Sand Aggregation by Methanotrophic Bacteria and Marine Microbial Hydrocarbon Degradation Under Differing Biogeochemistries

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Abstract

Methanotrophic bacteria, bacteria that can metabolize methane, are ubiquitous, EPS-producing, and capable of degrading hundreds of contaminants (Hazen, 2010). This dissertation looks at the possibility of using methanotrophic bacteria in sand surface aggregation and oil spill responses. Sand aggregation practices are widely used from infrastructure improvements to reversing desertification. In chapter 2, two selected methanotrophic cultures had their growth curves measured and their extracellular polymeric substances (EPS) production estimated. This second chapter tested and rejected two null hypotheses; the first null hypothesis was that there is no difference in EPS production rates over a culture's growth curve, showing that the exponential phase had the largest EPS production rates for both *Methylocystis sp.* and *Methylosinus sporium*. In chapter 3, two bench-scale experiments were set up to test the sand bioaggregation capabilities of methanotrophs. This third chapter had two null hypotheses, both of which were rejected, the first stating that there is no difference in aggregation between sand treated with different methanotrophic cultures, and the second null hypothesis is that there is no difference in aggregation based on when in their growth curve cultures are applied to sand. It was found that bacterial cultures applied during their stationary phase created the most stable aggregates as compared to those applied in their exponential phase. The fifth chapter aimed to determine how enriched microbial communities and their hydrocarbon degradation rates reacted to changes in biogeochemistry and methane amendments. There were three null hypotheses for the fifth chapter, all of which failed to be rejected, the last of which states that there is no difference in the methanotrophic percentage of the taxa plot with the addition of methane amendments. Overall, chapter 5 found that enriched microbial consortia had slightly better polyaromatic hydrocarbons (PAH) degradation in media that more closely mimicked their natural biogeochemistry and that the enriched consortia did not see an increase in hydrocarbon degradation.

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Chapter 1

Review of Current Bioaggregation of Surface Treatments

Surface aggregation practices have the potential to be utilized in many engineering situations, from erosion control, soil strengthening, dust control, desert reclamation, and aquaculture sealing. Aggregation techniques reduce potential infrastructure and environmental issues, while making life more comfortable for the public. Dust presents a danger to human health by itself and is a carrying agent for pollutant dispersal (Ivanov & Stabnikov, 2016; Viktor Stabnikov et al., 2013). Soil Loss is common through rainfall or wind events (Chung et al., 2021). Even small levels of aggregation can prevent dispersion (V Stabnikov et al., 2013). Traditional methods have focused on chemical aggregation but have fallen out of favor due to high economic and environmental costs (DeJong et al., 2010; Karol, 2003). They have been replaced with research based on biotechnology, which utilizes microorganisms and their byproducts. Ivanov and Stabnikov (2016) break down biotechnology into different types in their book on Construction Biotechnology. While many kinds of biotechnology overlap with one another, in the case of surface aggregation, the focus is on four aspects of biotechnological processes: bioaggregation, biocrusting, bioclogging, and biocementation.

The term bioaggregation is often used as a catch-all referring to increasing the size of soil particles. Biocementation's goal is the significant increase of the soil strength by filling porous spaces with insoluble compounds, often calcite. Biocrusting creates a crust on the soil surface from bacterial polysaccharides or biocementation. Bioclogging fills pore spaces in soil and rock, usually to control hydraulic conductivity, and is an alternative to chemical grouts (Ivanov & Stabnikov, 2016). Biocement and biogrout have a lower viscosity than their inorganic counterparts, allowing deeper surface penetration (Stabnikov et al., 2016; Stabnikov et al., 2011).

Microbial-Induced Calcite Precipitation

Microbially Induced Calcite Precipitation, MICP, utilizes urease-producing bacteria (UPB) to facilitate the production and deposition of calcite in porous media. MICP is arguably the most widely studied biocementation method (Reddy, 2013; Viktor Stabnikov et al., 2013). The process has three main elements: urease active bacteria, urea solution, and a calcium ion source (V Stabnikov et al., 2013). The urease enzyme

facilitates the hydrolysis of one-mole urea into one-mole ammonia and one-mole carbonate (Dhami et al., 2016):

$$(NH_2)_2CO + 3H_2O \xrightarrow{urease} 2NH_4^+ + HCO_3^- + OH^-$$

Spontaneous hydrolysis follows, forming one-mole carbonate (Reddy, 2013):

$$CaCl_2 + HCO_3^- + OH^- \rightarrow CaCO_3 \downarrow + H_2O + 2Cl^-$$

Resulting in an overall reaction of:

$$(NH_2)_2CO + 2H_2O + CaCl_2 \rightarrow CaCO_3 \downarrow + 2NH_4^+ + 2Cl^-$$

MICP is often studied in sand applications (Cheng et al., 2013; Cui et al., 2017; DeJong et al., 2006; Dhami et al., 2016; Kim & Youn, 2016; Naeimi & Chu, 2017; Riveros & Sadrekarimi, 2020; Sharma et al., 2022; Viktor Stabnikov et al., 2013; Stabnikov et al., 2016; Stabnikov et al., 2011) but has uses in many sediments and is also studied for infrastructure and cement repair. Surface applications of MICP include dust control, pond sealing/permeability reduction, soil stabilization, erosion control, and soil strength improvements.

Background

To the best of our knowledge, the first introduction of microbial ureolysis and calcite precipitation to alter sand was by Ferris et al. (1992) in their 1992 conference paper titled ‘Bacteriogenic Mineral Plugging’ and later in their 1996 Article of the same title, presented in the Journal of Canadian Petroleum Technology. At the time, Ferris et al. (1996) referred to this technology as Bacteriogenic Mineral Plugging; later research would coin the current terminology of ‘Microbial Induced Calcite Precipitation’, or MICP. Other references to MICP utilize broad naming as biomineralization of calcite (Reddy, 2013), biocalcification (DeJong et al., 2006), and biocementation (Stabnikov et al., 2011).

Bacteria Selection

DeJong et al. (2006) laid out the criteria that bacteria for MICP should be capable of consuming urea, aerobic, non-pathogenic, capable of CO_2 production in high pH, and preferably native to the environment. Initial focus and much of the published MICP research has focused on *Sporosarcina pasteurii* - formerly named *Bacillus pasteurii* (Chung et al., 2021; Cui et al., 2017; Gao et al., 2019; Kannan et al., 2020; Liu et al., 2020; Salifu et al., 2016; Sharma et al., 2022; Stabnikov et al., 2016; Stabnikov et al., 2011); *Lysinibacillus sphaericus - formerly Bacillus sphaericus - (Cheng et al., 2013; Sharma et al., 2022)* and *Sporosarcina ureae (Riveros & Sadrekarimi, 2020; Whitaker et al., 2018)* have also been of high interest. Other studied and potentially suggested bacteria include different strains of *Bacillus (Dhami et al., 2016; Kim & Youn, 2016; Naeimi & Chu, 2017; Viktor Stabnikov et al., 2013; Stabnikov et al., 2011), Sporosarcina (Kim & Youn, 2016; Riveros & Sadrekarimi, 2020)*, *Spoloactobacilus, Desulfotomaculum*, and *Staphylococcus* (Kim & Youn, 2016). It should be noted that higher urease enzyme production in a species does not correlate well with calcite precipitation amounts (Kim & Youn, 2016), so it should not be the only factor in choosing a strain.

Components

Bioaugmentation and MICP require three components: a urease-producing bacteria culture, urea, and a source of calcium - often $CaCl_2$. It is recommended that the bacterial solution be applied at an optical density, OD600, of 0.8-1.2 for higher urease activity (Okwadha & Li, 2010). Urea and $CaCl_2$, the mixture often referred to as cementation solution, are suggested to be at a concentration each of 0.5 mol/liter or higher (Harkes et al., 2010). Few studies combined both the bacterial and cementation solutions before application (Chung et al., 2021); most techniques apply the bacterial solution first and allow bacteria time to 'cure' and attach themselves to soil particles before application of the cementation solution (Cui et al., 2017; Gao et al., 2019; Liu et al., 2020; Salifu et al., 2016; Stabnikov et al., 2011). Time intervals ranged between 1 hour (Liu et al., 2020) and 8 hours (Cui et al., 2017). A key concern in application methods is creating the most homogenous distribution of calcite crystals possible in the

biocementation area, which differs for differing geotechnical areas. With sand, Cui et al. (Cui et al., 2017) tested a two-step bacteria application process, the first application of pure bacterial culture and the second application of a mixed solution of bacterial culture and $CaCl_2$, which created a 'relatively' uniform diffusion.

Application

For this review and maintaining the focus on surface applicable results, MICP treatment methods discussed here are those that apply to surface conditions; bulk stabilization alone and deep grouting techniques are not mentioned as they do not precipitate well onto surface applications and are instead focused on ground improvements. A commonly studied application technique for dust suppression and other surface MICP uses is spray application (Gao et al., 2019; Liu et al., 2020; Naeimi & Chu, 2017; Viktor Stabnikov et al., 2013; Stabnikov et al., 2016). Spraying solutions are a convenient and relatively easy-to-apply method (Liu et al., 2020) that can utilize smaller dosages than conventional aggregation methods (V Stabnikov et al., 2013). Other top-down applications include pouring, dripping, and premixing the soil and solutions. Cementation solution is commonly applied multiple times, with intervals between each application, depending on conditions and the amount of cementation desired. In order to optimize seepage control of biocemented sandy soil Gao et al. (2019) tested multiple application techniques, including surface spraying alone, surface spray after bulk stabilization - ground injections, and immersion. They found that surface spray after bulk immobilization had over 40 times the seepage control and 50% more penetration resistance which will be discussed more in the results section.

Analyses and Monitoring

Progression of the calcite precipitation and bioaggregation can be monitored from the soil samples themselves or through effluent monitoring. When available, effluents can be monitored for cation and ammonia concentrations (Salifu et al., 2016). Cation analyses measure whether calcium ions from the cementation solution have been utilized or are flowing through and still present in the effluent. The presence of ammonia in the effluent shows that the reaction has proceeded, as ammonia is a

byproduct of urea hydrolysis. Soil samples are monitored through calcite measurements, Scanning Electron Microscope (SEM) imaging, and X-ray analysis (XRD, XRF, EDX) (Cui et al., 2017; Riveros & Sadrekarimi, 2020; Sharma et al., 2022). Calcite is soluble in acid, and as such, its concentrations can be easily measured through acid titrations, like those used in the calcimeter, or by mass balance measurements (Chung et al., 2021; Cui et al., 2017; Gao et al., 2019; Salifu et al., 2016). The presence of new precipitated calcite indicates MICP is occurring. SEM imaging provides surface views of calcite crystal distribution and morphology (Cui et al., 2017), showing the structure of calcite-calcite and calcite-sand bonds (Sharma et al., 2022). Preparation of SEM samples differs from publication to publication as some samples are oven-dried beforehand (Riveros & Sadrekarimi, 2020), and others are dried and coated in platinum (Chung et al., 2021; Kim & Youn, 2016), carbon (Cui et al., 2017), or gold (Sharma et al., 2022). Stabnikov et al. (Stabnikov et al., 2011) prepared their SEM samples by fixing them with glutaraldehyde for critical point drying. The energy dispersive x-ray analysis inside (EDX) inside of SEMs and the X-ray fluorescence analysis (XRF) can be used to verify the chemical composition of precipitated crystals.

Measurements to determine the success of biocemented samples varies with the purpose of the experiments. For example, the mass of samples lost is valuable in dust suppression and erosion studies since the focus is not on the strength of the crust formed but on the retention of the starting soil mass. Other studies, for example, researching the sealing of aquaculture ponds, focus on measurements of hydraulic conductivity. Penetration resistance can be measured via a penetrometer and correlates to the unconfined compressive strength (Chung et al., 2021; Gao et al., 2019).

General Takeaways and Trends

MICP was found to be an effective surface bioaggregation method for dust control (Naeimi & Chu, 2017), seepage control, penetration resistance (Chung et al., 2021), and general strength improvement. These MICP treatments all required multiple applications of cementation solution. Penetration resistance increases with every application until it reaches a plateau (Chung et al., 2021); this is due to MICP being

most effective at particle-particle contact (Cheng et al., 2013), and once particle contacts are cemented, strength gains are limited. Biocementation creates a film on top of the soil (Naeimi & Chu, 2017; Salifu et al., 2016), and the efficiency decreases with depth from the application (Dhami et al., 2016). However, even a weak crust can significantly reduce fine particle and dust emissions (Naeimi & Chu, 2017). Biocementation with MICP can greatly reduce hydraulic conductivity (Dhami et al., 2016; Liu et al., 2020; Stabnikov et al., 2016; Stabnikov et al., 2011) while maintaining some porosity (Salifu et al., 2016). MICP is resistant to damage from freeze-thaw damage (Cheng et al., 2013). The amount of calcite precipitated was generally positively correlated to shear strength (Sharma et al., 2022; Tian et al., 2018) and brittleness index (Cui et al., 2017). It is exponentially positively correlated to unconfined compressive strength (Cheng et al., 2013; Salifu et al., 2016), stiffness (Cheng et al., 2013) and effective cohesion (Cheng et al., 2013; Cui et al., 2017). Calcite content has a positive linear relationship with friction angle (Cui et al., 2017). Compared to coarse grains with the same calcite content, fine sands have a higher cohesion and lower friction angle (V Stabnikov et al., 2013). Smaller grain soils are considered more efficient at MICP (Cheng et al., 2013; Dhami et al., 2016) and require less cementation solution and calcite precipitation to increase their strength (Dhami et al., 2016; Naeimi & Chu, 2017; Soon et al., 2014; Whiffin et al., 2007). At low saturation, calcite crystals contribute more to cohesion than friction angle (Cheng et al., 2013), as lower saturation restricts the locations of crystals. The degree of saturation is negatively correlated with unconfined compressive strength and stiffness, with past the 80% degree of saturation and higher having little effect (Cheng et al., 2013).

Select Results

Many MICP studies focus on subsurface applications, biogrouting, and structural repairs, and while the chemical reactions are the same application, results are not included in this review. General MICP and ground improvement reviews are available by Dhami et al. (2013) and Naveed et al. (2020). Regarding flow rate, in the Dhami et al. (2016) study, their 0.2 mm grain columns decreased from a 10.2 ml/min flow rate to 5.8 ml/min on day four and down to 3.4ml/min on day 10. Meanwhile, their 0.5 mm grain

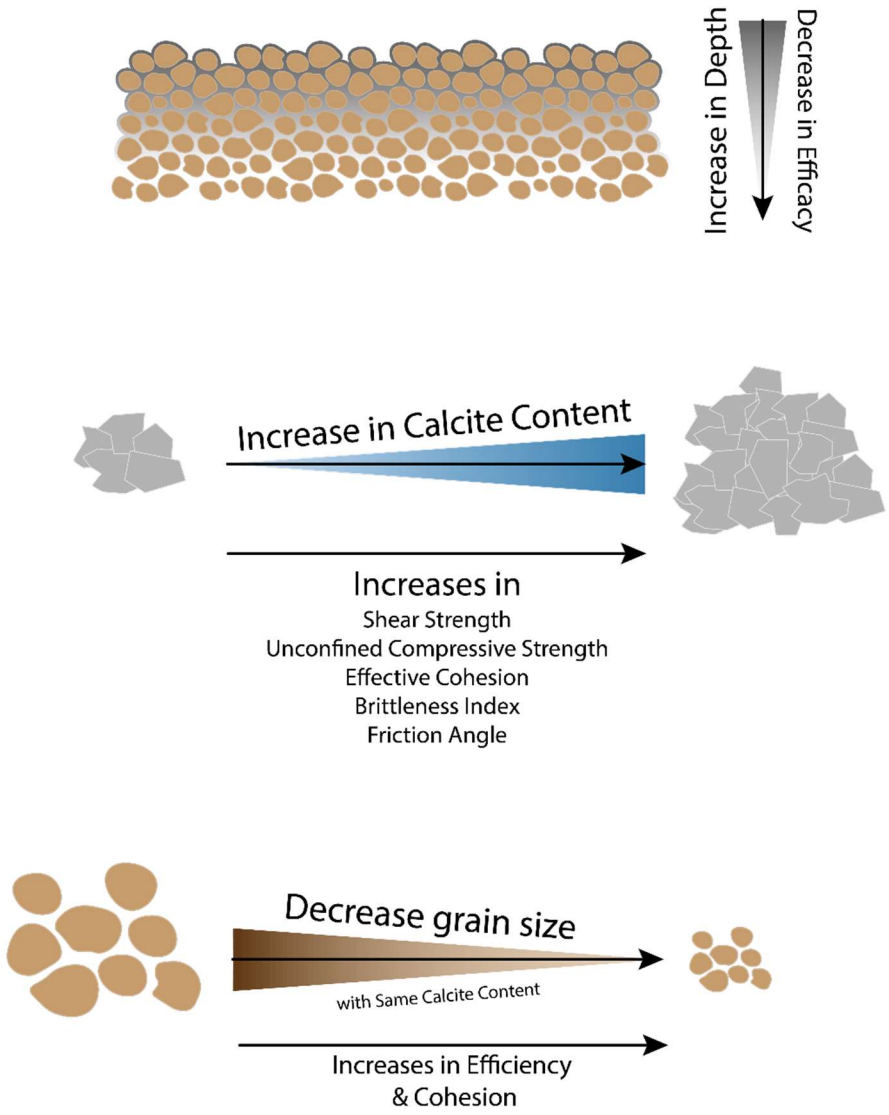

Figure 1.1 Generalized MICP Trends

columns decreased from an initial starting rate of 12.2 ml/min to 5.6 ml/min on day four and then to 1.4 ml/min on day 10. Larger-grained columns had a slower reduction in the beginning but picked up as they began following the trends of the smaller-grain columns. Sharma et al. (2022) saw a 95% reduction in flow rate after 18 days of MICP cycles. Gao et al. (2019) saw seepage rates reduced 379 times at their best conditions (spray and bulk stabilization) down to eight times reduction for spray applications alone. It is interesting to note that while seepage showed massive differences based on the application method, other characteristics (strength, calcite conversion, crack resistance, etc.) showed less drastic but significant differences from application methods. Penetration resistance was increased from 6N to a range of 35N for spray application alone to 55N for spray and bulk application (Gao et al., 2019). In general, strength tests are hard to generalize for MICP as one of the main advantages of the technique is being able to adjust the number and concentration of applications to get the desired result (Sharma et al., 2022). The sand crust was brought to ‘rock-like' strength and strain behavior, where it reacted to triaxial stress tests in a similar fashion to limestone (Sharma et al., 2022; Stabnikov et al., 2011).

Calcite Locations and Crystals

Calcite deposits on grains, in bridges connecting grains, and in pore space. Cui et al. (Cui et al., 2017) differentiate between what they call 'effective calcite crystals', calcite directly related to the bonding of two particles, and 'non-effective calcite crystals’, those only attached to one particle or other calcite crystals. Calcite initially deposits on grain surfaces where bacterial cells have attached; bacteria preferentially attach to the surface of soil particles (Gao et al., 2019). Cell walls are negatively charged, creating an attraction for the positively charged calcium ions (Whitaker et al., 2018), allowing cells to provide nucleation sites for crystal formation. The grain connection depends on the amount of calcite deposited, with lower amounts not creating enough bridging between crystals to be strength effective. Therefore, bridging calcite crystals are preferred (Chung et al., 2021). Effective crystals create biocementation, while non-effective crystals provide bioclogging. Both biocementation and bioclogging aid in the strength benefits of bioaggregation. When initially produced, calcium carbonate starts in a highly

soluble but unstable form; it then passes through the transitional polymorph vaterite to become the thermodynamically stable calcite (Riveros & Sadrekarimi, 2020). Calcite can take many forms, and it is unclear what leads to polymorph selection in MICP (Whitaker et al., 2018) or what effect the selection has on strength. Rhombohedral crystals were commonly found in MICP studies (Cheng et al., 2013; Chung et al., 2021; Sharma et al., 2022); other forms included rosette (Whitaker et al., 2018) and acicular polymorphs (Liu et al., 2020). Whitaker et al. (2018) and Liu et al. (2020) are of interest here as the same treatment saw two types of calcite crystals. In desiccation crack repair. Liu et al. (2020) found acicular-shaped calcite at points of particle contact. However, the surface cracks themselves were dominated by rounded calcite crystals multiple times the size of those seen at particle connections. Whitaker et al. (2018) theorizes that fast nucleation and crystallization lead to the amorphously shaped calcite deposits they found, as compared to the rosette formations they saw.

