrounding the environment.

CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF MICROORGANISMS FROM ALUMINUM ALLOY 2024

INTRODUCTION

Attachment to hard substrates in aquatic environments provides microorganisms with an advantage of being able to access resources more readily than planktonic microorganisms. Some organisms are able to attach to surfaces and influence the integrity of that surface, especially in regard to metal alloys that are submerged in aquatic systems, since structural integrity is of extreme importance (McNamara et al., 2005, Rajasekar & Ting, 2010). Of the microorganisms that can attach to metal surfaces, most are able to produce biofilms or spores to withstand changes in the surrounding environment and maintain attachment (Davey & O'toole, 2000).

Microorganisms that attach to aluminum (Al) alloy surfaces, and penetrate the passive oxide layer, encounter an influx of Al and other heavy metals at higher concentrations than experienced before (Rajasekar & Ting, 2010). Previous research shows microorganisms like *Pseudomonas* sp. can tolerate from 0-50 mM Al salt (Appanna et al., 1994, Appanna & St. Pierre, 1994, Appanna & St. Pierre, 1996). A microorganism isolated from either Al alloy 2024 or 7075 is hypothesized to be tolerant of the same range mentioned above. In order to determine how additional stressors may affect these isolate's tolerance in the environment, the isolates will be grown at different temperatures under increasing amounts of Al.

When bacteria encounter stressors in their environment, they must adapt to overcome or be outcompeted by others that can. The microbial communities present on Al alloys in an aquatic system, especially in estuary environments, are sensitive to fluctuations like temperature and

salinity which may affect the microbe's ability to tolerate aluminum due to the additional stress added to the cell (Faust et al., 1975, Fu et al., 1991, Gikas et al., 2009, Li & Torres, 1993, McMeekin et al., 1987, Price & Sowers, 2004). Microorganisms attached to Al in the environment are expected to grow, in the lab, at similar conditions to the environment they were isolated from. It should be noted that salinity and water temperature fluctuate during the environmental study, so the one reading taken for each sampling trip is used as an estimate because it is not representative of the variability they experienced *in situ.* To date, there have been no studies observing how a bacteria's tolerance to Al is influenced by additional stress like changes in temperature and salinity.

The goal of experiment 2 was to isolate microorganisms from Al 2024 in the presence of aluminum chloride and characterize their aluminum tolerance as well as other growth conditions to help elucidate what these organisms could be doing in the environment. When microorganisms attach to Al alloys, they experience an influx of aluminum oxide. Aluminum disassociates rapidly and forms complexes with surrounding chloride ions in the water, creating aluminum chlorides (Rao & Rao, 2004) which is why aluminum chloride was chosen for this study. The two isolates focused on in this study were identified as *Bacillus* sp. and *Pseudoalteromonas* sp. As found in chapter 1, temperature is an important environmental factor that influenced attachment and with this potential increase in exposure of aluminum chloride due to corrosion, it is necessary to understand how these dual stressors could affect how these microbes' function. I hypothesize that decreasing the temperature 5°C below the isolate's optimum growth temperature will negatively affect growth and aluminum tolerance. I expect to see less growth (as measured by optical density OD600) as I decrease temperature and increase the aluminum chloride concentration. Characterizing these isolates and their tolerance of Al will

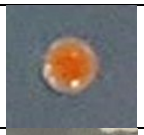
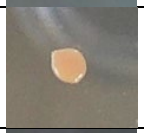
be used to make further hypotheses about what environments these organisms can be found in and help elucidate what they are doing at the surface of the alloy. Since estuaries are a dynamic environment, with temperature and salinity fluctuating mildly, microorganisms attached to Al alloys in estuary environments are posed with endless threats that potentially effect tolerance and overall attachment to Al surfaces.

METHODS

Isolation of Bacteria from Aluminum Alloys 2024 & 7075

To isolate bacteria from both Al alloy types, 100 µL of coupon material was plated on different agar types in the presence of Al chloride hexahydrate ($AlCl_3 \bullet 6H_2O$). Al chloride was chosen because it is the most representative salt of what the organisms experience in the environment (MacLeod, 1983). Aluminum ions dissociating from the Al alloy combine with chloride ions from the surrounding aquatic environment and create Al chloride. Nutrient agar (NA), estuary media agar (EMA), diluted marine agar (DMA), with 0.5 mM $AlCl_3 \bullet 6H_2O$, were used to select for organisms capable of surviving on micromolar concentrations of Al. Twelve colony types were selected—based on their unique colony morphology—and isolated via a streak for isolation until a pure culture was obtained and confirmed via epifluorescence microscopy (Table 3). Once a pure culture was confirmed, frozen stocks were made for later revival and leftover culture was transferred to an Al-free broth twice consecutively, and after incubation the second culture was poured into a 50 mL conical and pelleted via centrifugation at 4,000 x g for 15 minutes. The supernatant was removed until ~5mLs left, and the pellet was stored at -80°C for later use in nucleic acid extractions.

Table 3: Isolated bacteria from Al alloy coupons, their colony morphology, and putative identities based off of 16S rRNA sequencing. Agar types: Nutrient agar (NA), Estuary media agar (EMA), Diluted marine agar (DMA).

Isolate #	Site	Al alloy	Agar Type Isolated From	Can Grow On	Colony Morphology (color, size, form, margin, elevation, texture)		Preliminary ID (genus)
1	P3	Al 2024	DMA	DMA & EMA	Red, small, circular, entire, convex, hard		*Pseudoalteromonas*
4	P7	Al 7075	DMA	DMA & NA	White, small, circular, entire, convex, mucousy		*Photobacterium*
5	P7	Al 7075	NA	NA	Cream, large, circular, entire, crateriform, hard		*Bacillus*
6	P3	Al 7075	NA	NA	White, large, irregular, undulate, crateriform, hard		*Bacillus*
7	P3	Al 2024	DMA	DMA & NA	Tan, small, circular, entire, raised, hard		*Shewanella*
10	P7	Al 2024	NA	NA	White, small, circular, entire, raised, mucousy		*Enterobacter*
11	P3	Al 2024	NA	EMA & NA	Yellow, small, circular, entire, flat, hard		*Staphylococcus*
12	P3	Al 2024	NA	EMA & NA	White, large, irregular, undulate, crateriform, hard		*Bacillus*

Isolate Identification

Pellets of the Al isolate cultures were used to extract DNA using the DNeasy Powersoil kit (Qiagen, Inc., Carlsbad, Ca). The 16S rRNA was amplified via PCR using the universal primers 8F and 1492R and subsequently purified using QIAquick PCR Purification kit (Qiagen, Inc., Carlsbad, Ca). The purified PCR products were quantified and aliquoted for subsequent

Sanger sequencing at ECU Genomics Core Facility. Universal primers 8F, 1492R, 533F, and 907R were sent along with the purified PCR products for use in sequencing. The sequence results were aligned using Sequencher and analyzed using BLAST to determine what the closest identity of the isolates are. Of the aforementioned 12 isolates, two (isolates 1 & 12) were chosen for further characterization in this study based on their identities and previous research recognizing their ability to produce biofilms in harsh environments. These isolates were identified as *Bacillus* sp. and *Pseudoalteromonas* sp. Leftover DNA retrieved from the isolates was cleaned up using the QIAquick PCR Purification kit (Qiagen, Inc., Carlsbad, Ca) and sent off for whole genome sequencing (Dalhousie University) to confirm the identity of the isolates and characterize their genomic capabilities.