Concerns and Limitations

The most apparent concern comes from the urease hydrolysis reaction, which forms ammonia. Unless managed, ammonia remains in the environment and is a highly toxic compound. Many of MICP's limitations are based on consistency and geotechnical conditions at the site. The uniform distribution of the application solutions and the precipitated calcite is essential but difficult to achieve (Cui et al., 2017; Liu et al., 2020; Salifu et al., 2016; Sharma et al., 2022). When not uniformly distributed, calcite can cause local differences in shear resistance (Cui et al., 2017). Unfortunately, real-world soil for in-situ application is rarely uniform, creating differences in solution penetration and pathways. Treatment solution will seek the path of least resistance when percolating through the soil (Liu et al., 2020). MICP's effectiveness is directly related to the composition of soils, making some locations inhospitable for biocementation through MICP. Organic Matter content in the soil can inhibit calcite precipitation by adsorbing the needed calcium ions (Rowley et al., 2018) and can block nucleation sites, inhibiting growth (Hoch et al., 2000). Relative density, pore size, and grain size all have effects on the ability of MICP to proceed, as bacteria must be able to pass through. For example, DeJong et al. (2010) noted that the small pore throat spaces in clay restrict the ability of

bacteria to move freely. A 20% change in relative density can cause a 50% change in the amount of calcite precipitation in the soil. Limitations can be seen in study recommendations as V Stabnikov et al. (2013) recommend in their dust suppression application to be applied on a 'not hot,' 'not sunny' and humid day, while Naeimi and Chu (Naeimi & Chu, 2017) recommend theirs be used for “sandy remote disturbed areas." Spray application alone cannot penetrate far and evenly enough to create thick layers of crust, and MICP remains restricted to the top few millimeters of soil (Dhami et al., 2016). Surface spray applications are often prone to cracking (Gao et al., 2019), especially when the thickness of the bio-sealing layer is insufficient (Stabnikov et al., 2016). Cracking for bioaggregation samples is less than the cracking by water addition alone (V Stabnikov et al., 2013). The stability of soil biocemented by MICP is also subject to environmental conditions.

Iron Biocement

Iron biocement, also referred to as Microbially Induced Ferric Hydroxide Precipitation by Stabnikov et al. (2016), utilizes the same process and bacteria as MICP but with a calcium source replaced with ferrous sulfate (Naeimi et al., 2016). Urease producing bacteria precipitate iron salt causing bioclogging and cementation. The equation is as follows (Stabnikov et al., 2016):

$$Fe^{2+} + 0.25O_2 + 4H^+ + 3(NH_2)_2CO + 5.5H_2O \xrightarrow{urease} Fe(OH)_3 \downarrow + 6NH_4{}^+ + 3CO_2$$

Ferric Hydroxide precipitation is not recommended for soil strengthening (Naeimi et al., 2016) since it does not cause a significant increase in the unconfined compressive strength of the soil (Stabnikov et al., 2016). Recommended uses include mine waste stabilization, mine rehab, repairing fractured rock, and pond sealing (Gagen et al., 2020; Stabnikov et al., 2016).

Microbial Extracellular Polymeric Substances and Biological Soil Crusts

Extracellular polymeric substances (EPS) are also referred to as extracellular polysaccharides (Costa et al., 2018), exopolysaccharides (Godinho & Bhosle, 2009),

exopolymers, and extracellular polymer secretions (Zheng et al., 2011). They are a secondary metabolite that aids in survival (Godinho & Bhosle, 2009) and are composed of polysaccharides, proteins, humic matter, and nucleic acids (Ding et al., 2015). EPS is probably most notorious for its role as a significant component in biofilm, which provides cell protection, adhesion and water retention (Chen et al., 2020; Godinho & Bhosle, 2009; Wu et al., 2014; Wu et al., 2013).

While EPS has multiple bioaggregation applications, much of the current surface bioaggregation research is focused on applications with Biological Soil Crusts, BSC, creation, and restoration (Delgado-Baquerizo et al., 2013; Schulz et al., 2016; Wang et al., 2009; Wu et al., 2013). BSCs can be composed of cyanobacteria, lichen, and moss crusts (Belnap, 2002) and include the uppermost portion of soil (Delgado-Baquerizo et al., 2013; Fattahi et al., 2020). These crusts are crucial in desertification prevention, erosion control, soil fertility, and nutrient cycling (Delgado-Baquerizo et al., 2013; Wu et al., 2013). While BSC can form and repair themselves naturally, it is estimated that it would take tens to thousands of years for a full recovery without human intervention (Belnap, 1993; Belnap & Eldridge, 2001). In addition, BSCs play an essential role in nitrogen cycling (Delgado-Baquerizo et al., 2013). One limiting factor for recovery growth in these arid regions is low levels of nitrogen (Madan et al., 2007; Zhao et al., 2010), which opens the door for augmentation with nitrogen-fixing bacteria.

Bacteria Selection and Monitoring

Cyanobacteria are a common component of crusts and are believed to be one of the first types of bacteria to appear in disturbed soils (Booth, 1941). Also referred to as blue-green algae, cyanobacteria are photosynthetic and known to be capable of producing large amounts of EPS (Wu et al., 2013). Cyanobacteria studied for inoculation in bioaggregation include *Mircocoleus vaginatus (Fattahi et al., 2020; Hu et al., 2003; Wang et al., 2009; Wu et al., 2013; Zheng et al., 2011), Scytonema javanicum (Hu et al., 2003; Wang et al., 2009; Wu et al., 2013)*, *Paenibacillus mucilaginosus* (Wu et al., 2014) and *Nostac punctiforme (Fattahi et al., 2020).* The application of extracted EPS from bacterial cultures (Godinho & Bhosle, 2009) and model biopolymers

(Ayeldeen et al., 2017; Chang et al., 2017; Chen et al., 2020) were also studied as a possible bioaggregation technique.

BSC quality can be measured through its chlorophyll a content, thickness, and compressive strength (Zheng et al., 2011). The pigments chlorophyll a, carotenoid, and scytonemin, produced by cyanobacteria, can be used to monitor crust formation with chlorophyll a concentration able to estimate algal biomass in the sampled aggregation (Wu et al., 2013). SEM imaging is used to view interparticle bonding and EPS structure (Fattahi et al., 2020).

EPS Results

Inoculation with cyanobacteria cultures is an effective restoration technique for BSC and can help promote bacterial biodiversity and vegetation with time. Formation of the crust is found to increase moisture, available nutrients, compressive strength, and shear strength (Chen et al., 2020; Wang et al., 2009; Wu et al., 2014; Wu et al., 2013). As the concentration of EPS increases, so does sand stabilization and soil cohesion (Chen et al., 2020; Godinho & Bhosle, 2009; Wu et al., 2014). Chlorophyll a is correlated with crust depth and time since inoculation (Wang et al., 2009; Wu et al., 2013).

Wang et al. (2009) saw 100% crust cover at 18 days post application which declined to 41.5 % at one year, 45.5% at two years, and 48.5% at three years; although the percentage of cover decreased, it should be noted that the crust thickness more than doubled with time. After 45 days of inoculation, Godinho and Bhosle (2009) found 1.5 to 3 cm long aggregates. At one-year post inoculation Wu et al. (2014) found their crust to be 3mm thick. Wu et al. (2013) found theirs to be 5.45 mm thick at year 7, with a chlorophyll a concentration of 24.38 ug/cm2, double the available phosphorous compared to year two and four times the available nitrogen.

For wind erosion control, Fattahi et al. (2020) tested *Nostac punctiforme* and *Microcoleus vaginatus* bioaggregated samples of sand at wind speeds of 6, 15, and 25 m/s and they showed no visible changes in the surface. *N. puntiforme* and *M. vaginatus* had erosion rates of 0.026% and 0.007% respectively, of untreated sand erosion rates. Crust formation and general surface bioaggregation studies vary widely with the desired

outcomes, environment, and many study variables; this makes the comparison of results, at best, confusing and, at worst, seemingly contradictory. Wang et al. (2009) mentions that their total carbon and total nitrogen measurements are inconsistent with other studies. Chen et al. (2020) used xanthan gum as a model for EPS in sand and found cohesion increased by a factor of 5.8 to 18.4 kPa for a concentration of 0.1% and by 93.3 to 298.4 kPa for a concentration 0.5% which does not match to other studies like Lee et al. (2017) who found no strengthening at 0.5%.

Table 1.1. Summary of Current Surface Bioaggregation Techniques

Surface Bioaggregation Technology	**Bacteria**	**Application Methods**	**Health and Safety Risk**	**Recommended Uses**	**Soil Types and Limitations**
Microbially Induced Calcite Precipitation	UPB	Spray or Injection	Ammonia byproduct	Soil Strengthening Pollutant Immobilization Aggregation and Clogging Permeability Control	Uniform Distribution is Difficult Stability is significantly affected by environmental factors
Iron Biocement	UPB	Spray or Injection	Ammonia byproduct	Stabilize mine waste Rehab mines Sealing ponds	
Biological Soil Crusts	Cyanobacteria	Spray		Fix mobile Sand Control desertification Restore fertility/ecosystems Wind Control	Best in well-graded sand or soil with low organic matter Not good in agricultural soil or mine reject

Chapter 2

Cell Growth and Extracellular Polymeric Substances Estimation Curves

Abstract

Extracellular polymeric substances (EPS) are created by a variety of bacteria and are used in many industries. Methanotrophic bacteria, bacteria that can metabolize methane as their carbon source, are known EPS producers while being able to utilize methane, a potent greenhouse gas, and cometabolize a variety of contaminants. The goal of this study is to find when, in their growth curves, two methanotrophic bacteria, *Methylocystis sp.* and *Methylosinus sporium,* have their maximum EPS production rates to determine the optimal time for EPS extraction or culture application. Growth curves were measured simultaneously with EPS quantities, which were determined from the phenol sulfuric acid extraction method. Both cultures saw the highest rate of EPS production during their exponential growth phases, while there were oscillations in production rates throughout all growth phases. These outcomes indicate that for maximum EPS amounts, EPS extraction or culture application should occur after the end of the exponential phase.

Introduction

Methanotrophic bacteria, a subset of methylotrophs, can be found in a myriad of environments and have the unique ability to utilize methane as their sole carbon source. Methanotrophs are well-known producers of extracellular polymeric substances (EPS) (Malashenko et al., 2001). EPS is a secondary metabolite and is a method of protection from unfavorable conditions (Bussmann et al., 2006; Cantera et al., 2018; Chiemchaisri et al., 2001; Linton et al., 1986), often mentioned in reference to biofilm production, where they make up a major component (Wingender et al., 1999). EPS production in methanotrophs was found to be correlated with methane oxidation rates (Chiemchaisri et al., 2001); however, accumulation of EPS can later cause a decline in methane oxidation rates as gas uptake becomes harder (Chiemchaisri et al., 2001; Strong et al., 2015).

EPS is utilized in various industries and biotechnologies, and methane is a potent greenhouse gas, opening the potential for methanotrophic bacteria to produce EPS for industry while simultaneously utilizing and lowering methane emissions. This study's purpose is to identify where in their growth curves do two methanotrophic bacteria

cultures produce the highest amounts of EPS. The first null hypothesis maintains that there is no difference in EPS production rates during differing parts of the culture's growth curve, while the alternate hypothesis says that there are higher rates of EPS production during some parts of the growth curve. The secondary hypothesis states that there is no difference in EPS production among different bacterial strains, and the alternate hypothesis claims that some bacterial strains produce more or less EPS than others. EPS production rates are determined by estimating EPS content over time through carbohydrate content, as EPS is mainly composed of polysaccharides with proteins and minimal amounts of other components (Van De Lageweg et al., 2018). Growth curves were measured through optical density at the same time scale as EPS content.

Materials and Methods

Bacterial Strains and Growth Conditions

Two bacterial strains were chosen for analysis: *Methylocystis sp.* (ATCC 49242) and *Methylosinus sporium* (DSM 17706). Bacteria were grown on Nitrate Mineral Salts media (Whittenbury et al., 1970) at 30C, 100rpm, and 20% methane in the headspace exchanged every 24 hours. At time 0, a 10% inoculum (100mL) was introduced.

Growth Curves and EPS Estimation Curves

For growth curves, one mL of culture was removed at every time point for optical density (OD) measurements at 600 nm (Zhang et al., 2015). For EPS estimation curves, 0.2 mL of culture was removed at every time point and used in the phenol sulfuric-acid method (DuBois et al., 1956), where 200 µL of culture was combined with 200 µL of phenol and 1 mL of sulfuric acid and measured at OD 490nm after allowing for reaction time and mixing. A standard curve for calibration was made with glucose concentrations from 50 µg/mL down to zero. The calibration standard curve was used to convert optical density at 490nm to the concentration of carbohydrates, which was assumed to be a good estimate for EPS concentration. Error bars for EPS estimates were calculated using the standard error of the mean of the calibration standard curve.

Results

Calibration Graph

Fourteen Standards were measured against a blank of milliQ water and plotted (Fig 2.1); the calibration equation was found to be:

$$Glucose\ Concentration\ (\mu g\ /mL)\ = 100.17(\text{Optical Density at } 490nm)$$

Error bars were calculated to be 5.11 µg/mL based on a standard deviation of 19.12 and a sample size of 14.

Growth and EPS Estimation Curves

The growth rate, µ, was calculated based on the optical densities during the exponential phase using the following equation:

$$\mu = \frac{(lnOD_2 - lnOD_1)}{(t_2 - t_1)}$$

The exponential growth rates for *Methylosinus sporium* and *Methylocystis sp.* are 0.047 ± 0.021 h^{-1} and 0.073 ± 0.016 hr^{-1}, respectively. *Methylosinus sporium*, shown in Figure 2.2, reached the stationary phase sooner and had a shorter transitional phase than *Methylocytis sp.,* shown in Figure 2.3, whose exponential phase was shorter. The EPS production rate for Methylosinus sporium maxed out at 1.07 µg/mL*h-1 during the stationary phase of the growth curve, which was slightly higher than the 0.98 µg/mL*h-1 seen during the exponential phase. *Methylocytis sp.* had a maximum EPS production rate of 1.56 µg/mL*h^{-1} during the exponential phase. While *Methylocytis sp.* had a higher maximum rate of EPS production, it had lower overall EPS concentrations during the experimental time period.

Discussion

EPS production is dependent on the methanotroph's growth conditions, and this experiment shows production under 30°C, 100 rpm, and 20% methane headspace. There was no predictable trend to EPS concentration, as both cultures saw oscillations in EPS production rates, other than to say both saw a larger-than-average increase during the exponential growth phase. The first hypothesis can be rejected as different EPS production rates were seen at different points along the growth curves. The second hypothesis can also be rejected as *Methylosinus* sporium produced a larger amount of

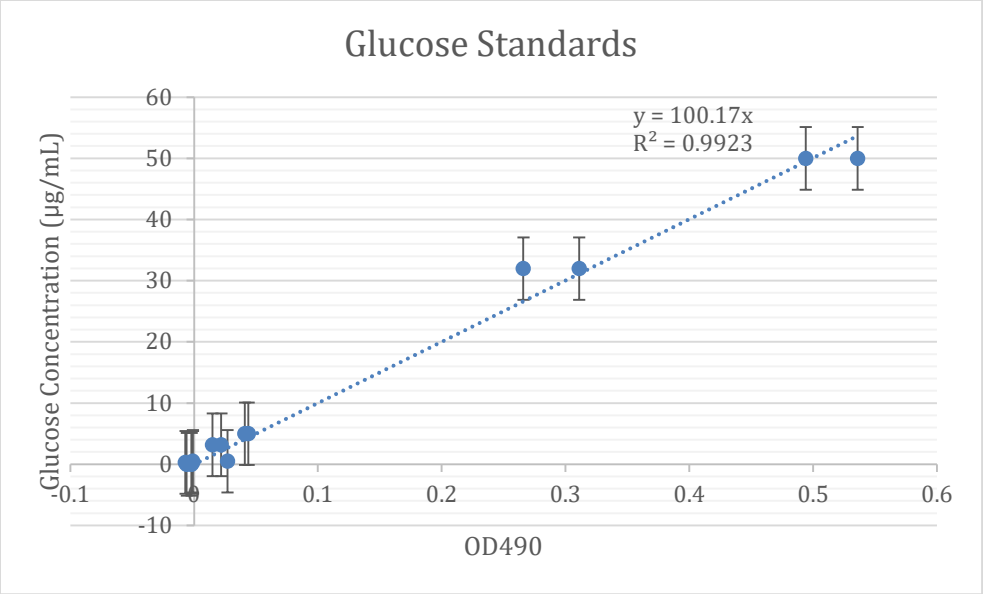

Figure 2.1. Calibration graph based on glucose standards

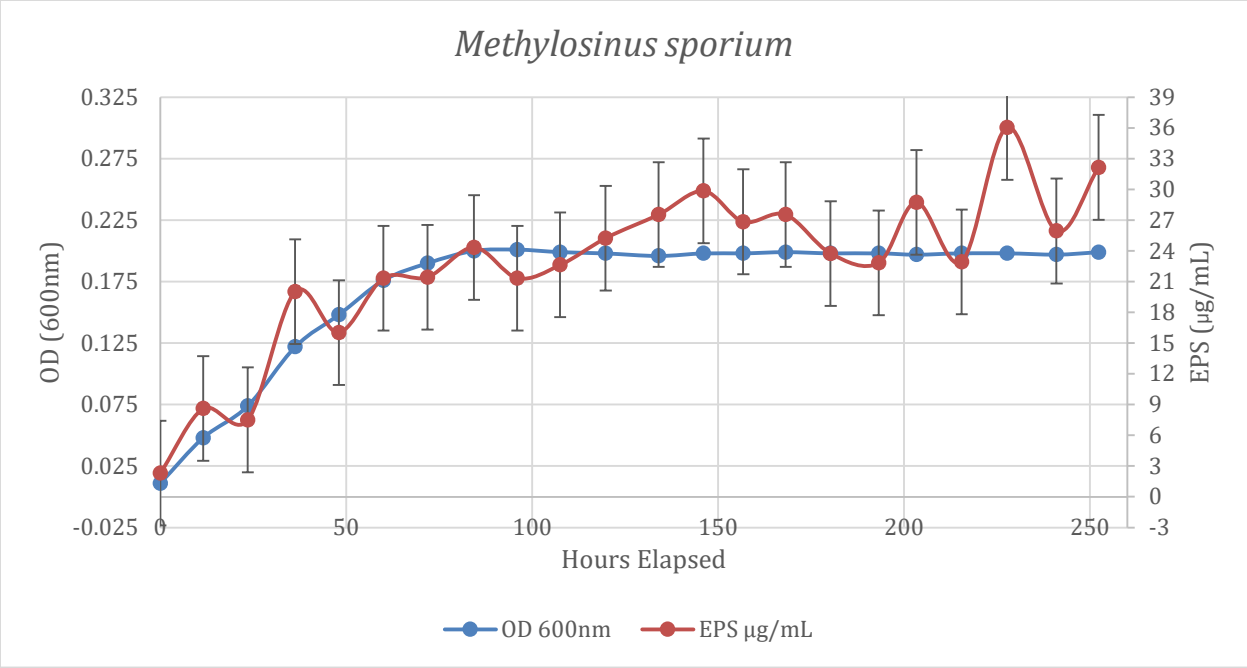

Figure 2.2. *Methylosinus sporium* growth and EPS production curves

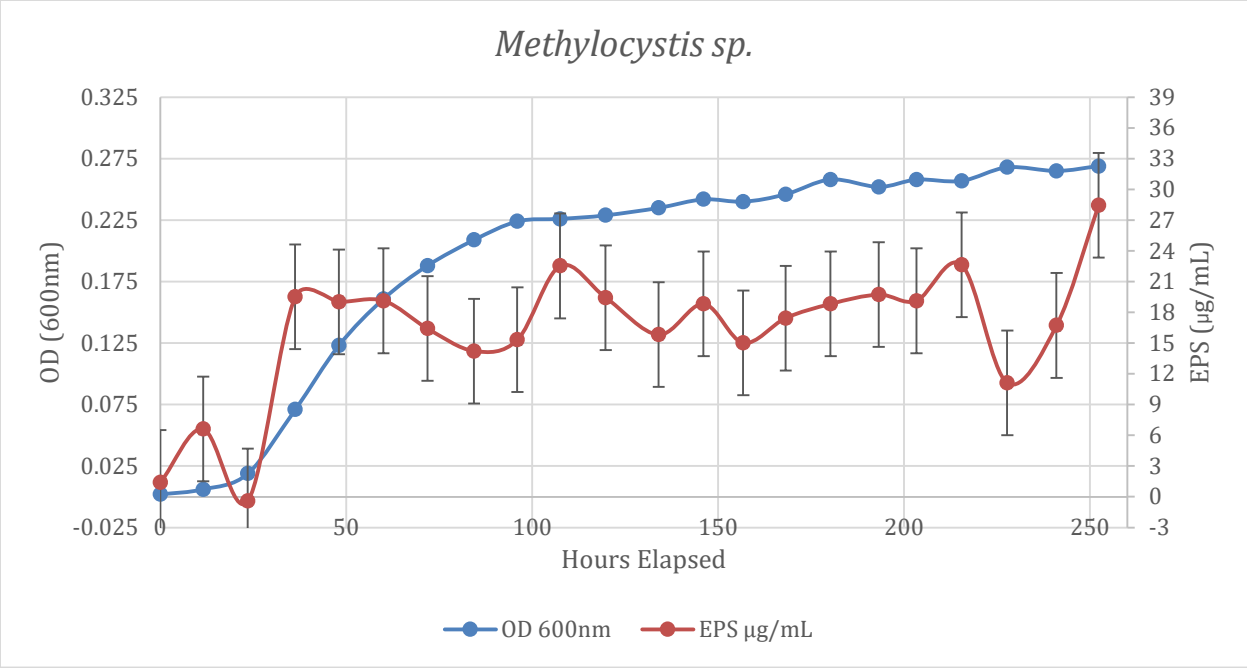

Figure 2.3. *Methylocystis sp.* growth and EPS production curves

EPS than *Methylocystis sp.* in the same time frame. Different times for application or extraction can be utilized depending on the intended use of the bacteria and EPS. EPS extraction for either of these bacterial strains can occur at any point during the stationary phase, while bacterial application might want to be applied during the lag phase so EPS can be produced in situ. Methanotrophs offer a unique ability to utilize methane, cometabolize contaminants, and produce EPS, creating interesting opportunities for methane mitigation or bioremediation while still creating EPS. Methanotrophic EPS production rates are lower than those of many other bacteria; however, methanotrophs have distinct advantages, especially in places like oil-producing deserts where methanotrophs and methane are abundant. The microbial cultures also saw EPS production increases during the stationary phase, where no increases in turbidity were seen; this is likely a response to stressors.

Conclusions

This study was designed to find EPS production rates throughout *Methylocystis sp.* and *Methylosinus* sporium's growth, at 30°C and 100 rpm shaking. While both cultures saw maximum rates during exponential growth, this doesn't indicate that this will be the case for other methanotrophs. Future studies are needed to elucidate which stressors and growth conditions maximize EPS production.

Chapter 3

Methanotroph Mediated Sand Aggregation

Abstract

Surface aggregation techniques are valuable for dust suppression and erosion control, among other things; one well-studied sand bioaggregation method utilizes extracellular polymeric substances (EPS), mainly from cyanobacteria, although they are not the only bacteria to produce EPS. These studies aim to evaluate the sand bioaggregation capabilities of methanotrophic bacteria, bacteria that can oxidize methane as its carbon source, and their EPS. Bacterial cultures were applied to silica sand either at their stationary phase – first study – or the beginning of their exponential phase – second study. Aggregation was quantified by dry sieve analysis and visualized with scanning electron microscopy (SEM) imaging. At 28 days post-application, the first study had visible crusting, and the fraction of sand larger than 0.425 mm after sieving increased from 1.04% in the control sand to up to 7.6% for the bacterially aggregated sand. At 15 days post application, the second study had visible but fragile clumping, and the fraction of sand larger than 0.25 mm after sieving went up from 14.7% in the control sand to up to 33.9% in the bacterially aggregated sand. SEM imaging saw clearer connections between sand grains in the first study, while the second study showed less. The results show that these four strains of methanotrophic bacteria are capable of some levels of sand aggregation and are a good starting point for future studies.

Introduction

Bacterial extracellular polymeric substances (EPS) are a secondary metabolite consisting primarily of polysaccharides and proteins. Traditionally, EPS is also referred to as extracellular polysaccharides, exopolysaccharides, and exopolymers; it's most notably known for biofilm or slime production. While not their only use, EPS play a role in cell cohesion and surface adhesion (Godinho & Bhosle, 2009; Wolfaardt et al., 1999). EPS adsorbs and provides connections between mineral surfaces while increasing the soil's ability to retain moisture (Henao & Mazeau, 2009; Roberson & Firestone, 1992). They are widely studied for their bioaggregation and stabilization capabilities in both sand and soil (Costa et al., 2018; Godinho & Bhosle, 2009; Hu et al., 2003; Van De Lageweg et al., 2018; Wu et al., 2014). EPS plays a large role in biological soil crusts

(BSC) and desert soil reclamation. The bacteria studied are often cyanobacteria; however, many different types of bacteria produce EPS.

Methanotrophic bacteria, bacteria that can utilize methane as their sole carbon source, are commonly researched for their EPS production in waste and landfill soils (Chiemchaisri et al., 2001; Wei et al., 2015). Methanotrophs are ubiquitous EPS producers, with Type I methanotrophs producing EPS more readily than type II (Malashenko et al., 2001).Type I methanotrophs are gamma-proteobacteria that use the ribulose monophosphate pathway to take in carbon, while Type II methanotrophs are alpha-proteobacteria that use the serine pathway (Strong et al., 2015; Vasudevan et al., 2023). Methanotrophs utilize methane monooxygenase (MMO) enzymes to catalyze the oxidation of methane; these enzymes have a broad substrate range, which also allows them to co-metabolize heavy metals and organic pollutants (Pandey et al., 2014). This opens the possibility for bioremediation coupled with bioaggregation or a methane sink source with bioaggregation.

The idea of utilizing EPS is not new to the world of bioremediation, as biomass plugging is used in permeability reduction, bioremediation, and enhanced oil recovery, among other things. EPS can bind to many types of metal species (Gadd, 2010).

The goal of these bench-scale studies was to see if methanotrophic strains of bacteria could bioaggregate sand. The first null hypothesis states there is no difference in sand aggregation between applications of differing bacterial cultures, while the alternate hypothesis claims there is a difference in aggregation between applications of different bacterial cultures. This is tested by applying three different bacterial cultures to sand and evaluating their aggregation capabilities against untreated sand based on grain size analysis and scanning electron microscope (SEM) imaging. The second null hypothesis states there is no difference in aggregation based on when in their growth curve bacterial cultures are applied to sand, and the alternate hypothesis is that there are differences in aggregation based on when, in their growth curve, bacteria are applied. The second hypothesis is tested by having the study done in two separate phases, one – trial one – with bacteria applied at their stationary phase and one – trial two – with bacteria applied at the beginning of their exponential phase. This is, to our knowledge, the first study of its kind, except for two 1982 USSR Inventor’s Certificates

(no 962594 and 973869) applied for by Gretsinger et al., which related to the dust-binding capabilities of methanotrophic EPS in coal mines.

Materials and Methods

Sand Selection and Preparation

U.S. Silica 100 Mesh - Hydraulic Fracturing Sand was purchased from U.S. Silica for the experiment. The sand was then subsampled, washed, autoclaved, and dried in preparation.

Bacterial Strains and Controls

For the first study, three bacterial strains were chosen: *Methylocystis sp., Methylosarcina quisquiliarum*, and *Methylomonas methanica*. For the second study, *Methylocystis sp.* and *Methylosarcina quisquiliarum were kept, and Methylosinus sporium* was added in place of *Methylomonas methanica.* Bacteria were initially grown in Nitrate Mineral Salts media (Whittenbury et al., 1970), with a methane concentration in the headspace, shaking at 100 rpm at 30℃. Two negative controls were chosen in addition to the bacteria: the first negative control was sand with NMS media added, and the second was sand with nothing added.

Petri Dishes

For plate samples, Fisherbrand 47mm diameter disposable petri dishes were chosen. These were polystyrene and tight-fitting to prevent moisture loss while still allowing gas transfer

Experimental Setup

For the first study, 13 grams of sand and 3.5 mL culture/media were placed in Petri dishes. Cultures were applied during their stationary phase. After set-up, samples and controls for T1-T5 were incubated for 28 days in a glove bag filled with a 2.5% methane in atmosphere gas mixture and stored in a 30℃ hot room. The glove bag was opened, and samples were removed at every time point and then resealed. Headspace was exchanged every three days whether samples were removed for time points. Each time point and condition had 4 Petri dishes that were placed in a 75℃ drying oven, 3 for dry sieve analysis, and 1 for SEM imaging.

For the second study, plates were filled with 5 grams of sand and dry weight was measured; then, the three bacterial conditions and the NMS control were amended with 1.35 mL of culture or media, and wet weight was measured. Cultures were applied at the beginning of their exponential phase. Samples and controls for T1-T3 were placed in a glove bag and treated the same as in the first study. One plate from each condition was freeze-dried for SEM imaging for each time point. The other six were placed in a 75℃ drying oven for 3 days until the samples were dry. Dried samples were then stored at 30℃ until ready for Dry Sieving (four plates), SEM Analysis (one plate), and EPS estimation (one plate).

Dry Sieve Analysis

For the first study, three dishes and for the second study, four dishes from each timepoint and condition were used for dry sieving analysis. Dried sand from each condition at each time point was run through four sieves: US size 10, size 40, and size 60. Sieving was run for five minutes using a shaker.

SEM Analysis

Microscopy access for the second study was provided by the Institute for Advanced Materials & Manufacturing (IAMM) at the University of Tennessee, Knoxville. SEM imagining was performed on a Zeiss EVO SEM.

Freeze Drying

Freeze drying was completed in a Labconco FreeZone Freeze Dryer.

First Study Results

Dry Sieve Analysis

Grain size analysis graphs for the first study are presented in Figure 3.1. The largest differences from control sand were seen in sieve size 40 (0.425 mm); the fraction of sand larger than 0.425 mm was 4.2% to 7.6% for the bacterially aggregated sand, while only 1.04% for the control (untreated) sand. *Methylomonas methanica* had the largest percent retained on sieves 40 and 10. Figure 3.2 shows the mass retained on the sieve size 10 (2 mm opening) by the 28-day time point of *Methylosarcina quisquiliarum*, even though mass measurements show 100 percent finer than the size 10 sieve.

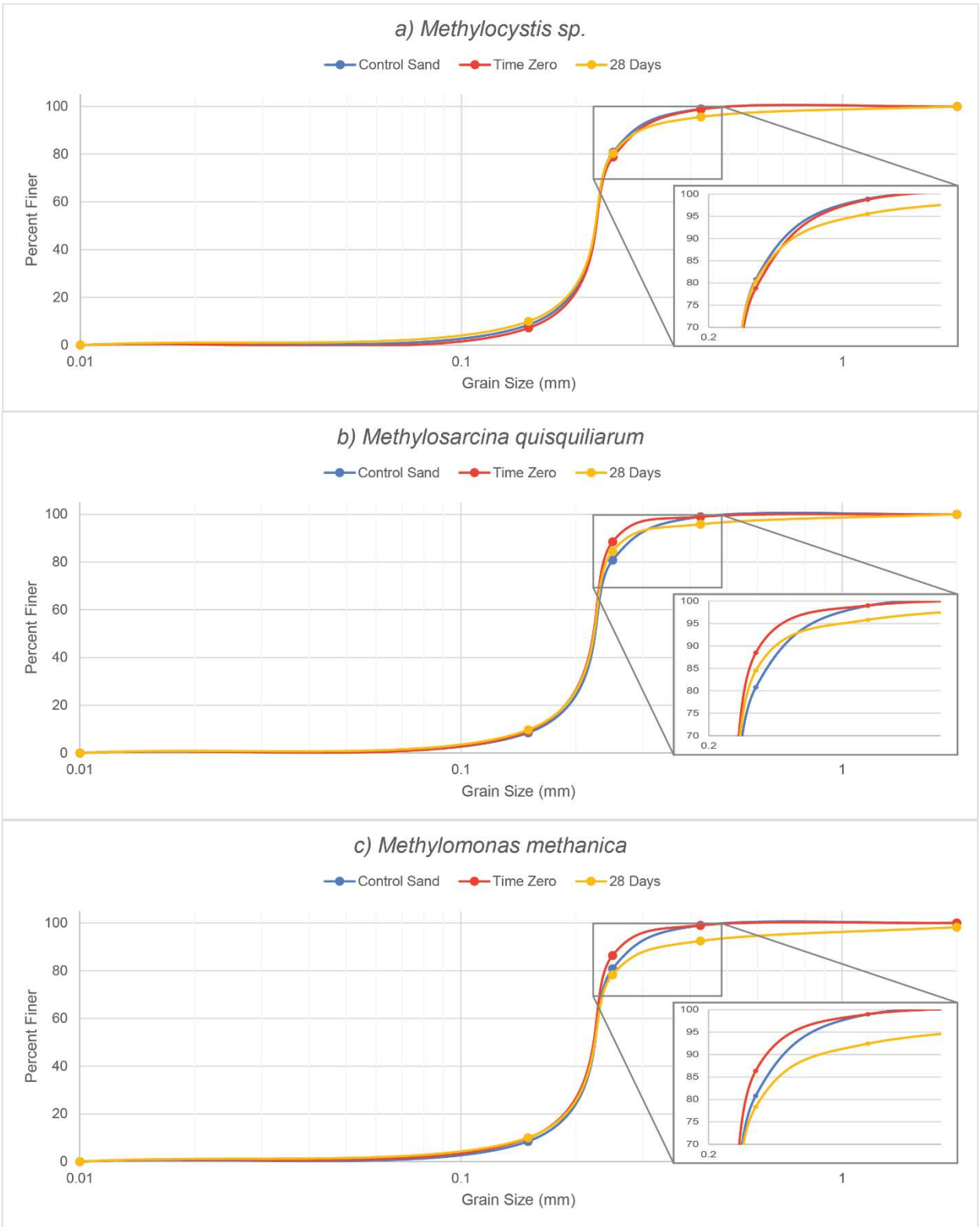

Figure 3.1. First Study Grain Size Analysis a) *Methylocystis sp.* treated sand, b) *Methylosarcina quisquiliarum* treated sand, and c) *Methylomonas methanica* treated sand

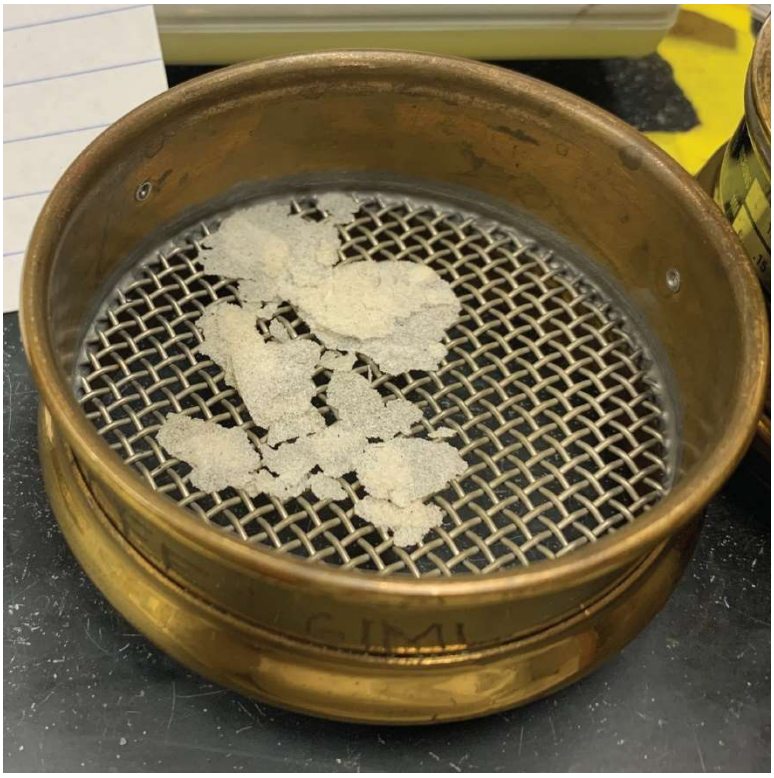

Figure 3.2. Sand Aggregated by *Methylosarcina quisquiliarum* retained on sieve size 10 (2 mm openings) at 28 days

SEM Analysis

All three bacterial treated conditions showed signs of bacterial attachment under SEM imaging. Figure 3.3 shows the untreated sand (control condition) with no bacteria. Figure 3.4 a-d displays *Methylocystis sp.* treated sand, showing the two variations of bacterial and EPS structures created by *Methylocystis sp*. Figures 3.4 a and b show the clusters of disk-like formations and Figures 3.4 c and d show a smoother bond between sand grains. Figure 3.5 a-d displays *Methylosarcina quisquiliarum* treated sand, showing some of the broken-up crust that was present as a film over the sample. At all points, the crust formed by *Methylosarcina quisquiliarum* consisted of sheets of sphere-shaped collections. Figure 3.5 b is a magnified view of the underside of the crust, with its fibrous connections to the sand grains. Although showing the largest aggregation in sieve analysis *Methylomonas methanica*, Figure 3.6 a-d, showed the least presence of bacteria and EPS in SEM imaging. *Methylomonas methanica* had elements of both previous bacterial applications with both clusters, Figure3.6 b, and crusting, Figure 3.6d.

Second Study Results

Dry Sieve Analysis

Grain size analysis graphs for the second study are present in Figure 3.7. *Methylocystis sp.* treated sand shows almost no change from the control sand over the period of the study. *Methylosarcina quisquiliarum* and *Methylosinus sporium* treated sand have distinct differences from the control sand at 15 days, specifically in the percent of mass retained on the #60 sieves. The fraction of sand larger than 0.25mm was 22% to 33.9% for those two cultures as compared to the 14.7% for the control sand. The culture-treated sands showed almost no change from control at time zero and more aggregation at the 15-day mark, which makes sense as the second study had cultures applied at the beginning of their growth as compared to the first study where bacteria was applied at their stationary phase.