Metabolic Characterization of Two Isolates

The two isolates, *Bacillus* and *Pseudoalteromonas*, used in this study were characterized in terms of their ability to grow with or without oxygen and using a series of biochemical tests, their potential to carry out the catabolism of specific carbohydrates and proteins. To determine if the isolates' grow with or without oxygen, their respective optimal agar types (NA for *Bacillus* and MA for *Pseudoalteromonas*) were inoculated with one single line—in triplicate—then placed in either an anaerobe bag or an open box to be incubated at their optimal growth temperature (30°C for *Bacillus*, 25°C for *Pseudoalteromonas*) for 48 hours. A thioglycolate broth was also inoculated by stabbing with an inoculating loop for each isolate and incubated at their optimal growth temperature for 48 hours to validate the plate results. A series of biochemical tests were performed in triplicate on both isolates to provide a simple characterization of the isolates' catabolic abilities based on commonly used biochemical testing methods (Andrews, 2019). The following agar plates were inoculated with each isolate by

streaking a single line through the middle of the plate: starch agar plate and spirit blue agar plate. The following tests were inoculated by stabbing the tube with an inoculating loop: oxidation/fermentation (OF) glucose test, triple sugar iron (TSI) agar test, Simmons citrate agar test, gelatin hydrolysis test, and ornithine decarboxylase test. Two of the OF glucose test tubes were inoculated and one of them, as well as the ornithine decarboxylase test tube, were overlaid with mineral oil to create an anoxic environment in the tubes. The following tests were inoculated by pipetting 200 µL of the isolates culture: sucrose and lactose fermentation tests and urea broth test. All of the inoculated biochemical tests were incubated at the isolates' optimal growth temperatures for 48 hours prior to reading results. The gelatin hydrolysis test tubes were placed in the fridge for 15 minutes prior to reading their results to solidify any undegraded gelatin as it becomes a liquid regardless at the incubation temperatures.

Determining Growth Conditions and Ranges on Solid Media

A growth/no growth study was performed to narrow down the temperature range used to incubate the isolates in. The temperatures used were expected to span their growth range and beyond. When *in situ* these isolates experienced between 27-35°C, based on the endpoint measurements collected for the environmental study. In triplicate, a streak for isolation was performed for each isolate on their respective favored agar type and incubated in either 4, 10, 20, 30, or 45°C to characterize the isolates' growth range based on temperature. Using the results from this experiment, temperatures 4, 15, 25, 30, and 35°C were used to test the growth of the isolates in a factorial experiment with $AlCl_3 \cdot 6H_2O$. Both nutrient agar and marine agar plates were made with differing concentrations of $AlCl_3 \cdot 6H_2O$ to use for the factorial experiment: 0, 0.1, 0.5, 1, 5, 10, and 20 mM. These concentrations were chosen based on previous research isolating bacteria and fungi from aluminum environments (Appanna & Pierre, 1996, Fischer et

al., 2002). In triplicate, 100 µL of each isolate was spread plated on the various agar types ensuring there was triplicate of each Al concentration for each temperature being tested. The spread plates were incubated in all of the previously mentioned temperatures to characterize the isolates ability to grow in the presence of $AlCl_3 \cdot 6H_2O$ while experiencing temperature stress.

Temperature Effects on Al Tolerance in Liquid Medium

One of the two isolates, the *Bacillus* sp., was chosen to use in a spectrophotometric growth study in order to evaluate the isolates' ability to grow in the presence of micromolar concentrations of Al chloride at its optimal growth temperature (30°C) and 5°C lower (25°C). A 96-well plate was set up in conjunction with spot plates to assess the optical density at 600 nm (OD_{600}) and original colony forming units/milliliter, respectively, for each condition. The following concentrations of $AlCl_3 \cdot 6H_2O$ were used in this study based off of the previous experiment qualitatively assessing their Al tolerance using spread plates: 0, 0.1, 0.5, ,0.75, 1, and 1.5 mM. Each condition was performed in triplicate with negative controls. Absorbance measurements, which correspond to optical density, were taken at 600 nm every 10 minutes over a 24-hour period. The absorbance data was analyzed first by subtracting the negative controls from all the isolate treatments, then averaging the triplicates together and plotting the absorbances over time. The maximum absorbances for each treatment was then identified and used to make a bar graph showing the maximum OD_{600} for each treatment at the two temperatures. This study was performed three times for each of the two temperatures, and the data averaged together to increase statistical power. A t-test was performed on the maximum OD_{600} both between the treatments in the same temperature run and the different temperature, to determine statistical significance of results.

RESULTS

Isolation and Identification of Bacteria from Aluminum Alloys 2024 & 7075

Eight of the 12 isolates were sent off for Sanger sequencing and identification since they were confidently deemed pure cultures, while the other four were not. These eight isolates were successfully identified at the genus level as a variety of microorganisms including *Pseudoalteromonas* sp.*, Photobacterium* sp., *Bacillus* sp., *Shewanella* sp., *Enterobacter* sp., and *Staphylococcus* sp. (Table 3). One of the *Bacillus* sp. isolates and the *Pseudoalteromonas* sp. isolate were chosen to use for the rest of the study because of their interesting colony morphology when grown in the presence of Al or under heat stress, use in previous studies on aluminum alloys (Mansfeld, 2007, McNamara et al., 2005, Rajasekar & Ting, 2010, Zhang et al., 2019), and advantageous physiological properties. This *Bacillus* isolate was isolated from the control plate, without Al. The *Bacillus* isolate's colony morphology without Al present was characterized as white, large, irregular, undulate, crateriform, and hard (Figure 13A), but when Al was present was characterized as white, small, irregular, crateriform, and hard (Figure 13B). The largest difference between the morphologies on Al compared to off Al is the bacteria looked like it was growing on top of itself to get away from the solid Al agar surface. When *Bacillus* isolate was grown at 45°C (Figure 13D), 15°C higher than its optimal growth temperature (Figure 13C), it exhibited signed of heat stress. The colonies became mucous-like and the agar became a yellow pigment that fluoresced under UV light.

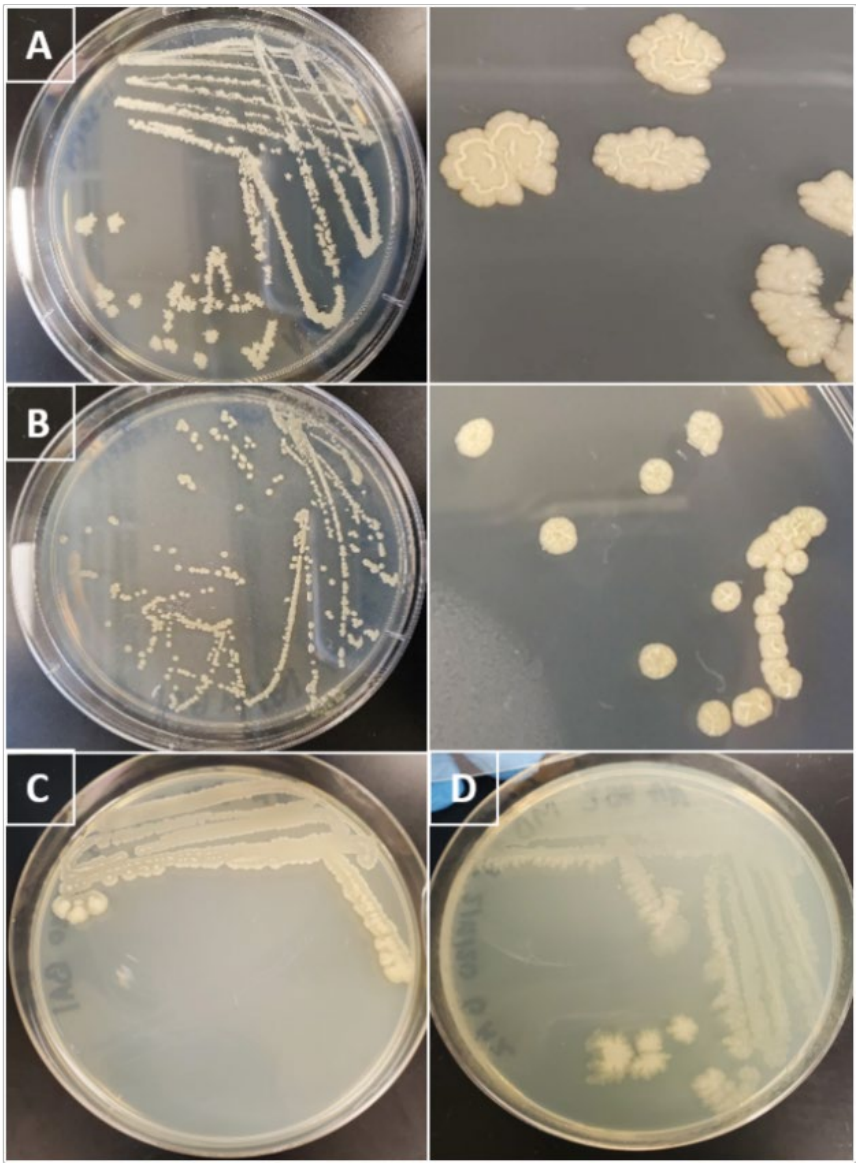


Figure 13: *Bacillus* isolate changes (A) colony morphology when under stress. When grown in the presence of Al (B) the colonies grow vertically rather than horizontally across the agar surface, and when grown in (D) higher temperatures than their (C) optimum, the colonies appear mucous-like and potentially secrete a secondary metabolite.