SEM Analysis

The second study's SEM images were all taken under variable pressure, high pressure, to help alleviate the sample charging. Although all showed visible signs of aggregation, there were minimal, if any, visible signs of EPS growth and aggregation

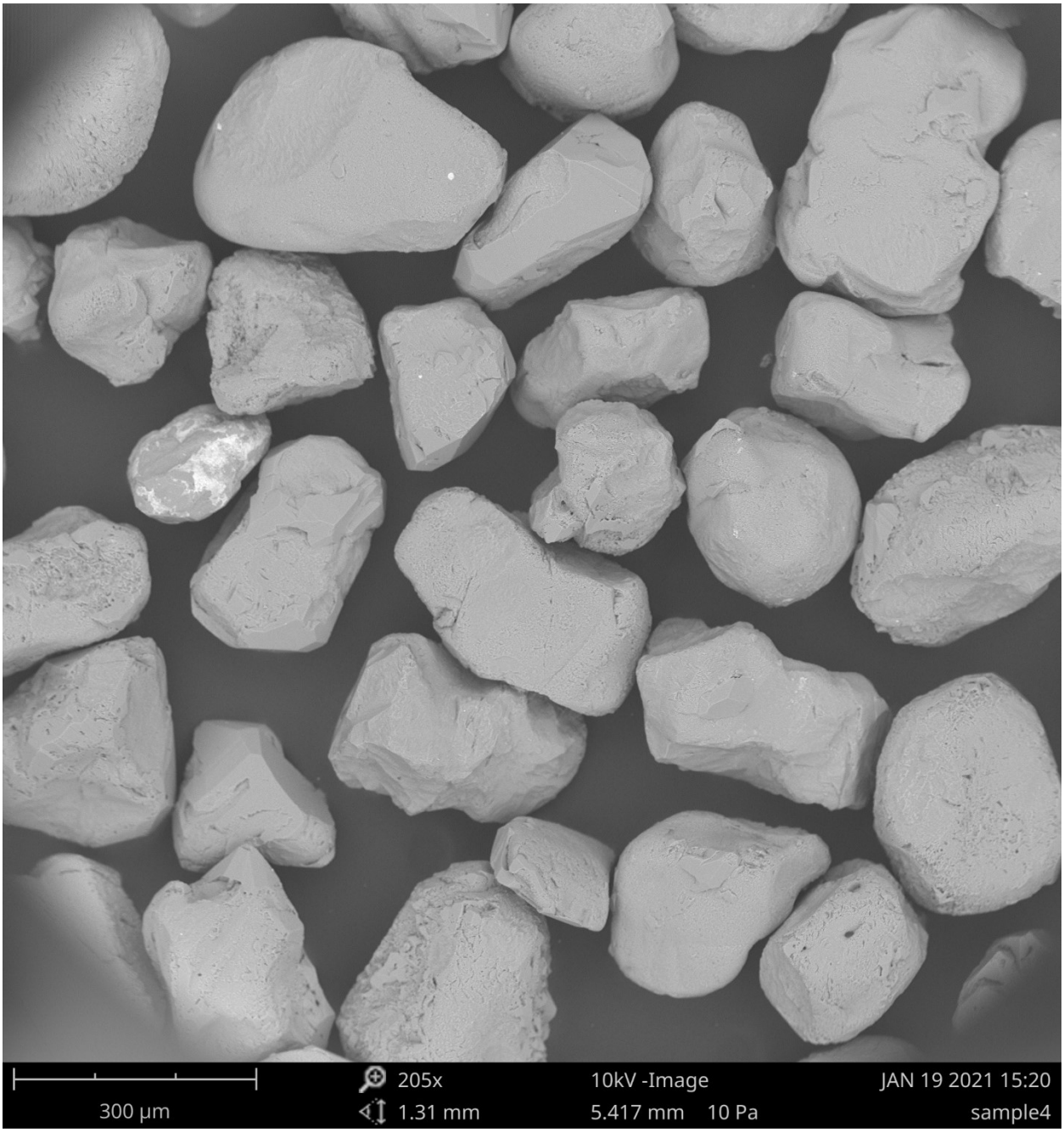

Figure 3.3. Untreated control sand

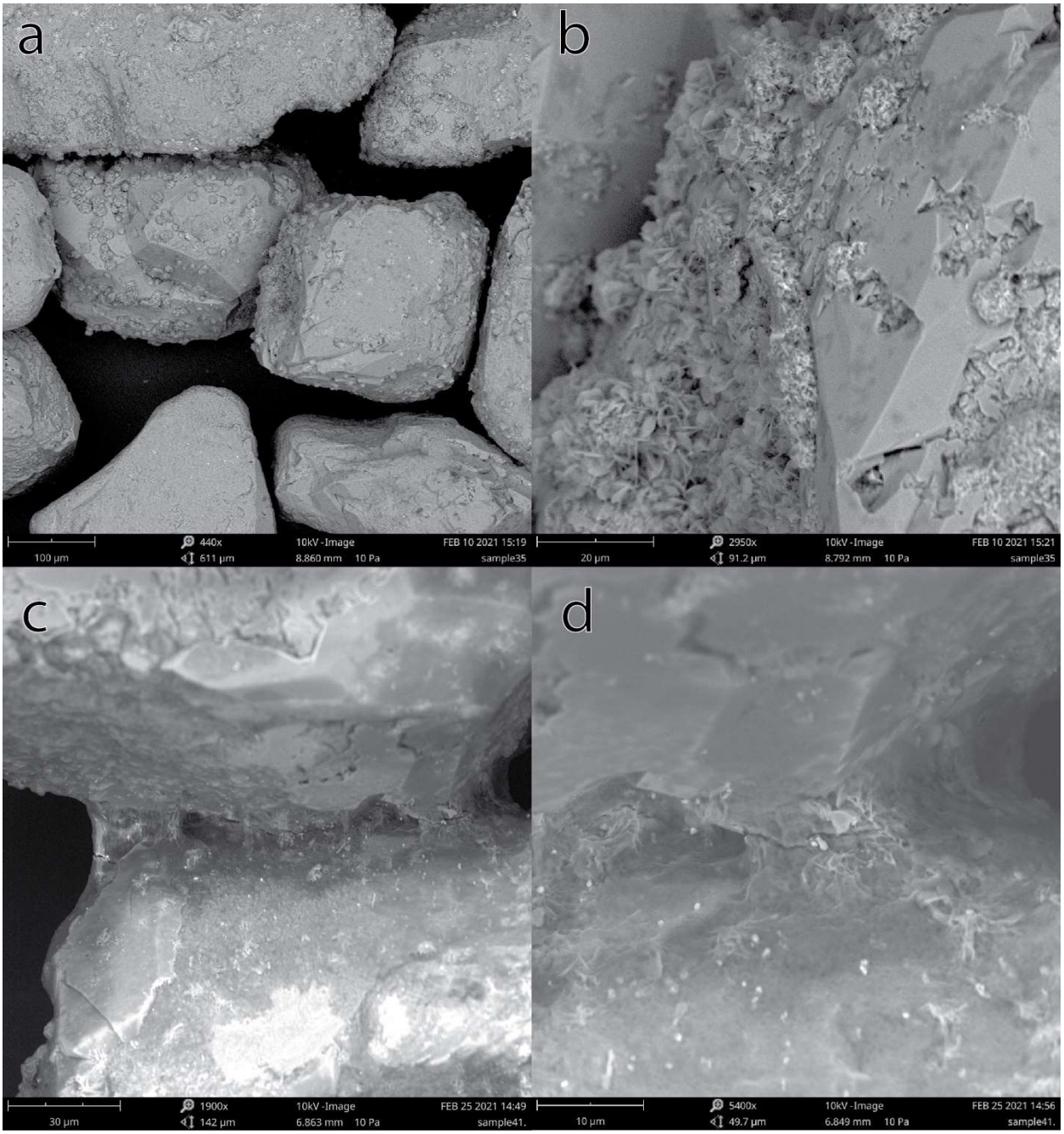

Figure 3.4. SEM images from *Methylocystis sp*. treated sand. a) Overview of sand at time 4, 18 days. b) Magnified view of the cell and EPS clusters on sand's surface at time 4, 18 days. c) Sand aggregate connection between two grains at time 5, 28 days. d) A more magnified view of the connection of Figure3.3c

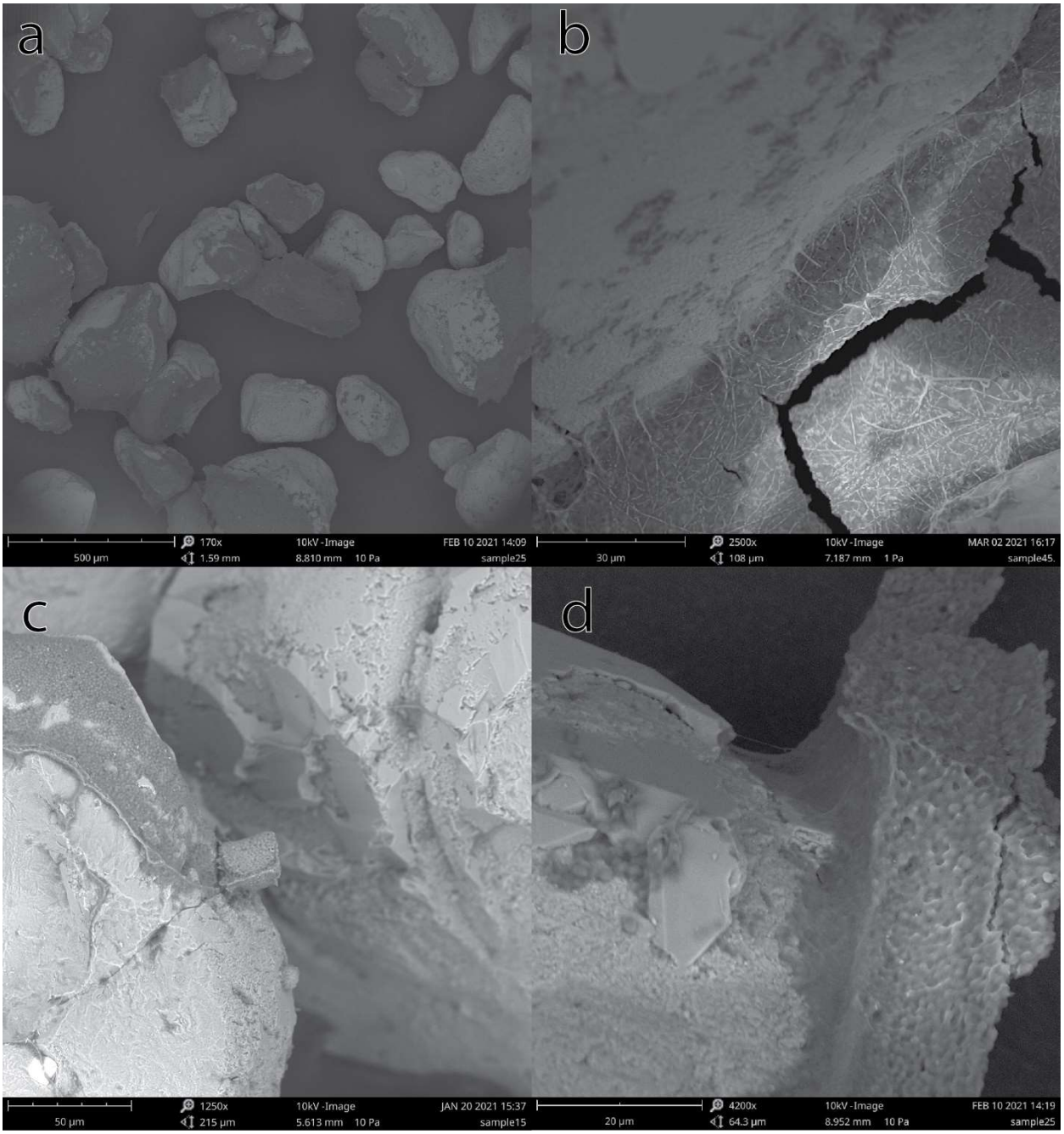

Figure 3.5. SEM images from *Methylosarcina quisquiliarum* treated sand. a) Overview of sand particles at time 3, 12 days. b) Underside of crusting between sand particles at time 5, 28 days. c) beginning of more crust formation at time 2. d) Close-up image of crusting at time 3, 12 days.

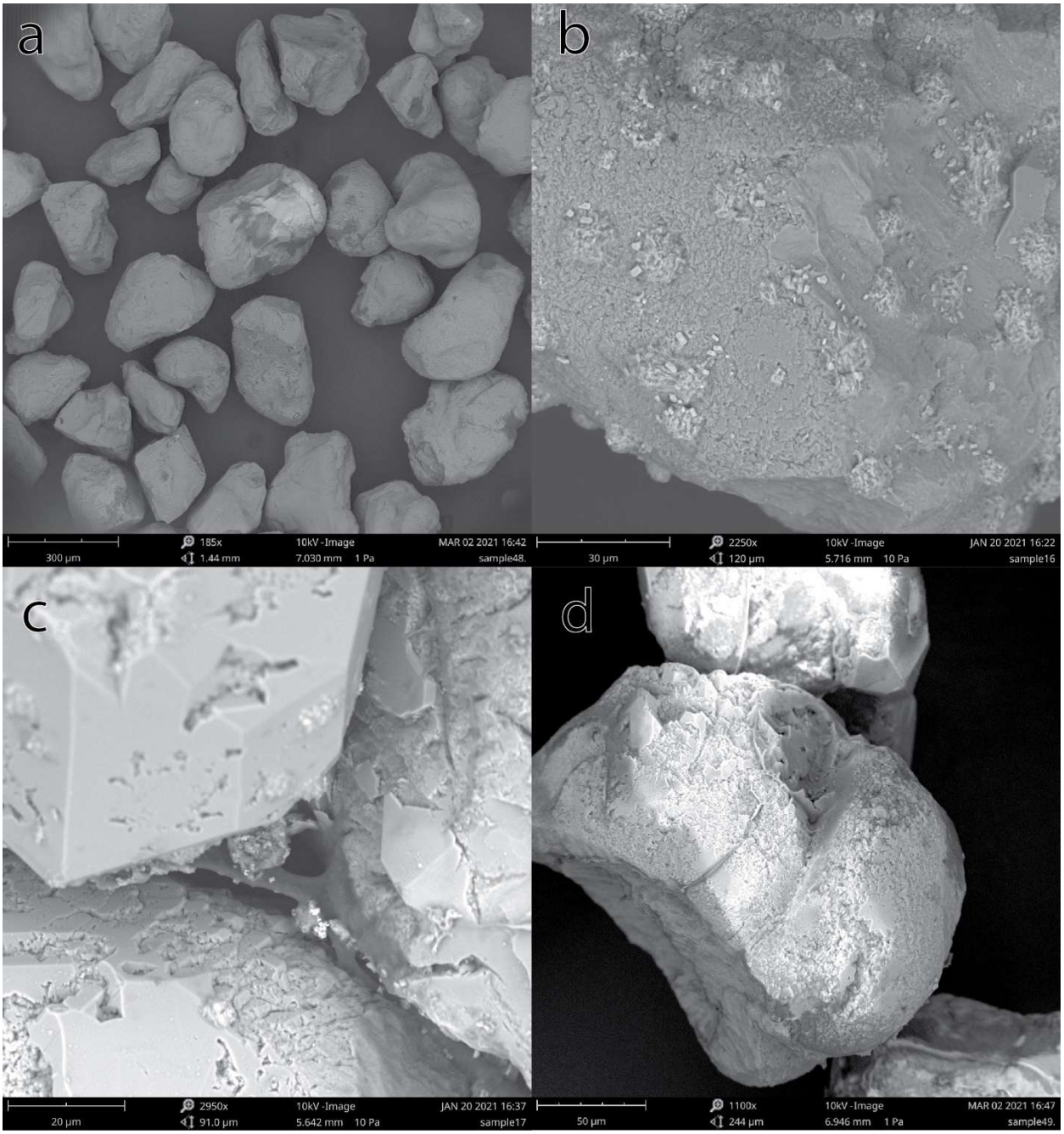

Figure 3.6. SEM images from *Methylomonas methanica* treated sand. a) Overview of sand particles at time 5, 28 days. b) Sand particle surface at time 2 showing bacterial and EPS clumps. c) Sand aggregation connections between particles at time 2. d) Sand particles with broken crusting at time 5, 28 days.

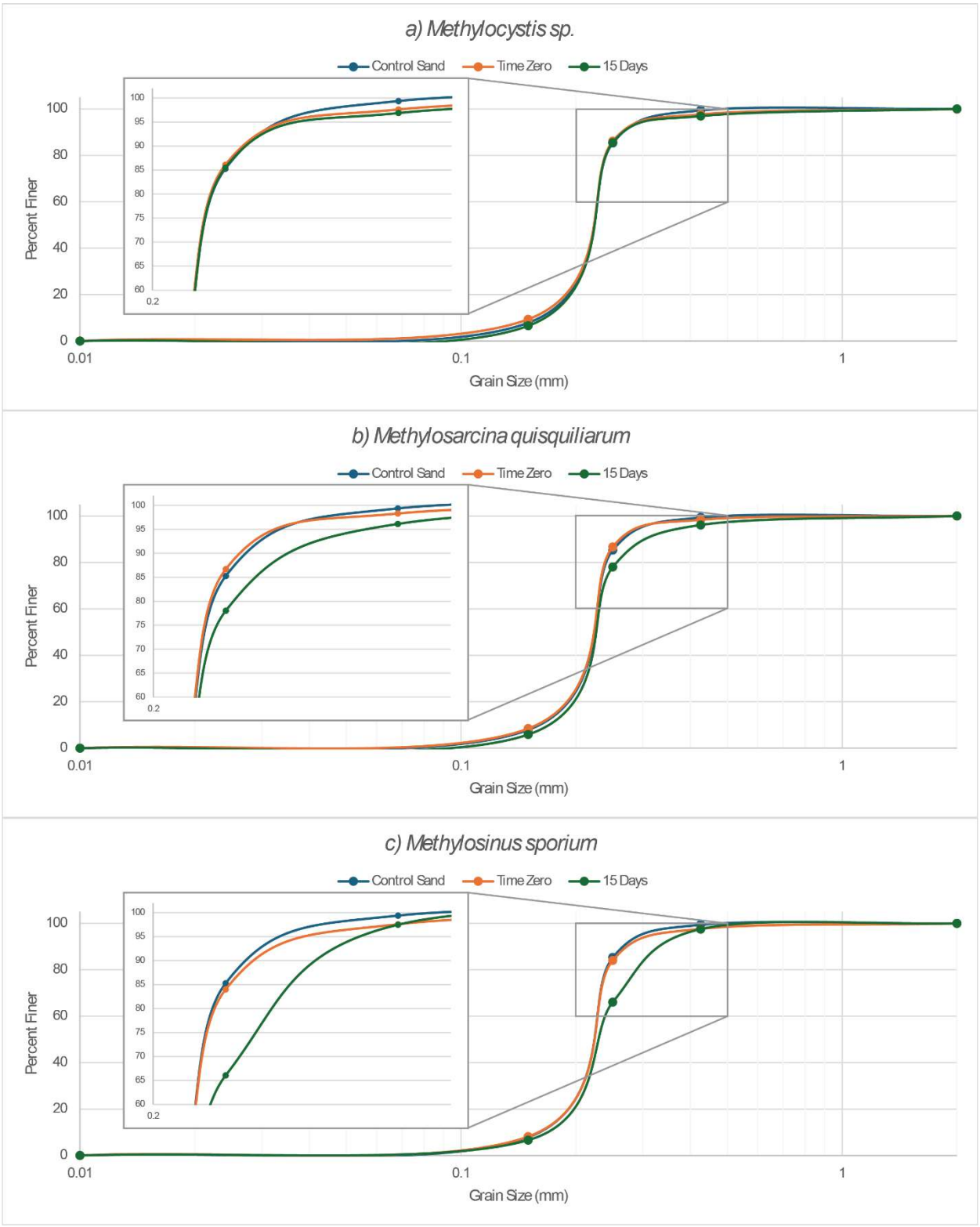

Figure 3.7. Second Study Grain Size Analysis a) *Methylocystis sp.* treated sand, b) *Methylosarcina quisquiliarum* treated sand, and c) *Methylosinus sporium* treated sand

except for in *Methylosinus sporium*. Figures 3.8 a and b show the *Methylocystis sp.* treated sand, which displayed some globules of bacteria and EPS adhered to the sand surface; these were quite similar, just less robust, to the ones seen in the first study and Figures 3.4 a-b. *Methylosarcina quisquiliarum* treated sand shows signs of some spherical bacterial and EPS attachments, Figure 3.9 b. Figure 3.10 a shows a broken-up aggregated clump of *Methylosinus sporium* treated sand, Figure 3.10 b is the same sand in a loose sample. None of the freeze-dried samples visually appeared to have any crust or clumping, which was confirmed under SEM imaging that showed little to no bacteria and EPS adhesion. Freeze-dried sample's SEM imaging is shown in Figure 3.11.