Pseudoalteromonas isolate's colony morphology was characterized as red, small, circular, entire, raised, and hard (Figure 14A). When grown in liquid medium, the entire culture turned pinkish-red (Figure 14B). After a week of growth, all colonies displayed bullseye coloration with the darker coloration gradually increasing towards the outer edge of the colonies (Figure 14C).

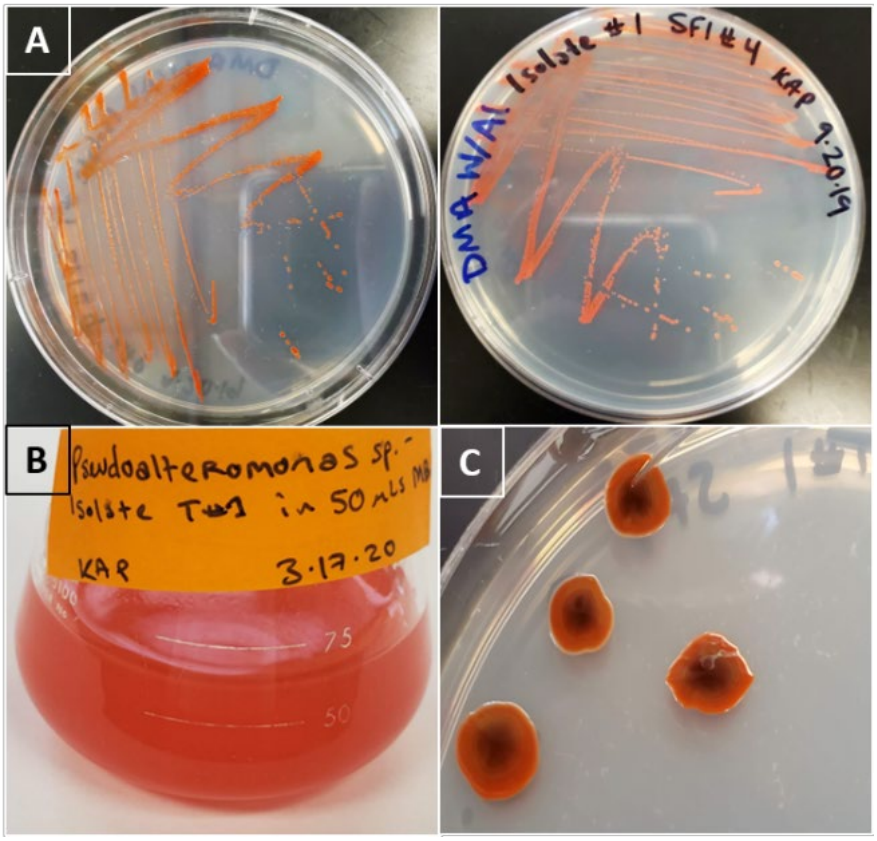


Figure 14: *Pseudoalteromonas* isolate produces a pigment both (A) on solid media and (B) in liquid media and the (C) colonies have a bullseye appearance to the coloration.

Metabolic Characterization of Two Isolates

Both isolates grew only on the aerobically incubated plate, as well as at the top of the thioglycolate broth suggesting they are obligate aerobes. From the biochemical tests used to characterize the catabolic potential of the isolates, it was determined that the *Bacillus* isolate is capable of fermenting sucrose with acid production only and the *Pseudoalteromonas* isolate is capable of decarboxylating ornithine. Both isolates were capable of producing the enzyme gelatinase to break down gelatin. All of the other biochemical tests were either negative or unable to be read because of the pigment produced by the *Pseudoalteromonas* isolate (Table 4; Figure 15).

Table 4: Results from biochemical tests performed on *Bacillus* and *Pseudoalteromonas* isolates. *Bacillus* isolate was found positive for sucrose fermentation and gelatin hydrolysis and *Pseudoalteromonas* isolate was found positive for ornithine decarboxylation and gelatin hydrolysis.

Biochemical Test	***Pseudoalteromonas* sp.**	***Bacillus* sp.**
Starch Hydrolysis Test	-	-
Spirit Blue Agar	-	-
OF Glucose Test	-	-
Lactose Fermentation Test	-	-
Sucrose Fermentation Test	- acid; - gas	+ acid**;** + gas
TSI Slant	- acid; - gas	+ acid**;** - gas
Simmons Citrate Test	-	-
Gelatin Hydrolysis Test	+	+
Urea Broth Test	-	-
Ornithine Decarboxylase Test	+	-

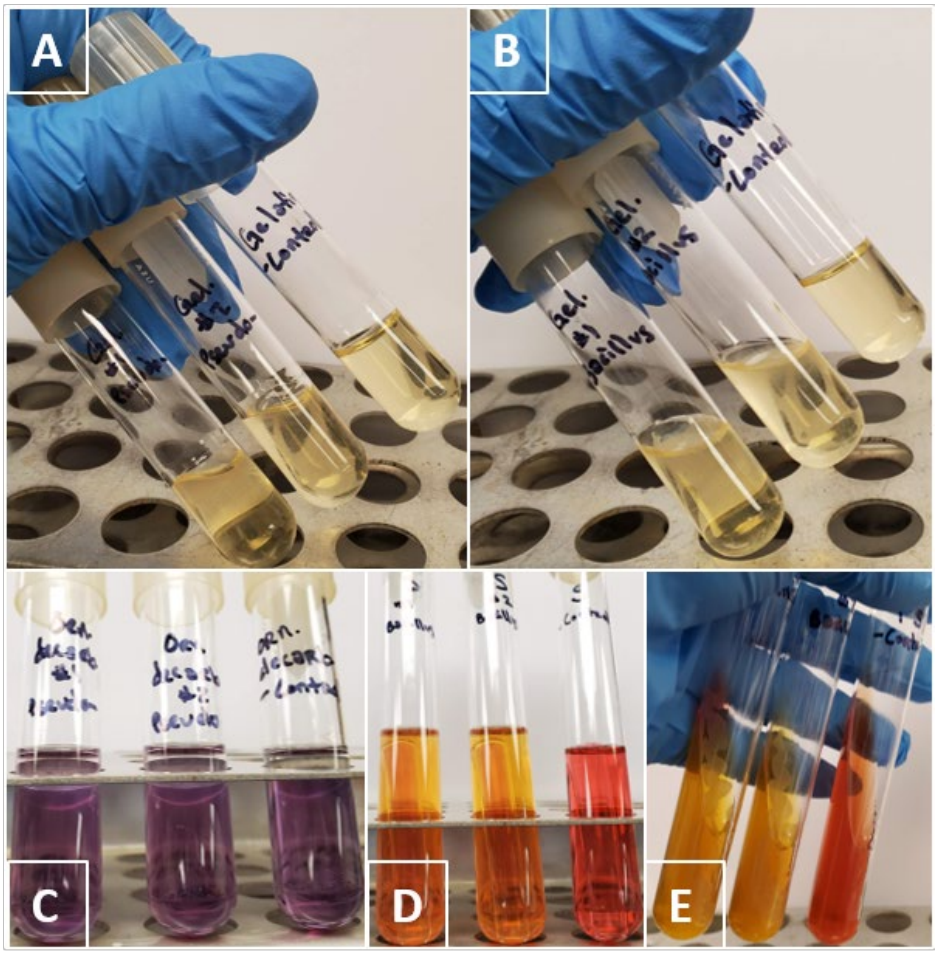


Figure 15: Images of positive gelatinase test results for (A) *Pseudoalteromonas* isolate and (B) *Bacillus* isolate. Image of positive (C) ornithine decarboxylase test and (D & E) sucrose fermentation.

Determining Growth Conditions and Ranges on Solid Media

Results from the growth/no growth study showed the *Bacillus* isolate grew at 20, 30, and 45°C within 48 hours and displayed signs of cellular stress due to the increase in heat such as a mucous-like appearance to the once hard colonies and yellow coloration to the agar, which fluoresces when exposed to UV light (Figure 11D). The *Pseudoalteromonas* isolate grew at 20, 30, and 45°C within 48 hours and at 10°C in 2 weeks. The higher the temperature, the darker the red pigment of the colonies became.

Results from the factorial experiment showed when both isolates were incubated at 30°C, in the presence of $AlCl_3•6H_2O$ concentrations spanning from 0-20 mM, growth was observed by both isolates between 0-1 mM but not at the higher concentrations. As the temperature increased, both isolates were able to grow more, as observed by cell density and pigment, in the presence of $AlCl_3•6H_2O$ (Table 5 & 6). At 15°C, the *Pseudoalteromonas* isolate grew but displayed less pigment (Figure 16A) than when grown at its optimum (Figure 16B) and was least at 0 mM, increasing as the Al concentration increased.

Table 5: Factorial experiment results for *Bacillus* isolate after 1 week. Growth was rated using the following designation: - (no growth), + (least growth), ++ (moderate growth), and +++ (most growth).

mM $AlCl_3•6H_2O$	4°C	15°C	25°C	30°C	35°C
0 mM	-	+	++	+++	+++
0.1 mM	-	+	++	+++	+++
0.5 mM	-	+	+	++	++
1 mM	-	+	+	++	++
5 mM	-	-	-	-	-
10 mM	-	-	-	-	-
20 mM	-	-	-	-	-

Table 6: Factorial experiment results for *Pseudoalteromonas* isolate after 1 week. Growth was rated using the following designation: - (no growth), + (least growth), ++ (moderate growth), and +++ (most growth). No pigment was produced in the highlighted cell.

mM $AlCl_3•6H_2O$	4°C	15°C	25°C	30°C	35°C
0 mM	-	+	++	+++	+++
0.1 mM	-	++	++	+++	+++
0.5 mM	-	++	++	+++	+++
1 mM	-	+++	++	+++	+++
5 mM	-	-	-	-	-
10 mM	-	-	-	-	-
20 mM	-	-	-	-	-

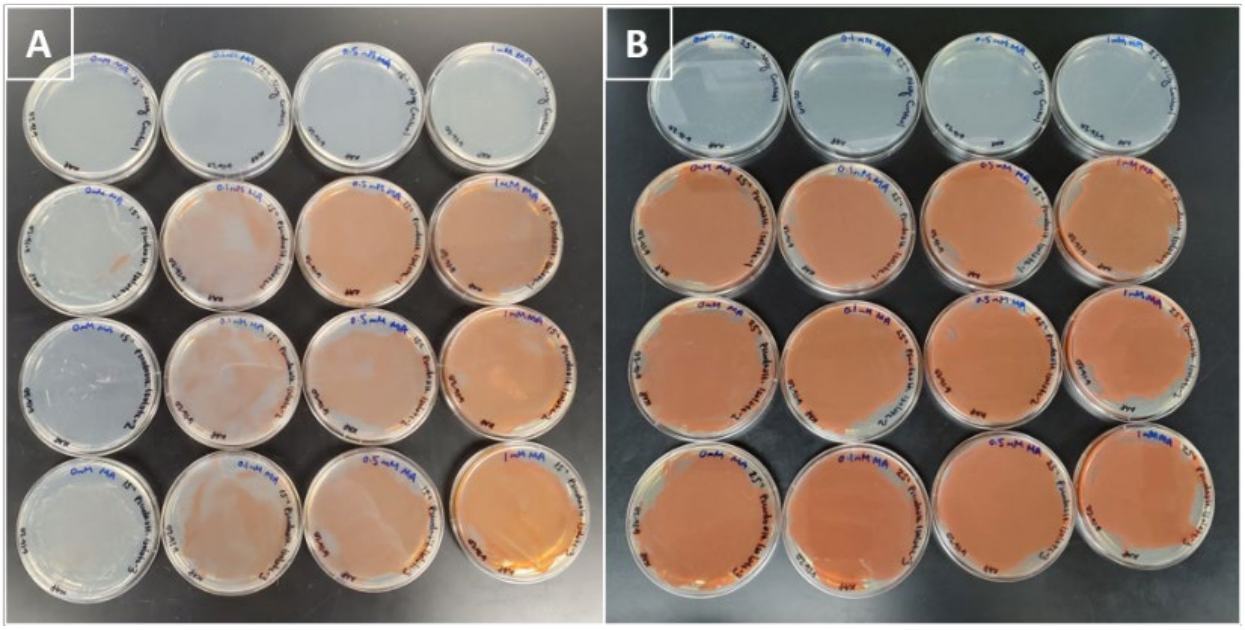


Figure 16: Pigment production decreases as a stress response by *Pseudoalteromonas* isolate. Top row of plates are all negative controls. $AlCl_3•6H_2O$ concentrations increase from left to right (0, 0.1, 0.5 and 1.0 mM, respectively). When grown at (A) 15°C, which is 10°C below its (B) optimum growth temperature, there is less pigment produced than normal. As $AlCl_3•6H_2O$ increases at 15°C, pigment production increases.

Temperature Effects on Al Tolerance in Liquid Medium

Since absorbance and cell growth have a linear relationship, when looking at the absorbances of *Bacillus* isolate over time, the biggest differences between the two temperatures tested was at 1.0 mM $AlCl_3•6H_2O$ (Figure 17) where the maximum optical densities were significantly lower at 25°C compared to 30°C ($p<0.05$; Figure 18; Table 7). The *Bacillus* isolate consistently showed the highest maximum optical density (OD_{600}) in the presence of 0.5 mM

$AlCl_3 \cdot 6H_2O$ at both 30 and 25°C ((0.50 ± 0.01 OD_{600} and 0.52 ± 0.02 OD_{600}, respectively) respectively ($p>0.05$). The lowest maximum OD_{600} (0.20 ± 0.11 and 0.16 ± 0.04) was seen in the presence of 1.5 mM $AlCl_3 \cdot 6H_2O$ at 30 and 25°C, respectively ($p>0.05$) (Figure 18). Within the temperatures tested, there was statistical significance when comparing the maximum OD_{600} between 0.1 mM and 0.5 mM mM $AlCl_3 \cdot 6H_2O$ and between 0.75 and 1.0 mM $AlCl_3 \cdot 6H_2O$ (both $p<0.05$). Within the 25°C trials, the jump from 0.5 to 0.75 mM $AlCl_3 \cdot 6H_2O$ was also considered statistically significant ($p<0.05$) (Table 7). Results from the spectrophotometric study support my hypothesis that decreasing temperature will negatively affect the isolate's ability to grow in the presence of $AlCl_3 \cdot 6H_2O$.