Discussion

These two studies aimed to investigate the potential for methanotrophic bacterial cultures to be applied to sand for bioaggregation. Aggregation was evaluated by dry sieving and SEM imaging. The main difference between the two studies was the point in their growth curves where the cultures were added to sand; the first study had cultures applied at their stationary phase, while the second study saw cultures applied at the start of their exponential phase. The idea behind this was that the first study would see the cultures applied when most of the EPS had already been produced, but the second would see cultures applied before most of the EPS production to see if EPS grown in the sand would have better bioaggregation capabilities. Other differences were the duration of the study – 28 days for study one and 15 days for study two – and the volume of sand tested on – 13 grams per dish of sand for study one and 5 grams for study two.

There were clear visual differences in the aggregated sand between the two studies. The first study saw thinner crust-like aggregation that was wide across the petri dish, but the second study saw thicker, less wide clumps of aggregated sand. While the second study saw deeper aggregates, they were more fragile, which may explain why none of the aggregates remained large enough to be retained on the #10 sieve (2 mm openings) after shaking. Despite that *Methylosinus sporium* treated sand in the second study had the largest percentage of sand larger than 0.25 mm after sieving.

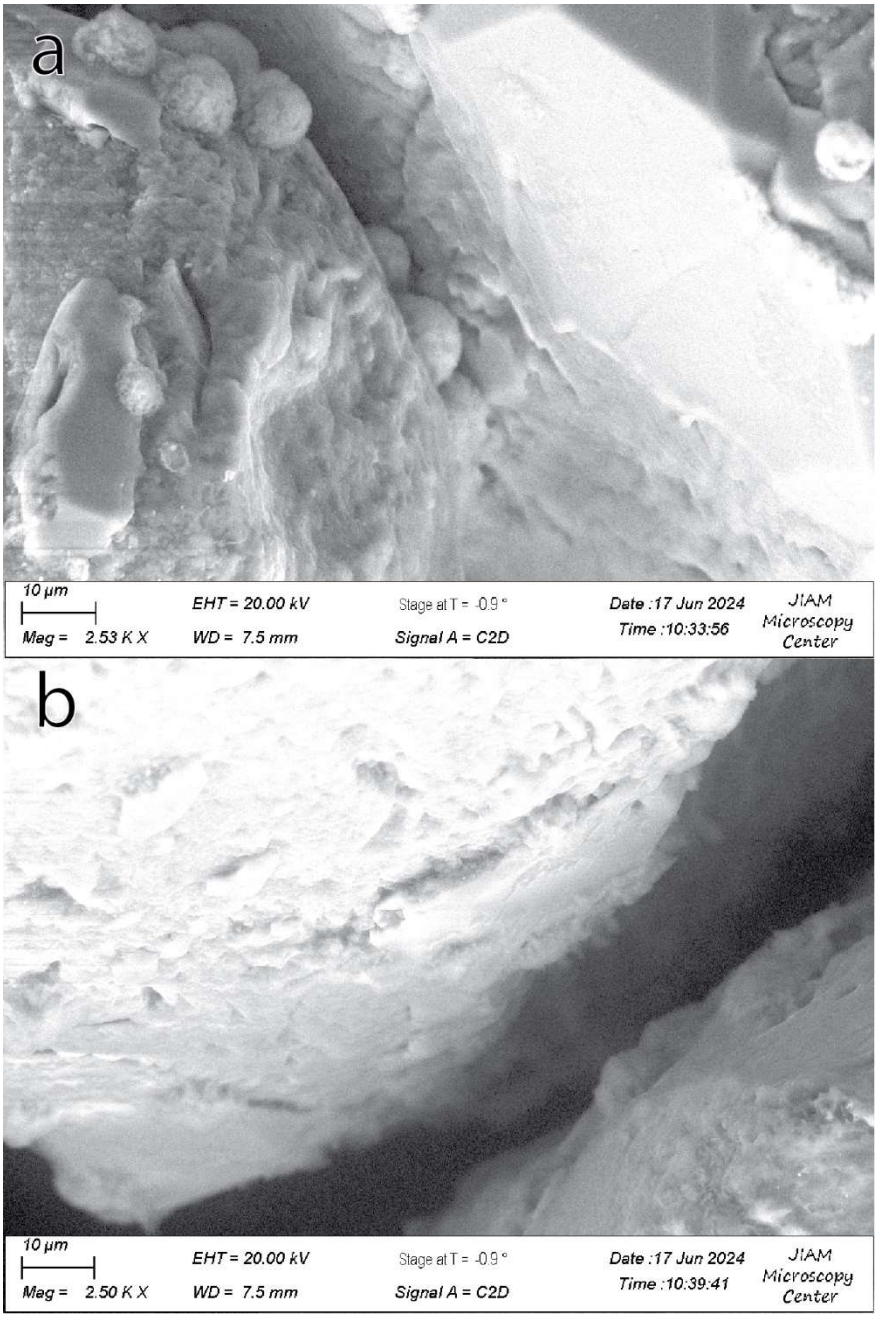

Figure 3.8. *Methylocystis sp.* treated sand SEM imaging at the final time point, 15 days. a) cell and EPS clusters between sand grains. b) sand grains with no noticeable bacterial adhesion

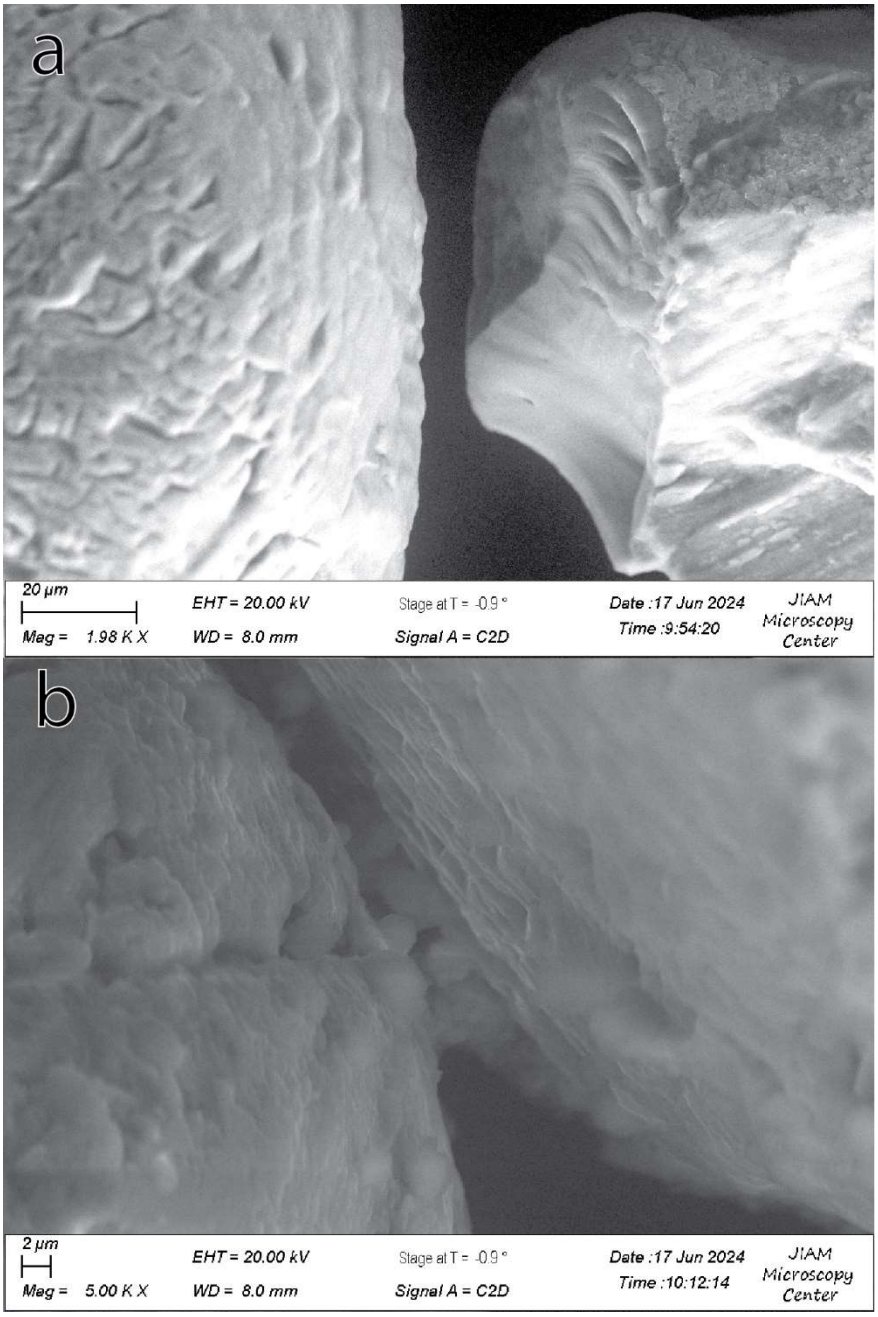

Figure 3.9. *Methylosarcina quisquiliarum* treated sand SEM Imaging at the final time point, 15 days. a) seemingly unaffected sand grains. b) Bacterial and EPS connection between sand particles

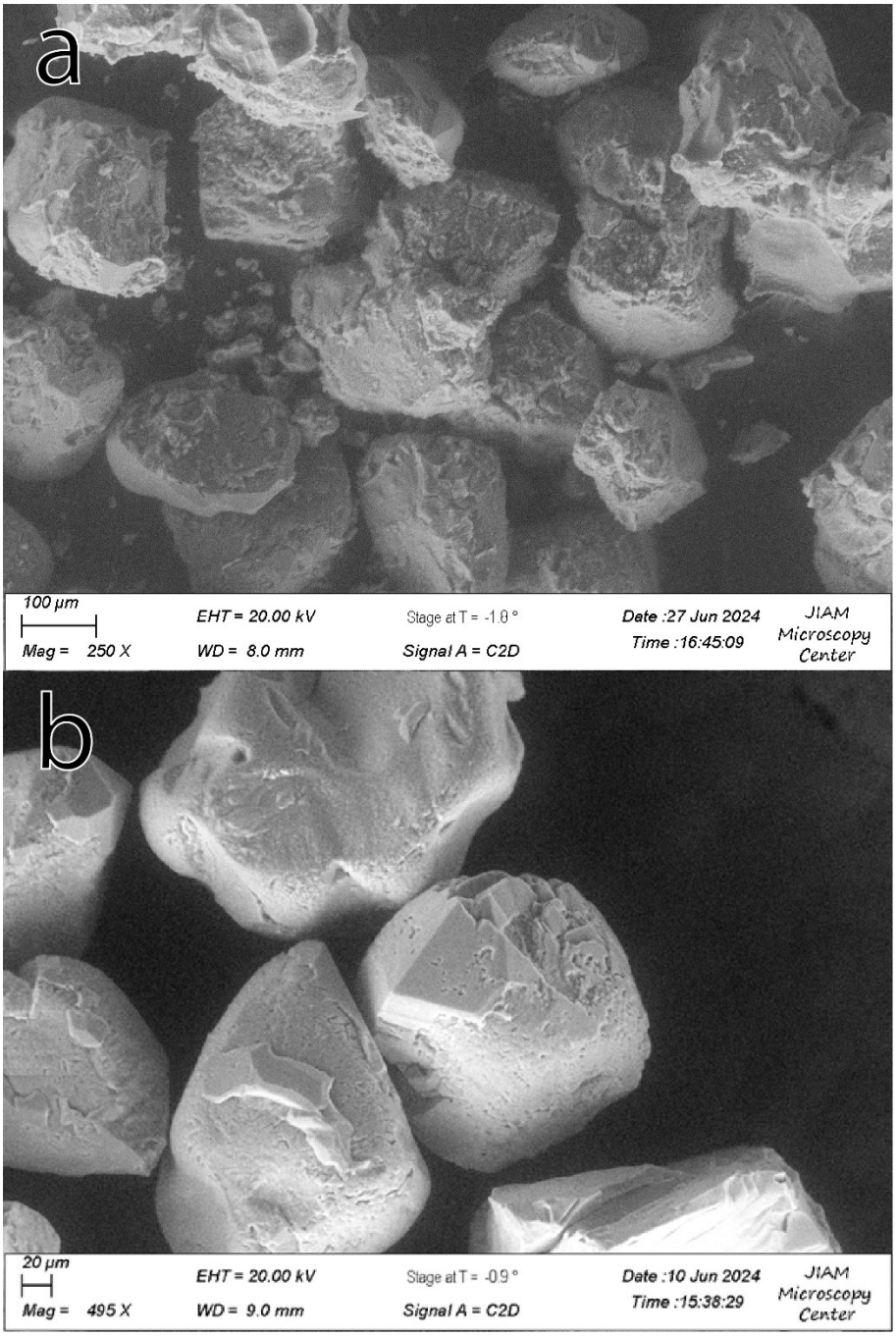

Figure 3.10. *Methylosinus sporium* treated sand SEM Imaging at the final time point, 15 days. a) aggregated sand grains. b) showing no signs of bacterial adhesion

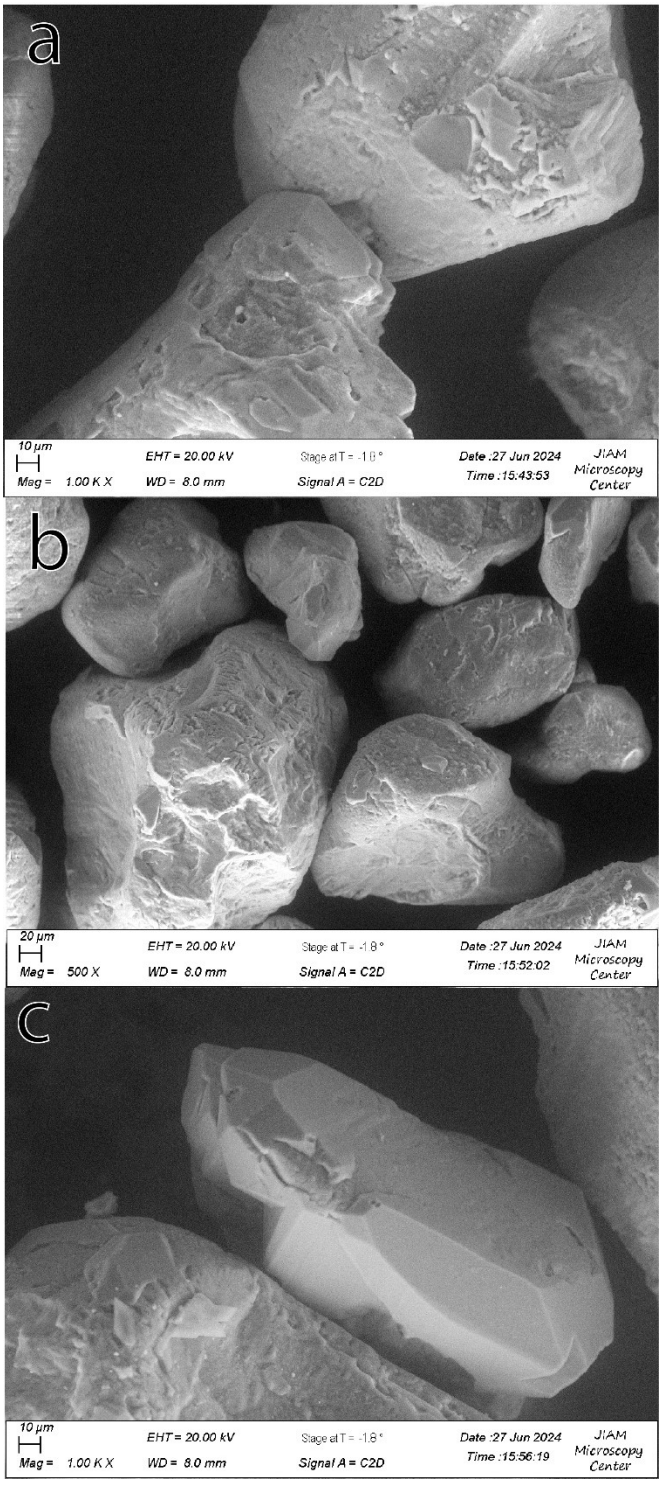

Figure 3.11. SEM images of freeze-dried bacteria treated sand a) *Methylocystis sp*. treated sand b) *Methylosarcina quisquiliarum* treated sand c) *Methylosinus* sporium treated sand

Interestingly, most of the SEM analysis for the second study showed little to no grain adherence; it is possible that bacteria and EPS attachments were weaker than the compressed air used for SEM sample preparation; which would explain the broken and blown-off particles seen stuck to the carbon tape in *Methylosinus sporium* treated sand (Figure 3.10a), however, if that were the case for all the bacteria it would be expected to see particles stuck to the carbon tape for the other two culture treated sands.

Methylocystis sp. and *Methylosarcina quisquiliarum* treated sands were used in both studies to differing results. *Methylocystis sp.* treated sand exhibited similar structures for both studies under SEM analysis to different extents - at 18 days, the first study had extensive amounts of sand surface covered by EPS and bacterial structures as well as grain-to-grain connections, but at 15 days, the second study showed minimal sand surface coverage and no visible grain connections. This was somewhat expected as in the second study *Methylocystis sp*. treated sand grain size distribution analysis had almost no difference between the treated sand and the control sand. In study one, *Methylosarcina quisquiliarum* treated sand had the most visible crusting and had almost the full first layer of sand covered in a crust made up of spheres stuck together along with fibrous attachments; this crust growth at 12 days was broken up for SEM imaging, Figure 3.5 a and d. This crusting was almost completely absent in the second study. The first null hypothesis can be rejected as different bacteria showed different aggregation through both grain size analysis and SEM imaging in both studies. The second null hypothesis can also be rejected as *Methylocystis sp.* and *Methylosarcina quisquiliarum*, the two cultures that were in both studies, showed differences in aggregation extent under SEM imaging between the first and second study.

It is posited that in the second study, the bacteria at the time of application were unencumbered by EPS and, therefore, able to penetrate further into the sand, which would explain why the aggregates went deeper, including sand adhered to the bottom of the Petri dishes. It is likely that bacteria had a more difficult time growing and producing EPS while in the sand, creating weaker grain-to-grain bonds. In the first study, it is believed that the bacteria, which were applied after growth, were blanketed in EPS and could not fully penetrate the sand's pore spaces, thereby blanketing the top layer and creating the crust that was seen.

In both studies, dry sieving—grain size analysis—was used to quantify the aggregation, but it could be argued that the sieves' shaking is more intense aggravation than would need to be withstood in nature for some surface aggregation needs. Future studies would benefit from multiple quantification methods selected based on the intended purpose of the application, for instance, wind resistance testing for dust suppression or erosion control studies.

Our research shows that EPS-producing methanotrophic bacteria can potentially be used in bioaggregation and that there are distinct differences in aggregation potential based on which bacterial strains are applied and when bacterial cultures are applied to sand in their growth curve. Methanotrophic bacteria are of interest for bioaggregation as they are ubiquitous, known EPS producers, and capable of Cometabolism of a myriad of contaminants (Hazen, 2010), giving the potential for simultaneous bioremediation and bioaggregation.