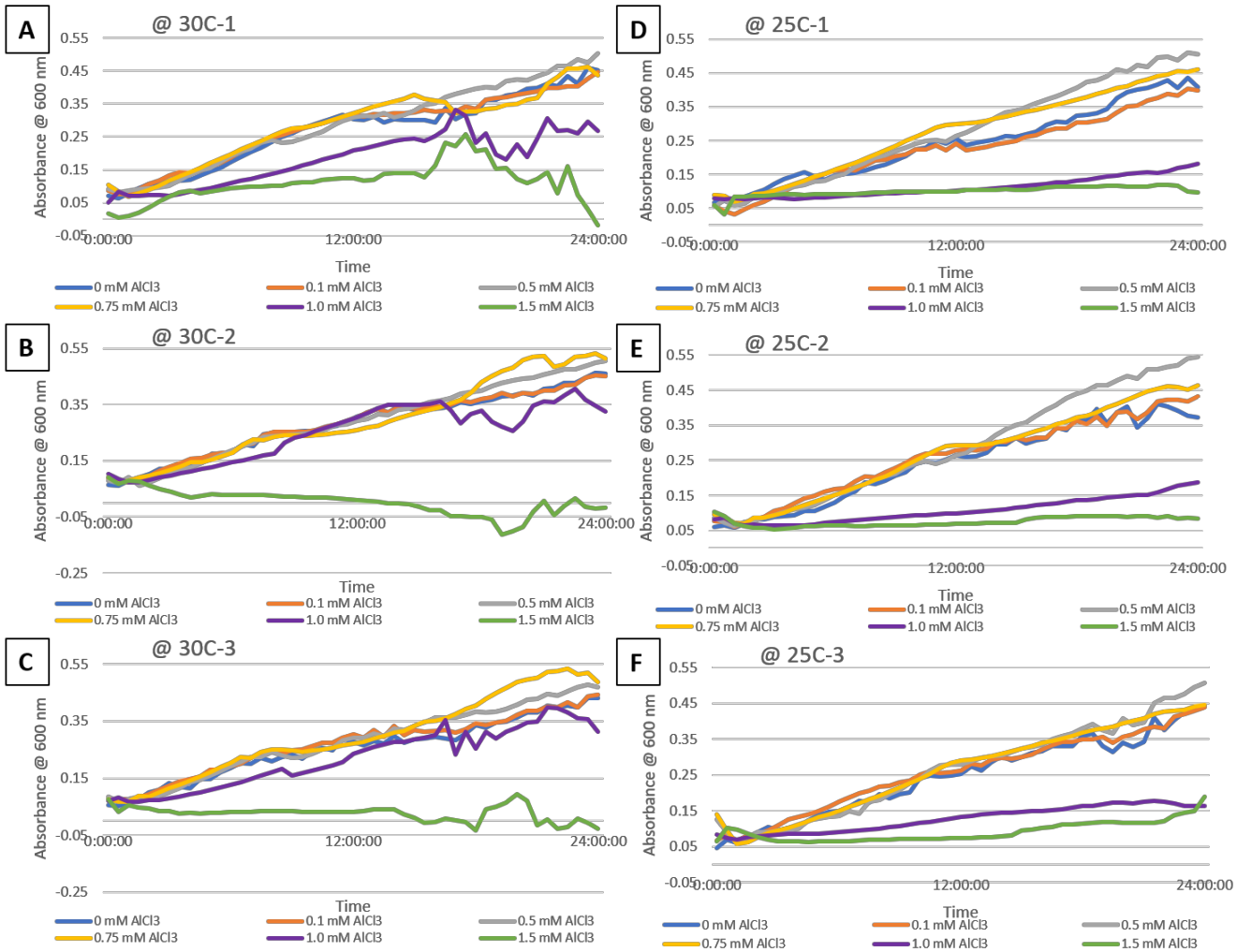


Figure 17: *Bacillus* isolate was grown in different concentrations of $AlCl_3 \cdot 6H_2O$ over a 24-hour period and absorbances were taken every 10 minutes at 600 nm. Treatments were performed in triplicate, averaged, and used to plot absorbances over time. Trials A-C were performed at 30°C, its optimum growth temperature, and trials D-F were performed at 25°C. The 5°C drop in temperature drastically affected the ability of the isolate to grow in the presence of 1.0 mM $AlCl_3 \cdot 6H_2O$.

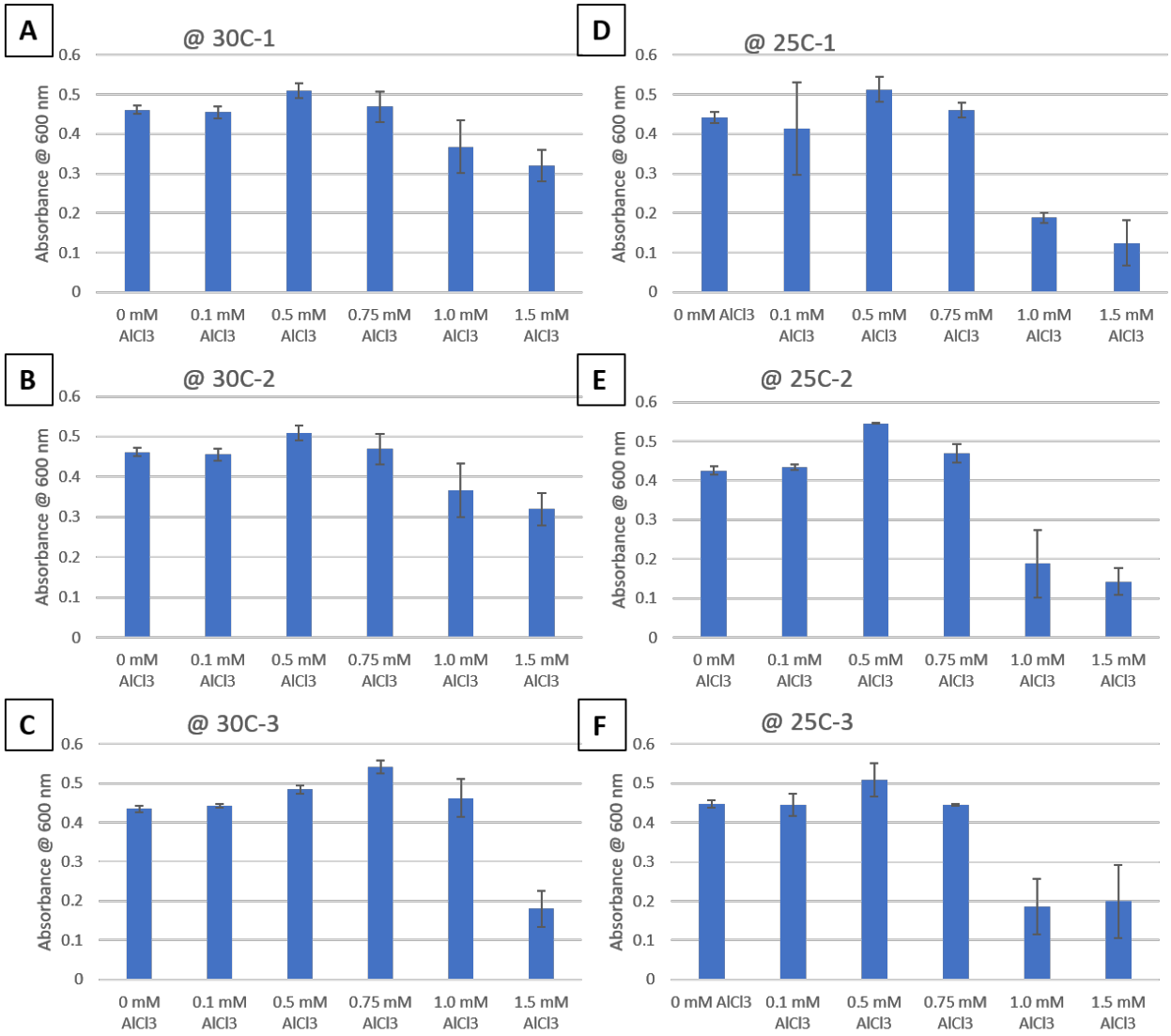


Figure 18: Average maximum optical density (OD_{600}) at 600 nm between different concentrations of $AlCl_3$•$6H_2O$. Trials A-C were performed at 30°C, its optimum growth temperature, and trials D-F were performed at 25°C. The maximum OD_{600} significantly decreased in the presence of 1.0 mM $AlCl_3$•$6H_2O$ from 30°C to 25°C.

Table 7: T-test results from pairwise comparisons of maximum OD_{600}. Under the p-value column, the bolded values were found to be statistically significant.

Pairwise Comparisons (Max OD_{600})	t-value	p-value
@30C--0:0.1 mM AlCl3	0.368522	0.7478366
@30C--0.1:0.5 mM AlCl3	12.70542	**0.0061377**
@30C--0.5:0.75 mM AlCl3	0.623597	0.5965337
@30C--0.75:1.0 mM AlCl3	-7.80303	**0.0160299**
@30C--1.0:1.5 mM AlCl3	-2.53742	0.1265062
@25C--0:0.1 mM AlCl3	-0.67888	0.5672387
@25C--0.1:0.5 mM AlCl3	6.329734	**0.0240619**
@25C--0.5:0.75 mM AlCl3	8.984609	**0.0121625**
@25C--0.75:1.0 mM AlCl3	45.76695	**0.0004771**
@25C--1.0:1.5 mM AlCl3	-1.39014	0.2989891
0 mM AlCl3--30C:25C	1.025572	0.4129324
0.1 mM AlCl3--30C:25C	-1.68747	0.2335656
0.5 mM AlCl3--30C:25C	2.221685	0.1564097
0.75 mM AlCl3--30C:25C	2.268831	0.1513625
1.0 mM AlCl3--30C:25C	8.161707	**0.0146822**
1.5 mM AlCl3--30C:25C	0.59905	0.6099573

DISCUSSION

Some of the microorganisms successfully isolated from Al alloys on agar made of 0.5 mM $AlCl_3 \cdot 6H_2O$ from Al 2024 and Al 7075 have been previously isolated from Al surfaces including *Bacillus* and *Shewanella* sp. (Mansfeld, 2007, McNamara et al., 2005, Rajasekar & Ting, 2010, Zhang et al., 2019). As previously described, due to their relevance in literature regarding bacteria in Al environments, their unique colony morphologies and presence in the microbial communities attached to Al alloys in the environmental study, one of the *Bacillus* sp. and the *Pseudoalteromonas* sp. were chosen for further characterization. *Bacillus* sp. are a genus of known spore- and biofilm-formers and can survive in a variety of extreme environments due to these characteristics. They have been isolated from biofilm attached to a variety of metal surfaces thought to both influence and inhibit corrosion depending on the species and environment (Zuo, 2007). *Pseudoalteromonas* sp. are a genus of known biofilm- and pigment-producing bacteria. Other studies have concluded their pigments are normally produced under

optimal growth conditions and have antimicrobial and antifouling properties when secreted in the presence of other organisms (Cai, 2006, Rao et al., 2005). Antifouling may be of particular interest in terms of microorganisms that attach to Al alloy surfaces. If this isolate is secreting compounds that inhibit the attachment of fouling organisms it may be useful to promote its growth within the community. If major environmental changes in water temperature and salinity affect the isolates ability to produce these substances, and ultimately attachment to the Al alloy surface, it will no longer be of use to inhibit deterioration of the metal.

The results from the biochemical tests suggest both isolates are obligate aerobes with the ability to produce gelatinase. Gelatinase allows gelatin to be broken down into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be used by the organism. Organic corrosion inhibitors (like gelatin and other polymers) have been used to protect Al surfaces for decades which suggests these isolates have an advantage of being able to gain essential amino acids even while attached to metal substrates (Abdallah et al., 2016). The *Pseudoalteromonas* isolate was capable of decarboxylating ornithine, which is essential for cell growth, allowing for polyamines to be created when necessary to stabilize newly synthesized DNA. Spermidine is most common polyamine in cyanobacteria and is known to promote gene expression and the replacement of damaged proteins under prolonged cold stress, suggesting this isolate may be using this method of protein catabolism to protect itself when temperature stress occurs (Zhu et al., 2015). Overall, results from the biochemical tests can help us understand the isolate's role in the environment.

Both isolates grew within the same temperature range (20-45°C) and in the presence of the same Al concentration range (0-1 mM $AlCl_3 \bullet 6H_2O$). The isolates used in this study were collected from the same site in the same month and isolated from the same Al 2024, which

supports the finding their optimal growth temperature is 30°C. The *Pseudoalteromonas* isolate did grow at 10°C after 2 weeks suggesting it grew slow due to the low temperature or the ability to adapt and survive under cold stress. This isolate also exhibited interesting pigment production during the factorial experiment with temperature and Al. At 15°C, the pigment was close to non-existent at 0 mM but increased as the Al concentration increased to 1 mM. This supports the idea that *Pseudoalteromonas* changes its pigment production in response to stress (Sakai-Kawada et al., 2019). From 25-35°C, the pigment produced by *Pseudoalteromonas* did not seem to be significantly affected between Al concentrations based on observed coloration. The *Bacillus* isolate displayed more translucent growth on all Al concentrations at 15°C but grew normally from 25-35°C with little variation in growth characteristics between Al concentrations. This reflects their environmental conditions in the summer months and microbial attachment to Al alloys under these conditions. Since the water temperature was slightly below 15°C in the winter, this suggests the *Bacillus* isolate may not be able to stay attached year-round, but the *Pseudoalteromonas* isolate can.

The 24-hour spectrophotometric growth study conducted on the *Bacillus* isolate provided fundamental knowledge on how decreasing temperature 5°C below it optimum, will affect the isolates ability to grow in increasing amounts of Al. The 5°C shift in temperature produced a significant difference in maximum OD_{600} at 1.0 mM $AlCl_3 \bullet 6H_2O$. At 30°C, the isolate's optimum growth temperature, the absorbances were higher at 1.0 mM $AlCl_3 \bullet 6H_2O$ compared to 25°C. The highest absorbance reading for both temperatures was at 0.5 mM $AlCl_3 \bullet 6H_2O$, suggesting either more growth, more cell death, or for the Al to absorb enough at 600 nm to cause an increase in absorbance. By looking at how the absorbance of each treatment increases

overtime and to what extent, further hypotheses can be made and tested about the limitations of the *Bacillus* isolate and other isolates taken from Al alloy surfaces and their ability to tolerate Al under environmental conditions that inevitably change. Based on the results of this study, the ability for the *Bacillus* isolate to attach to Al alloys would be negatively affected by a 5°C decrease in temperature, more specifically in the summer months since they may not be present in the winter.

These results provide further insight in the environmental role of the two isolates, as well as created a model to allow the implications of aluminum tolerance in an estuary to be understood in the lab setting. In an environment that is constantly changing, and in a world that is steadily getting warmer, it's necessary to understand not only the microbial communities that will attach to metal structures in estuaries, but also more about the particular microorganisms themselves. The findings from this study can be used to make hypotheses about the communities that would attach to Al 2024 in other environments across the globe because of the consistent presence of certain taxa like *Gammaproteobacteria,* regardless of their variation. *Gammaproteobacteria* were consistently present on the Al alloys between sites and were most abundant in the winter months, suggesting they are widespread and important in the microbial communities attached to Al alloys.

Microbial interactions with Al surfaces in estuaries are largely dependent on the surrounding environment, both in terms of salinity and water temperature, suggesting the communities that attach will vary globally and seasonally. Besides aircraft/marine wreckage and other aluminum structures, industrial runoff is a major contributor to the increasing amount of solubilized Al in the environment. By investigating the microorganisms that survive in these environments and evaluating the conditions that constrain their tolerance to Al, we can make

further predictions about the mechanisms they are using, the environments they can be found in, and expand upon the idea that microorganisms are constantly adapting to their surrounding environment.

CONCLUSION AND FUTURE DIRECTIONS

Chapter 1 of this thesis provided novel insights to the microbial communities that will attach Al alloys 2024 and 7075 in estuaries along the Pamlico River. Between the two Al alloys, Al 2024 generally exhibited lower diversity than Al 7075, with a mix of high and low diversity in the summer months (July) and the lowest diversity of all substrates, sites, and seasons, in the winter months (December). The microbial communities were found distinct in their composition between substrates in December only suggesting seasonal environmental changes, like a decrease in temperature, influences who can attach to all substrates. Why was there so much variation in microbial community diversity and composition between seasons on Al 2024 and not Al 7075? First, to get a better idea of the overall differences in the microbial communities that attach to Al 2024 and Al 7075, the number of sampling events as well overall time frame of the environmental study could be increased. This would allow for a better representation of how different locations and seasons influence microbial attachment to the Al alloys. More sampling sites could be added to the study, or another estuarine system could be used, which could be used to determine how the dynamic nature of estuaries influences attachment. Only discrete readings of the water temperatures and salinities from the environmental study were used for analysis. This did not allow for a complete picture to be drawn of the ever-changing environment these communities experienced. In order to better understand how the microbial community diversity and composition are influenced by these factors, temperature and salinity loggers could be used to gather continuous data, and then ultimately be used determine how constant fluctuations

influence attachment. This dynamicity could also be compared to a more static environment, like marine systems, to determine if these results are only representative of an estuarine environment and potentially elucidate a core aluminum microbiome between all the different environmental locations. To see how these small changes in chemical composition influence attachment, other Al alloys can be tested in addition to the Al 2024 and Al 7075. Al alloys are given a 4-digit number designation based on their chemical composition; the first number indicates the principle alloying element and also denotes the series it is in (there are 8 series of Al alloys). A representative study of all Al alloys would consist of one Al alloy from each of the 8 series available, allowing an understanding of how chemical composition influences microbial attachment to metal surfaces. Lastly, a third replicate could be added to the study to increase statistical power of the results.