Conclusions

This study represents the first of its kind, to our knowledge, to analyze the sand bioaggregation capabilities of *Methylocystis sp., Methylosarcina quisquiliarum*, *Methylomonas methanica,* and *Methylosinus sporium* cultures. It was shown through dry sieving analysis and SEM imaging that there was some bioaggregation but future research would benefit from different methods of aggregation analysis and strength testing to more realistically test what the sand would encounter in *in situ* applications. The bioaggregation results are highly dependent on starting bacterial culture and its pre-application growth conditions. Future research is recommended in different bacterial cultures and optimal growth conditions pre-application.

Chapter 4
Omics Of Oil Biodegradation

Harik, A.-M., Z. Griffiths, and **T. C. Hazen**. 2022. Omics of Oil Biodegradation. *Current Opinion in Chemical Engineering* 36:100800.
https://doi.org/10.1016/j.coche.2022.100800
Author Contributions: A-MGH: Writing, Visualization, ZG: Writing, Visualization, TCH: *Investigation, Methodology, Project administration, Supervision, Writing*

Abstract

Omics studies (metagenomics, transcriptomics, metabolomics, proteomics) for marine oil biodegradation research increased rapidly after the 2010 Deepwater Horizon (DWH) accident in the Gulf of Mexico. Since then, it has been demonstrated how omics techniques can be used to model and better understand pre-spill environments, monitoring during a spill and post-spill. Data that encompass everything from the ecosystem to the molecular level are needed for understanding the complicated process of petroleum biodegradation in marine environments. Consequently, using omics for monitoring oil in the ocean will help in developing more robust systems models and would make responses to spills much more defensible in terms of risks to the environment and people. Omics is enabling for a Systems Biology approach to oil spills which allows a search for hidden interactions and attributes at different trophic levels because 'the whole is greater than the sum of its parts'.

Introduction

Metagenomics, transcriptomics, proteomics, and metabolomics have played a significant role in environmental monitoring and bioremediation efforts in recent years. This trend was catalyzed by the marine oil biodegradation research after the 2010 Deepwater Horizon (DWH) spill in the Gulf of Mexico. Physical clean-up efforts were hindered by technical or logistical difficulties caused by the depth at which this spill occurred. DWH is the deepest oil well blowout (~1500 m below the surface) that has ever occurred. The uncertainty surrounding the fate of oil in deep-sea environments presented a unique research opportunity. The first studies demonstrated faster-than-expected hydrocarbon biodegradation rates in deep waters (Hazen et al., 2010). Based on field and lab studies, hydrocarbon composition changes were observed with distance

from the DWH well blow out which helped to reveal a variety of hydrocarbon-degrading microorganisms (Hazen et al., 2010). The indigenous microorganisms were adapted to natural seeps of crude oil in that marine environment (Lu et al., 2012). Rapid oil biodegradation was facilitated by water-soluble constituents in the crude oil and through the injection of subsea dispersant (Miller et al., 2020). The microbial community composition and dominant taxa changed with the degradation of certain components of the crude oil thus changing the petroleum composition through time. The more easily degraded components were consumed first by microbes that could grow faster during the initial environmental conditions, followed by microbes that could degrade more difficult to metabolize components and/or daughter products created by the initial biodegradation (Kostka et al., 2020).

Oil spills have severe effects on the ambient area around the release site (National Academies of Sciences & Medicine, 2019). These incidents may occur during oil and gas exploration or production-related activities, including accidents during storage or transport (National Academies of Sciences & Medicine, 2019). Oil biodegradation by microorganisms can be effective in reducing some of the immediate risks associated with oil spills while having minimal adverse effects on the environment (National Academies of Sciences & Medicine, 2019). Temperature, oxygen concentration, and available nutrients can influence the rate and extent of oil bioremediation (Miller et al., 2019). The selection of the most appropriate response option(s) following an oil spill typically involves the consideration of many factors and trade-offs, for example, urgency related to nearness and sensitivity of risk factors and long-term effects on the environment.

Marine microbial communities are a vital component of global carbon cycling, and numerous studies have shown that populations of known oil-degrading bacteria are ubiquitous in marine environments. Omics technologies will prove useful in establishing baseline communities and activities for example, during natural exposure from underwater oil seeps. Understanding the microbial community composition and diversity aids in the prediction of petroleum biodegradation by microbial communities in situ and is therefore an important component of oil spill response decision-making process. Successive blooms of taxonomically distinct indigenous microbial populations as the oil

weathers and labile components are sequentially degraded, leaving less-readily degraded components to feed subsequent blooms of microbes is critical to understanding the best alternatives for long-term spill response.

Microbial Communities Across Marine Basins

The presence of marine petroleum hydrocarbon pollution has both natural and anthropogenic sources. Anthropogenically, the growing demands for fossil fuel and its derivative products over the last millennia have resulted in increased extraction and exploration which in turn increased offshore drilling, pipelines, and shipping spills globally. This creates potentially hazardous situations as it increases the risk of large-scale marine pollution events worldwide. Natural seeps provide points of entry for hydrocarbons as oil and gas are slowly released from cracks or fissures on the ocean floor over time. Global estimates suggest that 47% of oil released into marine environments comes from these natural oil seeps (National Academies of Sciences & Medicine, 2019). Primarily, seeps have attracted attention as an indicator of a subsurface accumulation of petroleum, but, they can also be used to identify potential hydrocarbon degrading bacteria and their catalytic mechanisms (Hassanshahian & Cappello, 2013). The microbiota residing in the waters near these natural seeps are primed for hydrocarbon degradation through repeated exposure, forming a memory response to this contaminant (Hazen et al., 2016). Valentine et al. (Valentine et al., 2012) found that the memory response of these oil degrading microbes led to a rapid increase in their abundance and accelerated oil biodegradation during the Deepwater Horizon (DWH) Spill (Kujawinski et al., 2020).

The following deep-sea basins have received some attention as their drilling and exploration activities have increased the need for appropriate spill response measures: Gulf of Mexico, Eastern Mediterranean's Nile Deep-Sea Fan, Central Mediterranean and the Sirte Basin, North Sea, Caspian Sea, Angola, Trinidad and Tobago, Great Australian Bight, and Brazil's Amazonian Deep-Sea Basin. These basins are found in different geographic locations which are impacted by water masses that have different temperatures, salinities, dissolved oxygen (DO) concentrations and limiting nutrients from land surfaces and diagenesis of hydrocarbon deposits in the deep (Hazen et al.,

2016). Geochemical gradients exist within these basins ranging from warmer, well oxygenated surface waters to cold temperatures, darkness, high pressures, and possible anaerobic conditions at deeper depths. Distinct communities of hydrocarbon degraders thrive in the deep ocean and surface waters with varying metabolic capabilities (Hazen et al., 2010; Hazen et al., 2016; Kostka et al., 2020; Miller et al., 2020; Redmond & Valentine, 2012). Environmental factors in these basins influence the microbial community composition and the abundance of potential hydrocarbon degrading microorganisms (Miller et al., 2020). The effect of temperature, depth, and the physical properties of the native oil have an impact on the microbial community response and their degradative capabilities (Hazen et al., 2018; Meng et al., 2016).

Though seawaters generally have high microbial abundance and diversity, hydrocarbon contamination will support the proliferation of a set of specialized obligate hydrocarbon utilizers known as obligate hydrocarbonoclastic bacteria (OHCB) (Hassanshahian & Cappello, 2013; Meng et al., 2016). After the DWH spill, the microbial community responded in different ways based on depth. Though surface and deep waters were both dominated by Gammaproteobacteria, the genera belonging to this class differed at these depths. Oceanospirillaceae, Pseudoalteromonas, Pseudomonas, Vibrio, Acinetobacter, and Alteromonas were dominant in the warm surface waters, whereas the prominent organisms in the colder, deeper waters were Oleispira, Colwellia, and Cycloclasticus (Meng et al., 2016; Miller et al., 2019; Miller et al., 2020; Redmond & Valentine, 2012). This is consistent with other reports of probable hydrocarbon degrading genera found in other deep sea oil basins (Hazen et al., 2018). The Oceanospirillales family, are frequently associated with hydrocarbon pollution and biodegradation of aliphatic hydrocarbons and are ubiquitous across marine basins, with variation in specific genera amongst basins (Miller et al., 2020; Valentine et al., 2012). Oleispira and Colwellia have been previously isolated from Arctic environments with hydrocarbon pollution and are psychrophilic microorganisms. These microbes have adapted to the limitations on biodegradation imposed by lower temperatures to produce cold-adapted enzymes (Miller et al., 2020; Ribicic et al., 2018).

Omics Role in Pre-Spill Response Planning

The Spill Impact Mitigation Assessment (SIMA) model utilizes natural in situ biodegradation as a baseline response and for comparison of efficiency and hazards related to other remediation techniques (Hazen, 2020). It is considered as the starting point of 'no intervention' for the SIMA model (https://www.ipieca.org/resources/awareness-briefing/guidelines-on-implementing-spill-impactmitigation-assessment-sima/ IPIECA, 2018). Natural degradation of oil is dominated by bacterial biodegradation, as such the in-depth knowledge provided by omics is crucial for decision making and targeted response plans (Hazen et al., 2016). Omics data is a main component used in the Structured Learning in Microbial Ecology (SLiME) model to predict biodegradation rates (Smith et al., 2015). Having a basic understanding and local microbial sampling done before contamination to establish a baseline community leads to more informed predictions of hydrocarbon transport and natural transformation rates (Love et al., 2021).

SIMA utilizes the Oil Spill Contingency and Response (OSCAR) model which take some level of microbial data into account but could be greatly enhanced by site specific bacterial community data (Table 4.1). The abundance and diversity of potential hydrocarbon degrading bacteria have been shown to vary significantly by both location and water depth (King et al., 2015; Miller et al., 2019; Miller et al., 2020) making site-specific omics data crucial to better model fate of hydrocarbons. Though potential microbial hydrocarbon degraders are widespread, variation in environmental pressures will result in differences in these microbes, even among the same groups of bacteria. Thus rates of degradation and microbial response cannot be treated as constant across basins and depths (Hazen et al., 2016). During DWH it was found that the biodegradation rates of Macondo oil varied from total petroleum half-life's of 1.2–6.1 days (Hazen et al., 2010), while the TPH half-life's in Eastern Mediterranean, Great Australian Bight, Central Mediterranean, Angolan Coast and Caspian varied from 11 to 21 days for indigenous oil.

Omics data can inform the model if the local microbial community contains any known hydrocarbon degraders, via genomics; if the bacteria present are expressing oil

Table 4.1. Showing targets of enzymes commonly found among hydrocarbon-degrading microbes. Enzymes involved in anaerobic biodegradation are marked in italics

Hydrocarbon class	Enzyme	Action on hydrocarbons	References
Aliphatics	Alkane 1-monooxygenase	C5–C16 alkanes, alkyl benzenes, cycloalkanes	(Abbasian et al., 2015; Varjani, 2017; Varjani & Upasani, 2017)
	Alcohol dehydrogenase	Oxidation of hydrocarbon intermediates to fatty acids	(Abbasian et al., 2015; Baburam & Feto, 2021)
	Soluble/particulate methane monooxygenase	C1–C8 alkanes, C1–C5 halogenated alkanes, alkenes, cycloalkanes	(Abbasian et al., 2015; Varjani, 2017; Varjani & Upasani, 2017)
	Eukaryotic cytochrome P450 hydroxylases	C10–C16 alkanes, Fatty acids	(Al-Hawash et al., 2018)
	Bacterial cytochrome P450 hydroxylases	C5–C16 alkanes, cycloalkanes, C15–C30 alkanes	(Abbasian et al., 2015)
	Dioxygenases	C10–C30 alkanes	(Abbasian et al., 2015)
	Alkyl-succinate and arylalkyl-succinate synthases	n-alkanes	(Rabus et al., 2016)
Aromatics	Naphthalene 1,2-dioxygenase Benzene dioxygenase Toluene dioxygenase	Addition of molecular oxygen to low molecular weight aromatic hydrocarbons to form alcohol intermediate	(Lee et al., 2019; Xu et al., 2018)
	Ring-cleaving dioxygenases (intradiol and extradiol dioxygenases)	Ring cleavage of catecholic compounds (main intermediate in the biodegradation of aromatic hydrocarbons)	(Ghosal et al., 2016; Vaillancourt et al., 2006)
	Eukaryotic cytochrome P450 hydroxylases	Transformation of aromatic hydrocarbons to trans-dihydrodiol	(Ghosal et al., 2016; Vaillancourt et al., 2006)
	Ethylbenzene dehydrogenase	Hydroxylation of aromatic hydrocarbons	(Abbasian et al., 2015; Rabus et al., 2016)
	Alkyl-succinate and arylalkyl-succinate synthases	Fumarate addition to aromatic hydrocarbons, alkyl-aromatic hydrocarbons	(Abbasian et al., 2015; Rabus et al., 2016)

degrading genes, via transcriptomics; and further down to proteins, metabolites, and so on, (Table 4.2). Omics samples should not stop at genomics (DNA analysis); while knowing which potential degraders are present is important, it does not fully account for cometabolic degradation and biosurfactant producers (Hazen et al., 2016). Omics also provides the benefit of not needing laboratory cultivation, as many of the potential taxa are not successfully cultured (Dubinsky et al., 2013). Microcosm studies are subject to bottle effects (changes in nutrient composition in enclosed systems over time result in shifts of the microbial community that cause variations from what was present in the in situ environment at the time of collection) which limits their use in mimicking in situ degradation (Hazen, 2020). Lack of culturing ability and communities subject to bottle effects necessitates in situ data. A systems biology approach to planning for potential incidents is ideal in analyzing the effects of hydrocarbon pollution at the molecular, cellular, and community level to provide insight on the innate degradative capabilities that can be employed for bioremediation. Modeling from a systems biology approach in conjunction with nutrient data should help elucidate limiting factors in the hydrocarbon degradation pathways to better plan for nutrient amendments and dispersants (Geng et al., 2021; Smith et al., 2015). Models like SLiME (Smith et al., 2015) can be used with rapid sequencing techniques to provide results in one day that predict how an oil plume will change and how fast it will degrade. After amendments and engineering controls have been applied to ameliorate the extremes of the oil spill, samples can be taken monthly to monitor hydrocarbon concentrations, increasing time between samples as it proceeds until contamination is below detectable limits for critical risk receptor concerns.

The DWH oil spill in the Gulf of Mexico was the first large scale spill where omics were implemented (Hazen, 2020; Kostka et al., 2020), and the data acquired opened new potential monitoring methods for the clean-up process. Genomic data regarding microbial community composition indicated a correlation between changes in the community structure and dominant taxa to the hydrocarbon composition of the oil as it changes during the degradation process (Dubinsky et al., 2013; Hazen, 2020; King et al., 2015; Kostka et al., 2020). Expanding on the knowledge gained from DWH, microbial omics data may be used as another stream of evidence in pre and post spill

Table 4.2. Showing application of omics and possible analyses from biodegradative processes

Biodegradation actions	Omics tracking/analysis
Progression of hydrocarbon degradation	Genomic community analysis is used to identify community and dominant taxa shifts through progression of hydrocarbon degradation (Dubinsky et al., 2013; Hazen et al., 2010; Hazen et al., 2016; IPIECA, 2018)
Microbially produced biosurfactants	Functional gene analysis can show which bacteria are producing biosurfactants, and metagenomic analysis can show which organisms have the potential to create biosurfactants (Hazen et al., 2016)
Memory response	Identification of genes/organisms consistently associated with hydrocarbon degradation for use as biomarkers in the detection of hydrocarbon contamination (Hazen et al., 2016; Techtmann & Hazen, 2016)
Co-metabolic degradation	Identification of unique or unusual degradation pathways with real-time analysis of community metabolic state (Ghosal et al., 2016; Hazen et al., 2018)

monitoring to identify: (1) best practices for spill response, (2) the presence of hydrocarbon contamination, and (3) how far have hydrocarbons been degraded. Building off the omics tracking data that began with DWH monitoring, the monitoring and omics data from future spills and contamination events can be fed back into upgraded models — creating a feedback loop for machine learning to build the best predictions and spill response plans that allow the models to be rapidly updated for a real time response for those in the field (Hazen, 2020).

Omics and Bacterial Data Limitations

Microcosm studies are subject to bottle effects (changes in nutrient composition in enclosed systems over time result in shifts of the microbial community that cause variations from what was present in the in situ environment at the time of collection) which limits their use in mimicking in situ degradation (Molina-Menor et al., 2021). Utilizing culture independent methods from omics technologies has helped to broaden our knowledge of microbial species that were previously deemed as ‘unculturable’. This was seen during the DWH spill where the integration of molecular based and culture-independent techniques increased the capacity for real time assessment of oil biodegradation (Hazen et al., 2018). The use of powerful omics technologies and bioinformatics methods, including metatranscriptomics, proteomics, metabolomics, and metagenomics allowed scientists to comprehensively examine the microbial community structure, and their dynamic and metabolic capabilities associated with a major oil pollution event. It better guided our assessment of: who are they and what are they doing? Genomic data helped to identify trends in microbial succession and transcripts associated with hydrocarbon biodegradation. The combination of these approaches allowed researchers to investigate the metabolic pathways taken by the microbial community to degrade oil at different stages. This information may have otherwise been overlooked if species were only analyzed individually.

Though our knowledge has increased through these culture-independent techniques, there are still limitations in sampling efforts that can introduce bias into the data analysis. As previously mentioned, the ‘bottle effect’ can affect the microbial community during transport and storage of samples. Culture-independent methods also

require large volumes of water to generate a sufficient cell mass for downstream analysis (Hazen et al., 2013). Filtration methods and materials may also introduce biases into what cells are retained versus destroyed based on their adherence. Biases can also be introduced through nucleic acid extraction kits and protocols. A major concern is the ability to lyse and extract the nucleic acids from a representative set of cells from the microbial community to capture the ecological diversity (Hazen et al., 2013).

Molecular methods must be processed through various bioinformatics pipelines to be converted into a format that can be analyzed. Hence, these pipelines have the potential to introduce biases into our final conclusions. The databases chosen for analysis can also introduce further biases during gene calling and annotations since different databases have varying standards for gene classification and functional analysis (Chandran et al., 2020). Awareness and anticipation of these biases will better prepare us to mitigate their effects on downstream analysis and conclusions. Some of these limitations can be overcome by using multiple techniques and pipelines to provide multiple lines of evidence for any conclusions made. The strength of the conclusions from this data is provided by a multi-omic analysis that not only allows us to assess the presence of hydrocarbon degrading species but also the genes, and biogeochemical pathways involved.

Conclusions

Since biodegradation is the ideal spill response, and the base line for hydrocarbon contaminant modeling, microbial data is essential for better preparation (Hazen, 2020). Including multiple lines of omics research before and after contamination will allow for more robust models and response plans. Studying natural hydrocarbon seepages will also better our understanding of the microbial response without having to wait for another event. These seepages were key in the microbial response to Deepwater Horizon and providing the microbial communities with a memory response (Hazen, 2020). Omics should be considered part of the baseline data taken when considering drill sites or during exploratory expeditions. Once active, each site should have personnel trained in properly collecting and storing microbial samples. Allowing for multiple lines of constant local omics data will improve current modeling capabilities.

Omics is a crucial step in biodegradation and cannot be ignored when preparing for spills.