Chapter 2 of this thesis investigated two microorganisms isolated from Al alloy 2024 that was previously submerged at site P3 along the Pamlico River from July to September. The investigation provides basic characterizations of their growth limits as well as sets up future experiments that can be used to determine how temperature and salinity affect the isolates ability to grow in the presence of increasing Al concentrations. Both isolates grew within the same temperature range (20-45°C) and in the presence of the same Al concentration range (0-1 mM $AlCl_3 \bullet 6H_2O$), which was comparable to the water temperatures experienced *in situ*. The 24-hour spectrophotometric growth study conducted on the *Bacillus* isolate provided fundamental knowledge on how decreasing temperature 5°C below it optimum, will affect their ability to grow in the presence of $AlCl_3$. The isolates retrieved from this study need to be further characterized, especially since they both have the ability to form biofilms and *Bacillus* sp. can form endospores under stressful conditions. Both of these traits could be used to adapt to Al

environments and provides a selective advantage over other organisms that cannot. Staining followed by microscopy or boiling the culture then seeing if they survive could work to determine if the *Bacillus* isolate can produce endospores. This stress response could also have affected the optical density of the isolate, making it appear higher than it should. Future experiments could be performed using liquid cultures and spot plating to determine colony forming units/mL so that results could be normalized to cell number and compared confidently. The genomes of both isolates still need to be analyzed to characterize the genomic potential of the organisms and further understand how they are able to tolerate Al surfaces and survive in Al cultures. Genes that could potentially be used to detoxify the cell of or sequester heavy metals have yet to be identified. To understand how the isolates are genetically interacting with Al, a transcriptional study could be performed looking at these particular genes and seeing if they're upregulated in the presence of increasing concentrations of Al. The dynamic nature of the environment they were isolated from could also influence gene expression, which could be elucidated by looking at specific genes used for osmoregulation and protection against reactive oxygen species.

Overall, the findings of this work contribute to the knowledge gaps found in research involved with microbial attachment to Al surfaces. Al is a toxic metal that is incapable of being used by biological cells, but with the increase of solubilized Al due to pollution and placement of Al structures in aquatic systems, microorganisms are faced with both a substrate for attachment and a selective pressure. Whether these microorganisms persist, depends on the surrounding environment and seasonal changes, suggesting microbial communities found in Al environments around the world will vary in their diversity and composition.

Literature Cited

Abdallah, M., Kamar, E. M., El-Etre, A. Y., & Eid, S. (2016). Gelatin as corrosion inhibitor for aluminum and aluminum silicon alloys in sodium hydroxide solutions. *Protection of metals and physical chemistry of surfaces*, 52(1), 140-148.

Andrews, K. (2019). *Laboratory Manual for Microbiology* (9th ed.). Plymouth, MI: Macmillan Learning Curriculum Solutions.

Appanna, V. D., & Hamel, R. (1996). Aluminum detoxification mechanism in Pseudomonas fluorescens is dependent on iron. *FEMS microbiology letters*, 143(2-3), 223-228.

Appanna, V. D., Kepes, M., & Rochon, P. (1994). Aluminum tolerance in Pseudomonas fluorescens ATCC 13525: Involvement of a gelatinous lipid-rich residue. *FEMS microbiology letters,* 119(3), 295-301.

Appanna, V. D., & St. Pierre, M. (1994). Influence of phosphate on aluminum tolerance in Pseudomonas fluorescens. *FEMS microbiology letters*, 124(3), 327-332.

Appanna, V. D., & Pierre, M. S. (1996). Aluminum Elicits Exocellular Phosphatidylethanolamine Production in Pseudomonas fluorescens. *Appl. Environ. Microbiol*., 62(8), 2778-2782.

Bruckard, W. J., Calle, C. M., Davidson, R. H., Glenn, A. M., Jahanshahi, S., Somerville, M. A., ... & Zhang, L. (2010). Smelting of bauxite residue to form a soluble sodium aluminium silicate phase to recover alumina and soda. *Mineral Processing and Extractive Metallurgy*, 119(1), 18-26.

Cai, H. (2006). Microbiologically influenced corrosion and titanate conversion coatings on aluminum alloy 2024-T3. *University of Rhode Island*.

Colombani, N., Osti, A., Volta, G., & Mastrocicco, M. (2016). Impact of climate change on salinization of coastal water resources. *Water resources management*, 30(7), 2483-2496.

Crump, B. C., Hopkinson, C. S., Sogin, M. L., & Hobbie, J. E. (2004). Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. *Appl. Environ. Microbiol*., 70(3), 1494-1505.

Dahlman, L., & Lindsey, R. (2020, August 17). Climate Change: Ocean Heat Content: NOAA Climate.gov. Retrieved October 07, 2020, from https://www.climate.gov/news-features/understanding-climate/climate-change-ocean-heat-content.

Davey, M. E., & O'toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev*., 64(4), 847-867.

Emsley, J. (2011). *Nature's building blocks: an AZ guide to the elements*. Oxford University Press.

Enning, D., Venzlaff, H., Garrelfs, J., Dinh, H.T., Meyer, V., Mayrhofer, K., Hassel, A.W., Stratmann, M., Widdel, F. (2012). Marine sulfate reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. *Environ Microbiol* 14:1772–1787. https://doi.org/10.1111/j.1462-2920.2012.02778.x

Exley, C., & Mold, M. J. (2015). The binding, transport and fate of aluminium in biological cells. *Journal of Trace Elements in Medicine and Biology*, 30, 90-95.

Faust, M. A., Aotaky, A. E., & Hargadon, M. T. (1975). Effect of physical parameters on the in situ survival of Escherichia coli MC-6 in an estuarine environment. *Applied microbiology*, 30(5), 800-806.

Fischer, J., Quentmeier, A., Gansel, S., Sabados, V., & Friedrich, C. G. (2002). Inducible aluminum resistance of acidiphilium cryptum and aluminum tolerance of other acidophilic bacteria. *Archives of Microbiology*, 178(6), 554-558. doi:10.1007/s00203-002-0482-7.

Fu, B., Taoukis, P. S., & Labuza, T. P. (1991). Predictive microbiology for monitoring spoilage of dairy products with time-temperature integrators. *Journal of Food Science*, 56(5), 1209-1215.

Garrison, C. E., Price, K. A., & Field, E. K. (2019). Environmental evidence for and genomic insight into the preference of iron-oxidizing Bacteria for more-corrosion-resistant stainless steel at higher salinities. *Applied and Environmental Microbiology*, 85(14), e00483-19.

Gaylarde, C. C., & Videla, H. A. (1987). Localised corrosion induced by a marine vibrio. *International biodeterioration*, 23(2), 91-104.

Gikas, P., Sengor, S. S., Ginn, T., Moberly, J., & Peyton, B. (2009). The effects of heavy metals and temperature on microbial growth and lag. *Global NEST Journal*, 11(3), 325-332.

Guan, F., Zhai, X., Duan, J., Zhang, J., Li, K., & Hou, B. (2017). Influence of sulfate-reducing bacteria on the corrosion behavior of 5052 aluminum alloy. *Surface and Coatings Technology,* 316, 171-179.

Hatch, J. E. (1984). Aluminum: properties and physical metallurgy. *American Society for Metals, Metals Park, Ohio 44073, U. S. A,* 1984. 424.