Chapter 5

Microbial Hydrocarbon Degradation Under Differing Biogeochemistry Conditions

Abstract

Oil spills can have dire impacts on marine ecology and are a concern in all areas where oil drilling occurs. The Eastern Mediterranean, with its oil production potential, is an area of interest for oil spill response planning. The goal of this study is to examine how enriched microbial communities and their hydrocarbon degradation rates react to changes in biogeochemistry and methane amendments. Microcosms were created from two starting enriched consortia, one from surface water and one from deep waters, placed in three different conditions. Hydrocarbon analysis and 16S rRNA sequencing are combined to analyze the microcosms' response to different nutrient and methane concentrations over an 8-week period. Sequencing results and non-metric multidimensional scaling (NMDS) analysis indicated that microbial community structure grouped by starting inoculum rather than nutrient conditions or methane additions. While most of the conditions saw decent hydrocarbon degradation, there were no definite differences in degradation percentages between conditions. These results help elucidate the biodegradation capabilities of the Eastern Mediterranean's native microbial communities.

Introduction

The oil production potential of the Eastern Mediterranean Sea increases the chance of accidental oil spills (Liu et al., 2017), thereby increasing the need for spill response planning. Hydrocarbon bioremediation, the use of microbes to break down hydrocarbons, is an integral part of spill response plans and is often considered a baseline (Harik et al., 2022). Initial models for oil biodegradation failed in the Deep Water Horizon (DWH) oil spill by not taking into account psychrophiles (Bælum et al., 2012; Chakraborty et al., 2012; Hazen et al., 2010; Hazen et al., 2016). Later SINTEF created the Structured Learning in Microbial Ecology (SLiME) model, taking microbial community structure into account, which was able to correctly predict DWH deep oil plumes (Hazen, 2020). IPIECA – the global oil and gas industry association for environmental and social issues, API – the American Petroleum Institute, and IOGP – the International Association of Oil & Gas Producers – have created the Spill Impact Mitigation Assessment (SIMA), a four-stage process for selecting an oil spill response

which minimizes the environmental impact. The SIMA model considers ‘no intervention’, unaided in situ microbial biodegradation, as a starting point (https://www.ipieca.org/resources/guidelines-on-implementing-spill-impact-mitigation-assessment-sima IPIECA, 2017). This makes research into the local microbial community’s hydrocarbon degradation abilities imperative for spill response planning. Environmental and biogeochemical factors are known to affect microbial communities, including the presence of hydrocarbon degraders and rates of hydrocarbon degradation (Bælum et al., 2012; Liu et al., 2017; Steenbergh et al., 2014). As such, bioremediation calculations must take into account the local community structure and environmental conditions (McFarlin et al., 2014; Zahed et al., 2010).

The Eastern Mediterranean Sea is unique in its biogeochemistry as it has higher salinity, warmer deep-water temperatures, and lower deep-water nutrient concentrations compared to average ocean water (Krom et al., 2010; Liu et al., 2017; Moore et al., 2013; Skliris, 2014; Techtmann et al., 2015). The Eastern Mediterranean Sea water masses are divided into three layers by depth, with nutrient concentrations and bacterial diversity increasing with depth (Techtmann et al., 2015). Natural hydrocarbon seeps are also present (Techtmann et al., 2015).

The goal of this study was to provide insights into how the enriched microbial community structures and hydrocarbon biodegradation rates react to changes in biogeochemistry as well as methane amendments. The first null hypothesis states that there is no difference in microbial community structure when the enriched consortia are introduced to different nutrient conditions; the alternate hypothesis is that there is a change in microbial community structure when enriched consortia are introduced to different nutrient conditions. This is tested by introducing enriched consortia to two different media recipes and sequencing microbial communities, sequenced results are then visualized through nonmetric multidimensional scaling (NMDS) with 95% confidence interval ellipses showing overlapping or lack thereof between different conditions. The second null hypothesis states that there is no difference in hydrocarbon degradation capabilities between enriched surface consortia and enriched deep-sea consortia, while the alternate hypothesis is that surface and deep consortia show differences in hydrocarbon degradation amounts. Testing for the second hypothesis is

done through hydrocarbon quantification, at different time points, by gas chromatography-mass spectrometry (GCMS). The third and final hypothesis states that there is no difference in the methanotrophic percentage of the taxa plots when methane amendments are introduced; the alternate hypothesis states that methanotrophs make up a larger percentage of the taxa plots when methane amendments have been introduced. Third hypothesis testing is done by analyzing 16S rRNA sequencing data for the presence of methanotrophic bacterial taxa.

Materials and Methods

Media Preparation

In order to selectively mimic surface and near-bottom depth conditions from the Eastern Mediterranean Sea Nile Delta, two different media recipes were prepared. Each recipe altered the traditional ONR7a recipe (Dyksterhouse et al., 1995) based on previously collected geochemical measurements. In situ nutrient data collection and analysis is previously described in Techtmann et al. (2015), and select readings and their averages are shown in Table 5.1. Based on the average readings for each depth location, new media recipes were prepared with differing nitrate, iron, phosphate and ammonia concentrations. Since many iron readings were below detectable limits (5µg/L) which skewed averages, the station reading from where each microbial consortia enrichment was sampled was used for the final media recipes, shown below in Table 5.2.

Microbial Consortia

Two different enriched consortia glycerol stocks were acquired from samples collected and enriched on-ship during the BP Eastern Mediterranean Cruise aboard the MV Fuego Navigator. One consortium was from a surface sample, and one was from a near-depth sample. The glycerol stocks were grown in their coordinating media recipes and ambient temperatures for two weeks before being used to inoculate microcosms at 10% volume. Microbial community sequencing samples were taken from starting consortia as well as from microcosms.

Microcosm Set-Up

Microcosm Setup included 3 condition variables: two different media recipes

Table 5.1. *In Situ* Nutrient Analysis from stations in the Nile Delta Eastern Mediterranean Sea from Techtmann et al. (2015)

Sample ID	Station	Depth m	Temp ºC	Sulfate mg/L	Fe µg/L	Inorganic Phosphate µmol/L	Ammonia µmol/L	Nitrate µmol/L
Near Surface								
NDMS001	3	10	26.33	3486	<5.0*	0.171	0	0
NDMS009	1	50	19.2	3406	5.4	0.104	0	0
NDMS014	4	50	19.26	6544	<5.0*	0.063	0	0
NDMS024	5	50	19	7042	<5.0*	0	0	0
NDMS019	2	60	17.49	7183	<5.0*	0.033	0	0
	Average	44	20.26	5532.2		0.0742	0	0
Near Bottom								
NDMS002	3	495	13.96	2978	6.1	0.437	0	6.776
NDMS021	5	742	13.75	5523	<5.0*	0.298	0	6.315
NDMS011	4	972	13.75	4055	9.5	0.354	0	5.856
NDMS016	2	1055	13.76	3884	<5.0*	0.327	0	6.382
NDMS006	1	1210	13.79	2174	<5.0*	0.371	0.108	5.987
	Average	894.8	13.802	3722.8		0.3574	0.0216	6.2632
Deepest 5 Readings								
NDMS021	5	742	13.75	5523	<5.0*	0.298	0	6.315
NDMS007	1	824	13.77	3300	6.1	0.381	0	6.495
NDMS011	4	972	13.75	4055	9.5	0.354	0	5.856
NDMS016	2	1055	13.76	3884	<5.0*	0.327	0	6.382
NDMS006	1	1210	13.79	2174	<5.0*	0.371	0.108	5.987
	Average	960.6	13.764	3787.2		0.3462	0.0216	6.207

Table 5.2. Media Recipes

		1 L Surface Simulating Media	1 L Deep Simulating Media
SOLUTION ONE			
NaCl		22.79 g	22.79 g
Na2SO4		8.18 g	8.18 g
gly-gly		1 g	1 g
Solution1A Amount		1 mL	1 mL
	100 ml milli Q water (100X Stock)		
NaHCO3	3.1 g		
H3BO3	2.7 g		
NaF	260 mg		
KBR	4.8 g		
Solution1B Amount		5 µL	23 µL
	50 ml milli Q water (200,000X Stock)		
Na2HPO4 7H20	200 mg		
Solution1C Amount		0 µL	10 µL
	100ml milli Q water		
NH4Cl	50 mg		
KNO3	6.3 mg		
SOLUTION TWO			
MgCl2 6H20		11.18g	11.18g
CaCl2 2 H20		1.46g	1.46g
SrCl2 6H20		0.024g	0.024g
SOLUTION THREE		0 µL	1 mL
FECL2 4H20	0.015 g in 500 mL		
(Amount of solution three is dependent on sample location, for sample "03-E-002 SCG 1/2/13" was used, deep needs 6.1 ug/L and surface has 0)			
OIL:	50 ppm per bottle		

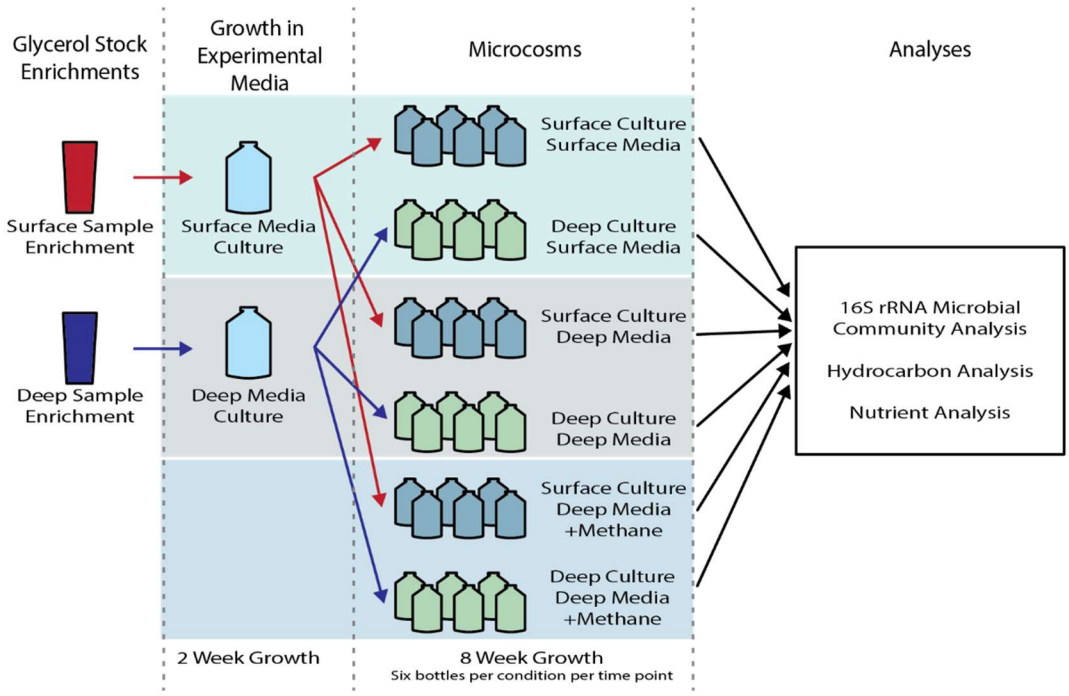

Figure 5.1. Microcosm Set-Up

(surface simulating and deep simulating), two head space make-ups (with and without methane gas amendments), and two different bacterial consortia enrichment inoculums (surface consortia and deep consortia), making for six different conditions. Conditions were Surface Consortia in Surface Simulating Media without the addition of methane, Deep Consortia in Surface Simulating Media without the addition of methane, Surface Consortia in Deep Simulating Media without the addition of methane, Deep Consortia in Deep Simulating Media without the addition of methane, Surface Consortia in Deep Simulating Media with the addition of methane, and Deep Consortia in Deep Simulating Media with the addition of methane. Each condition had 6 bottles per time point, along with negative controls at initial and final time points. At T0 each serum bottle was given 50ppm Norne blend oil, 90 mL of its corresponding media, and 10% (10 mL) of consortia inoculum. Afterward, bottles were plugged with vacuum caps and crimp sealed; bottles needing methane amendments had millimeters of headspace removed via syringe, and then the same amount of methane was added. Bottles with Surface consortia were stored in room temperature in a cabinet, approximately 22C, while deep consortia bottles were stored in an incubator at 14C. Twice a week head space for each bottle was allowed to be exchanged inside a biosafety cabinet, while conditions with methane had methane amended.

DNA Extraction and PCR Amplification

Three of the six bottles from each condition at every time point were chosen to be sampled for community analysis and nutrient analysis; the other three bottles were saved for hydrocarbon analysis. Approximately 30mL from each of the three community analysis bottles were vacuum filtered through 0.2μm filters, and filters were stored at -80℃ until extraction. Each filter was then cut into small pieces, and DNA was extracted using the FastDNA Spin Kit (MP Biomedicals). The 16S rRNA was amplified using 515F and 806R primers (Caporaso et al., 2012); the reverse primers included unique twelve base pair barcodes for each sample.

Sequencing and Analysis

Post-PCR samples were pooled into 9 pools based on gel electrophoresis band fluorescence strength. Gel slices of pools were cleaned using a Wizard SV Gel and PCR Clean-Up System (Promega Corporation). Cleaned pools were run on a

Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to assess the quality and fragment base-pair length. Initial pools were then quantified using a high-sensitivity assay on the Qubit (ThermoFisher Scientific), and from there, initial pools were combined to form a final library pool, which was diluted down to an estimated 4nM concentration. The final library pool concentration was quantified using qPCR with a NEBNext Library Quant Kit for Illumina (NewEngland Biolabs) on a Bio-Rad real-time PCR machine. Sequencing was run on an Illumina MiSeq with analysis conducted through qiime.

Nutrient Analysis

The remaining liquid in the first three serum bottles after removing samples for community analysis were combined in a plastic bottle and stored at -80℃. At a later date, samples were filtered in a 0.2μm filter and then refrozen in 50mL containers, which were sent to SOEST Laboratory (University of Hawai'i at Manoa) for Analytical Biogeochemistry for dissolved inorganic nutrient analysis.

Hydrocarbon Extraction and Analysis

The remaining three microcosm replicate bottles for each condition/time point were frozen and stored at -80C for use in hydrocarbon analysis. Bottles were sent to the Southeast Environmental Research Center at Florida International University for hydrocarbon processing and analysis. Samples were processed with liquid-liquid extraction in methylene chloride, concentrated, and analyzed with gas chromatography-mass spectroscopy (GCMS).

Results

Sequencing Analysis

16S rRNA sequencing led to the generation of 7,458,419 reads from 79 samples that were 250 base pairs long. Average number of reads per sample was 94,410. One of the triplicates of time point one, deep media and surface culture, was removed due to low reads and was excluded from further analyses. Deep Media with deep culture at time point six only had duplicates due to a broken bottle.

As seen in Figure 5.2 Starting Surface Microbial Consortia consisted mainly of *Kiloniellales*, *Oceanospirillales*, and *Pseudomonadales*. Starting Deep Microbial

Consortia was dominated by *Oceanospirillales*, with *Altermonadales* being the next highest percentage. Surface Consortia Communities saw initial increases in the relative abundance of *Alphaproteobacteria sphingomonadales* before a decrease at the final time point where *Alphaproteobacteria kiloniellales* dominated the communities. Deep communities saw an increase in *Gammaproteobacteria pseudomonadales* and *Alphaproteobacteria caulobacterales* while a decrease in *Gammaproteobacteria alteromonadales*.

Samples from the same starting consortia were grouped together on nonmetric multidimensional scaling (NMDS) plots (Figure 5.3), indicating that the community structure was similar across media conditions based on starting a community. Permutational multivariate analysis of variance (PERMANOVA) tests reinforced this, showing starting culture as the significant factor in relation to community structure (Table 5.3).

Nutrient Analysis

Total nitrogen, total phosphorus, phosphate, silicate, and ammonia were measured at all time points and conditions except timepoint 2 for Surface Media and Deep Culture was not used in nutrient analysis due to a lack of necessary volume. Nutrients were fit to the NMDS plot, and the only nutrient to show a significant ($p<0.05$) correlation with the NMDS plots was ammonia, where levels were higher for all surface cultures, especially during the later time points. Ammonia concentrations per time point are displayed in Figure 5.4.

Hydrocarbon Analysis

The average total aliphatic hydrocarbons saw a 19.7 to 40.3 percent reduction, shown in Figure A.5, over the eight-week time period for all conditions except for deep media surface culture with methane amendment, which only saw a 1.1% decrease. Deep media deep culture saw the largest reduction in total aliphatic concentration. Although not the highest rate of decrease, surface media deep culture was the only condition to see a significant ($p<0.05$) change in the averages from time point 0 to eight weeks through a paired t-test.

Polyaromatic hydrocarbons (PAHs) also generally saw a decrease in concentrations over the eight weeks, as shown in Figure 5.6, except for the deep media