Jenkins, A. (2014, October 02). With a pinch of salt – Climate Change: Vital Signs of the Planet. Retrieved October 07, 2020, from https://climate.nasa.gov/news/58/with-a-pinch-of-salt/

Jo, J., Jang, Y. S., Kim, K. Y., Kim, M. H., Kim, I. J., & Chung, W. I. (1997). Isolation of ALU1-P Gene Encoding a Protein with Aluminum Tolerance Activity from Arthrobacter viscosus. *Biochemical and biophysical research communications*, 239(3), 835-839.

Juzeliūnas, E., Ramanauskas, R., Lugauskas, A., Leinartas, K., Samulevičienė, M., & Sudavičius, A. (2006). Influence of wild strain Bacillus mycoides on metals: From corrosion acceleration to environmentally friendly protection. *Electrochimica acta*, 51(27), 6085-6090.

Lane, D. J. (1991). 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*, 115-175.

Li, K. Y., & Torres, J. A. (1993). Microbial growth estimation in liquid media exposed to temperature fluctuations. *Journal of food science*, 58(3), 644-648.

Little, B., Wagner, P., & Mansfeld, F. (1991). Microbiologically influenced corrosion of metals and alloys. *International Materials Reviews*, 36(1), 253-272.

Little, B., Wagner, P., & Mansfeld, F. (1992). An overview of microbiologically influenced corrosion. *Electrochimica acta*, 37(12), 2185-2194.

Liu, H., Zheng, B., Xu, D., Fu, C., & Luo, Y. (2014). Effect of sulfate-reducing bacteria and iron-oxidizing bacteria on the rate of corrosion of an aluminum alloy in a central air-

conditioning cooling water system. *Industrial & Engineering Chemistry Research,* 53(19), 7840-7846.

MacLeod, I. D. (1983). Stabilization of corroded aluminium. *Studies in conservation*, 28(1), 1-7.

Mansfeld, F. (2007). The interaction of bacteria and metal surfaces. *Electrochimica acta*, 52(27), 7670-7680.

Marty, F., Gueuné, H., Malard, E., Sánchez-Amaya, J.M., Sjögren, L., Abbas, B., Quillet, L., van Loosdrecht, M.C., Muyzer, G. (2014). Identification of key factors in accelerated low water corrosion through experimental simulation of tidal conditions: influence of stimulated indigenous microbiota. *Biofouling* 30:281–297. https://doi.org/10.1080/08927014.2013.864758.

McBeth, J. M., and Emerson, D. (2016). In situ microbial community succession on mild steel in estuarine and marine environments: exploring the role of iron-oxidizing bacteria. *Front. Microbiol*. 7:767. doi: 10.3389/fmicb.2016.00767

McBeth, J. M., Little, B. J., Ray, R. I., Farrar, K. M., and Emerson, D. (2011). Neutrophilic iron-oxidizing "Zetaproteobacteria" and mild steel corrosion in nearshore marine environments. *Appl. Environ. Microbiol*. 77, 1405–1412. doi:10.1128/AEM.02095-10

McMeekin, T. A., Chandler, R. E., Doe, P. E., Garland, C. D., Olley, J., Putro, S., & Ratkowsky, D. A. (1987). Model for combined effect of temperature and salt concentration/water activity on the growth rate of Staphylococcus xylosus. *Journal of Applied Bacteriology*, 62(6), 543-550.

McNamara, C. J., Perry IV, T. D., Leard, R., Bearce, K., Dante, J., & Mitchell, R. (2005). Corrosion of aluminum alloy 2024 by microorganisms isolated from aircraft fuel tanks. *Biofouling*, 21(5-6), 257-265.

N-Uptake, A. (1999). Primary production by phytoplankton and microphytobenthos in estuaries. *Estuaries,* 29, 93.

Öksüz, K. E., Bağirov, H., Şimşir, M., Karpuzoğlu, C., Özbölük, A., Demirhan, Y. Z., & Bilgin, H. U. (2013). Investigation of Mechanical Properties and Microstructure of AA2024 and AA7075. In *Applied Mechanics and Materials* (Vol. 390, pp. 547-551). Trans Tech Publications Ltd.

Örnek, D., Jayaraman, A., Syrett, B. C., Hsu, C. H., Mansfeld, F. B., & Wood, T. K. (2002). Pitting corrosion inhibition of aluminum 2024 by Bacillus biofilms secreting polyaspartate or γ-polyglutamate. *Applied microbiology and biotechnology*, 58(5), 651-657.

Örnek, D., Wood, T. K., Hsu, C. H., Sun, Z., & Mansfeld, F. (2002). Pitting corrosion control of aluminum 2024 using protective biofilms that secrete corrosion inhibitors. *Corrosion,* 58(9), 761-767.

Price, P. B., & Sowers, T. (2004). Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. *Proceedings of the National Academy of Sciences*, 101(13), 4631-4636.

Rajaram, R., Banu, J. S., & Mathivanan, K. (2013). Biosorption of Cu (II) ions by indigenous copper-resistant bacteria isolated from polluted coastal environment. *Toxicological & Environmental Chemistry,* 95(4), 590-604.

Rajasekar, A., & Ting, Y. P. (2010). Microbial corrosion of aluminum 2024 aeronautical alloy by hydrocarbon degrading bacteria Bacillus cereus ACE4 and Serratia marcescens ACE2. *Industrial & Engineering Chemistry Research*, 49(13), 6054-6061.

Rao, K. S., & Rao, K. P. (2004). Pitting corrosion of heat-treatable aluminium alloys and welds: a review. *Trans. Indian Inst. Met*, 57(6), 593-610.

Rao, D., Webb, J. S., & Kjelleberg, S. (2005). Competitive interactions in mixed-species biofilms containing the marine bacterium Pseudoalteromonas tunicata. *Appl. Environ. Microbiol.,* 71(4), 1729-1736.

Sakai-Kawada, F. E., Ip, C. G., Hagiwara, K. A., & Awaya, J. D. (2019). Biosynthesis and Bioactivity of Prodiginine Analogues in Marine Bacteria, Pseudoalteromonas: A Mini Review. *Frontiers in microbiology*, 10, 1715.

Sieburth, J. M. (1967). Seasonal selection of estuarine bacteria by water temperature. *Journal of Experimental Marine Biology and Ecology*, 1(1), 98-121.

Sterritt, R. M., & Lester, J. N. (1980). Interactions of heavy metals with bacteria. *Science of the Total environment*, 14(1), 5-17.

Total Materia. (2004, April) *Aircraft and Aerospace Applications: Part Two.* www.totalmateria.com/Article96.htm.

Total Materia. (2008, January). *Corrosion of Aluminum and Its Alloys: Forms of Corrosion*. https://www.totalmateria.com/page.aspx?ID=CheckArticle.

Turner, S., Pryer, K. M., Miao, V. P., & Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis 1. *Journal of Eukaryotic Microbiology,* 46(4), 327-338.

Videla, H. A., & Herrera, L. K. (2009). Understanding microbial inhibition of corrosion. A comprehensive overview. *International Biodeterioration & Biodeterioration*, 63(7), 896-900.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, 173(2), 697-703.

Zarasvand, K. A., & Rai, V. R. (2014). Microorganisms: induction and inhibition of corrosion in metals. *International Biodeterioration & Biodeterioration*, 87, 66-74.

Zhang, Y., Ma, Y., Duan, J., Li, X., Wang, J., & Hou, B. (2019). Analysis of marine microbial communities colonizing various metallic materials and rust layers. *Biofouling*, 1-14.

Zhu, X., Li, Q., Yin, C., Fang, X., & Xu, X. (2015). Role of spermidine in overwintering of cyanobacteria. *Journal of bacteriology*, 197(14), 2325-2334.

Zuo, R. (2007). Biofilms: strategies for metal corrosion inhibition employing microorganisms. *Applied microbiology and biotechnology*, 76(6), 1245-1253.

Zuo, R., Örnek, D., & Wood, T. K. (2005). Aluminum-and mild steel-binding peptides from phage display. *Applied microbiology and biotechnology*, 68(4), 505-509.