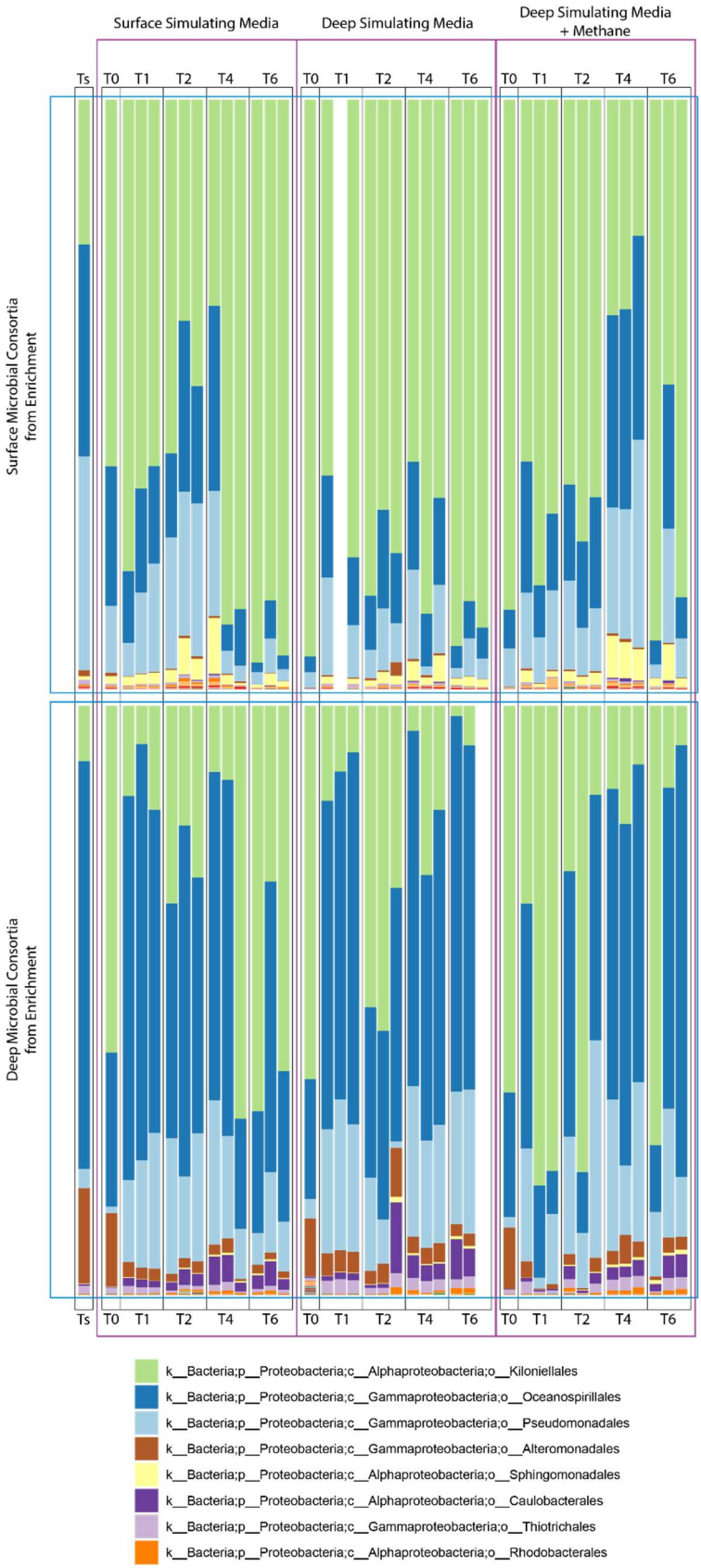

Figure 5.2. Taxa Plots of the Microbial Communities

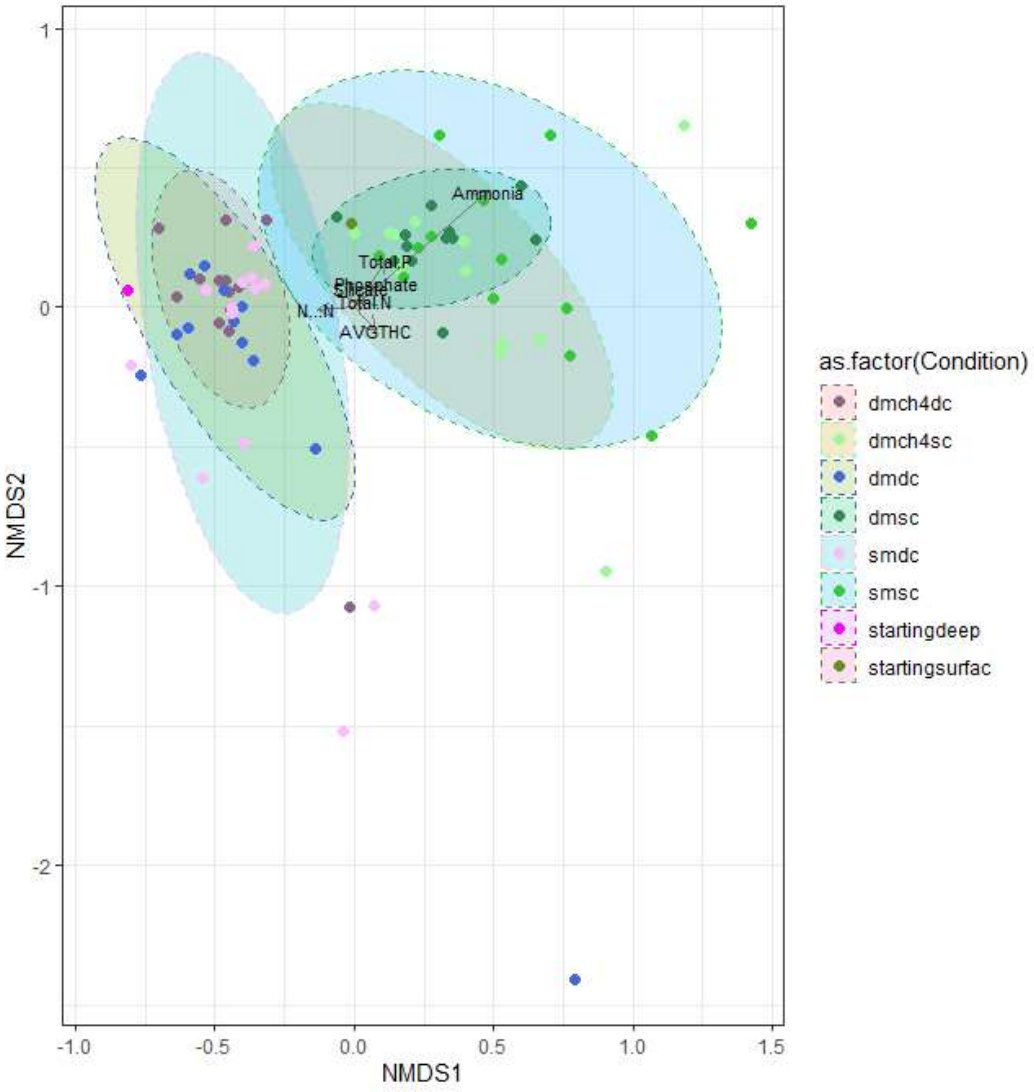

Figure 5.*3.* NMDS Plot

Table 5.3. PERMANOVA

	Df	SumOfSqs	R2	F	Pr(>F)	
Starting Culture	1	3.2918	0.35137	41.3854	0.01	**
Media	2	0.1906	0.02034	1.1979	0.18	
Residual	74	5.8860	0.62828			
Total	77	9.3684	1.00000			

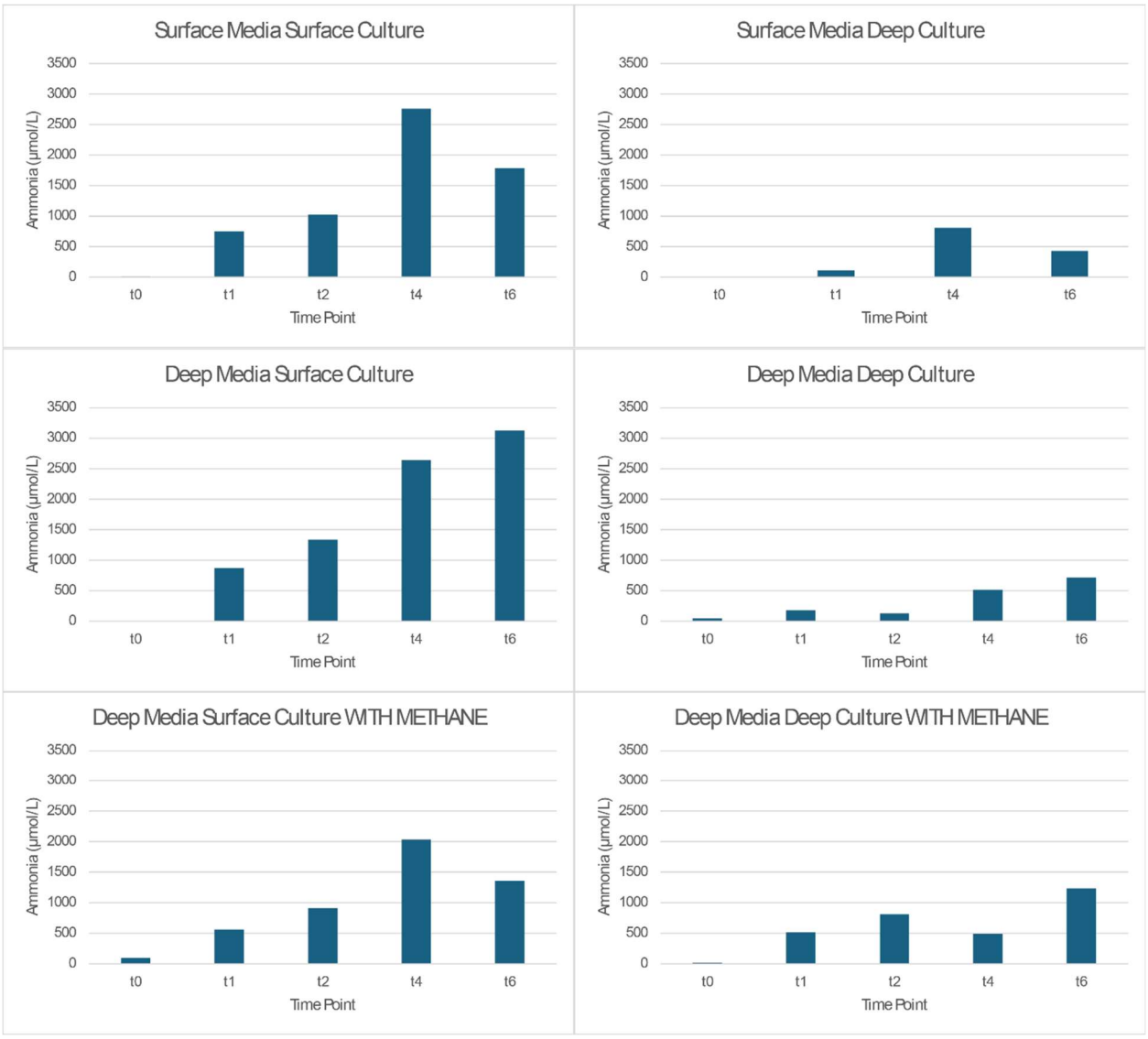

Figure 5.4. Ammonia Concentrations in µmol/L

surface culture with methane amendment, which saw both increases and decreases in PAHs. Both cultures had larger reductions in PAHs in their respective media recipes, surface culture doing better in surface media and deep culture doing better in deep media. Both deep media surface culture with methane amendment and deep media deep culture with methane amendment show essentially no reduction in naphthalene; this was due to a lack of naphthalene presence in the time zero bottles allowing for no room to be reduced; this was not the same for C1-Naphthalene, C2-Naphthalene, C3-Naphthalene, and C4-Napthalene which did see decreases from 38 to 84 percent over the two methane amended conditions.

Discussion

This study aimed to mimic the *in situ* conditions and characterize the previously enriched microbial communities' response to the presence of hydrocarbons, changes in ambient nutrients, and the amendment of methane gas using 16S rRNA sequencing and hydrocarbon analysis via gas chromatography-mass spectrophotometry (GC-MS). Microcosms were used to explore the potential for oil biodegradation under differing nutrient concentrations. The effects of methane amendments on community structure and hydrocarbon degradation are also reported in hopes of showing if methanotrophs can be enriched to aid in biodegradation. Microbial communities were limited in diversity based on the *in situ* microbial communities having been previously enriched with oil amendments on board the sampling cruise before being turned into glycerol stocks.

Enriched surface communities and enriched deep communities were different at all time points. Communities clustered by starting inoculum in the NMDS plot indicate the larger importance of starting a community structure than media conditions. The NMDS results indicate a failure to reject the first null hypothesis. Both communities were completely dominated by proteobacteria (99.10% to 100% of reads), as was expected, seeing as the starting enriched inoculum reads contained 99.76% proteobacteria for the surface community and 99.99% for the deep community. The top three microbial orders present in all conditions were Kiloniellales, Oceanospirillales, and Pseudomonadales.

Methane amendments to the microcosms did not notably increase methanotrophs or overall methylotrophs, indicating that the addition of methane did not

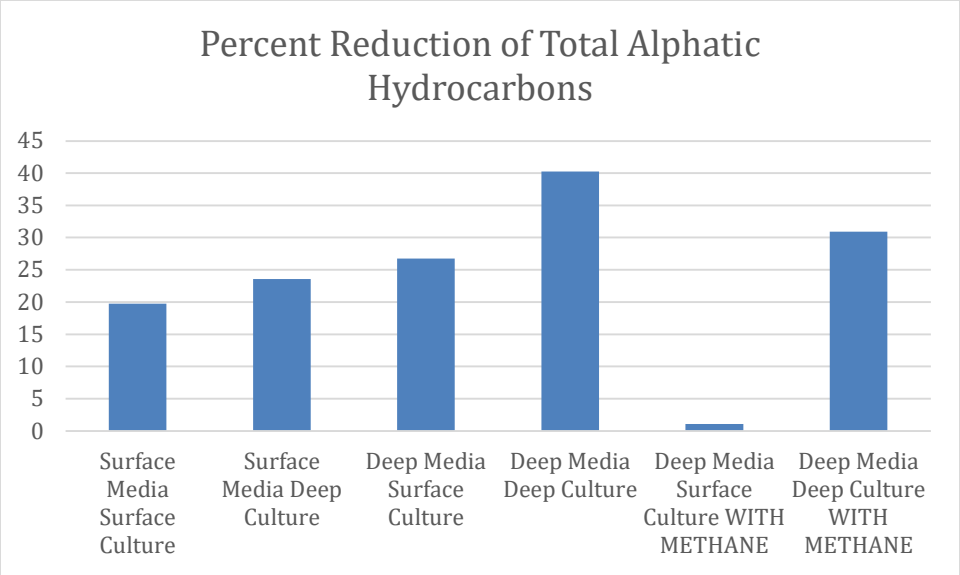

Figure 5.5. Percent Reduction in Total Aliphatic Hydrocarbons over 8 weeks

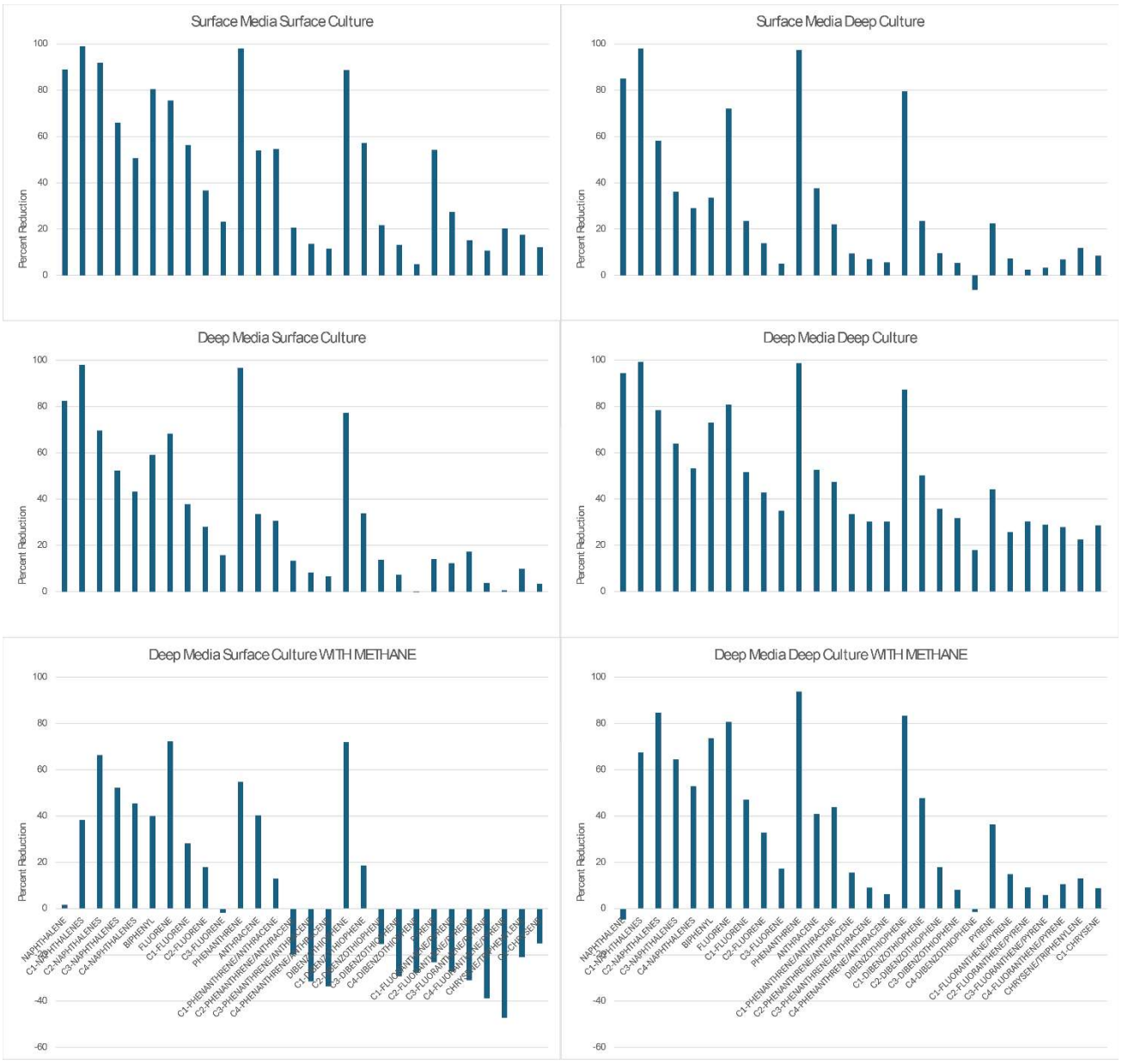

Figure 5.6. Polyaromatic Hydrocarbon Percent Reductions over 8 weeks

encourage a bloom in methanotrophs. Methanotrophic genus present in the samples included Methylosinus and Methylomonus, neither of which were present in the starting inoculums' reads. No functional gene analysis was conducted to detect if any methane monooxygenase enzymes were active; this would be helpful for future studies. The largest methylotrophic genus present is *Methylophaga*, which ranged from 0.03% to 2.40% of reads. Because of the lack of methanotrophs in taxa analysis, the third null hypothesis failed to be rejected. In contrast, Techtmann et al. (2015) reported the importance of methanotrophy in the deep waters of the Eastern Mediterranean, where the methanotroph *Methylococcales* was deemed an indicator species and comprised 5-20% of deep water reads.

In this study, previously crude oil enriched starting cultures were used to mimic the communities' post-spill response to nutrient variation and methane amendment. However, future studies may benefit from an inoculum more representative of the *in situ* community as our taxa plots, even at the starting time point, varied quite substantially from reported on-board microcosms sampling (Techtmann et al., 2015) and microcosms, (Liu et al., 2017). Most noticeable was the absence of Archaea and the stronger presence of Alphaproteobacteria in our community structures. As for microcosms, this is likely mainly due to the rapid nature of the testing in Liu et al. (2017) which covered 72 hours where the ending deep water results did see a trend toward fewer Archaea and the enlarged presence of Alphaproteobacteria, while our enriched starting inoculum was grown from glycerol stocks for two weeks prior to inoculation to provide a large enough volume for the microcosms.

The similarities between starting consortia across media continued across hydrocarbon degradation rates except for Deep Media Surface Community with Methane Amendment, which was an anomaly in both aliphatic and PAH degradation. Deep media surface community with methane amendments had almost no aliphatic degradation and saw increases in some PAHs over the 8-week period, while a decrease in others. As for the other conditions, aliphatic hydrocarbon degradation was slightly higher in deep media without methane amendments, with deep cultures performing better. PAH degradation was best for deep culture in deep media and best for surface culture in surface media, indicating that microbial communities are, understandably,

likely best suited for their natural nutrient conditions. The second null hypothesis failed to be rejected as aliphatic degradation was minimally different between starting consortia. This does not negate the need for future studies to examine nutrient amendments as a possible method for encouraging natural attenuation of oil spills, as this study only utilized minimal differences in phosphate, ammonium, nitrate, and iron to mimic known differences of in situ conditions. One potential area of interest may be phosphate, as the Eastern Mediterranean is known to have phosphate limitations (Krom et al., 2010).

This study reinforced the knowledge that native Eastern Mediterranean Sea bacteria have a good oil degradation potential while also illuminating some differences and similarities between the surface and deep-water hydrocarbon-enriched communities. These results help better understand the biodegradation abilities of native communities, which can shape oil spill response planning.

Conclusions

This study reinforced knowledge about the hydrocarbon degradation capabilities of the Eastern Mediterranean Sea's native bacteria; however, it is limited by the starting consortia microbial community structures, which diverge from *in situ* community structures as they have been previously enriched with oil and subject to bottle effects (changes in enclosed systems over time that cause variation in microbial community structure from initial sampling). Future studies with methane amendments are recommended to use inoculum more representative of *in situ* community structure as previous publications have noted the importance of methanotrophs in the Eastern Mediterranean, but they were absent from our starting consortia. Other future directions of interest include nutrient amendments, especially phosphate amendments that are larger than the difference seen in this study, which did not go above the highest phosphate levels of the Eastern Mediterranean which are still low comparatively to other marine basins.

Chapter 6
Dissertation Conclusions

This dissertation added to the knowledge of methanotrophic bacteria and their uses in both sand aggregation and oil spill response planning. Results from Chapter 3 indicate that methanotrophic strains and the EPS they produce are capable of being used for bioaggregation, especially in desert applications where methanotrophs are ubiquitous and have the benefit of being able to use methane. This study was limited by its own analysis techniques which could be more indicative of natural conditions. Future research is needed to study more methanotrophic bacterial strains' bioaggregation capabilities as well as growth conditions that encourage higher rates of EPS production rates. Results from chapter 5 reinforced some previously known hydrocarbon biodegradation knowledge about the *in situ* microbial community of the Eastern Mediterranean, as well as opening the door for further research into nutrient and methane amendments in oil spill response planning.

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