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The Effect of Nutrient Limitations on the Production of Extracellular Polymeric Substances by Drinking-Water Bacteria

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The Effect of Nutrient Limitations on the Production of Extracellular Polymeric Substances by Drinking-Water Bacteria

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Dedication

This thesis is dedicated to my parents, Mom and Doug.

Thank you for always listening and supporting me.

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Abstract

The Effect of Nutrient Limitations on the Production of Extracellular Polymeric Substances by Drinking-Water Bacteria

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Biological filtration (biofiltration) of drinking-water is gaining popularity due the potential for biodegradation of an array of contaminants not removed by traditional drinking-water processes. However, previous research has suggested that biomass growth on biofilter media may lead to increased headloss, and thus, greater energy and water requirements for backwashing. Research has suggested that the main cause of headloss might be due to extracellular polymeric substances (EPS) rather than the bacterial cells themselves. As EPS production has been shown to increase under nitrogen- and phosphorus-limited or -depleted conditions, the goal of this research was to add to the body of knowledge regarding biofiltration by studying the relationship between EPS production and nutrient limitations in drinking-water.

Batch experiments with a synthetic groundwater were run with a mixed community of drinking-water bacteria under nutrient-balanced (a molar carbon to nitrogen to phosphorus ratio [C:N:P] of 100:10:1), nutrient-limited (e.g., C:N:P of 100:10:0.1), and nutrient-depleted conditions (C:N:P of 100:0:1 or 100:10:0). After 5 days, growth was measured as the optical density at 600 nanometers (OD_{600}), and the concentrations of free and bound carbohydrates and proteins, the main components of EPS, were measured. In batch experiments with 2.0 and 0.2 g/L as carbon (mixture of

acetic acid, mannitol and sucrose) increases in EPS production per OD_{600} and decreases in growth were noted under nutrient-depleted conditions. When the same experiments were conducted with a pure culture of *Bacillus cereus*, bound polysaccharides normalized to OD_{600} increased under nitrogen- and phosphorus-depleted conditions.

Since previous research suggested that *Bradyrhizobium* would be an important player in EPS production in drinking-water biofilters, similar batch experiments were conducted with *Bradyrhizobium*. However, due to experimental challenges with *Bradyrhizobium japonicum* USDA 110, differences in EPS production under nutrient limitations could not be reliably assessed. Additional work is required with *Bradyrhizobium*.

Recommendations for future work include the replication of these batch conditions in steady-state chemostats containing biofilm attachment media and in bench-scale columns. Additionally, future work should include experiments at carbon concentrations as low as 2 mg/L to match typical carbon concentrations in drinking-water biofilters.

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CHAPTER 1: INTRODUCTION

1.1 Background

Biological drinking water filtration (biofiltration) is gaining popularity in the United States (US) due to the potential to economically biodegrade an array of contaminants not removed by traditional treatment processes. However, as very low phosphorus concentrations (<0.01 mg/L), an important nutrient required by bacteria, have been observed in biofilters due to upstream coagulation and sedimentation, a better understanding of how to effectively operate these filters is necessary.

Published literature on the role of nutrients in drinking-water biofilters is limited; however, there is a plethora of research regarding nutrient limitations in drinking-water distribution systems and wastewater systems. Due to concerns regarding regrowth, nutrients have been studied in drinking-water distribution systems. Phosphorus, especially, has been noted to be limiting in distribution systems; small increases in phosphorus are associated with significant increases in regrowth (Miettinen and Vartiainen, 1997; Sathasivan and Ohgaki, 1999; Lehtola et al., 2002; Chu et al., 2005; Fang et al., 2009). Further, research in activated sludge processes has shown that nitrogen- and phosphorus-limitations and changes in the carbon source cause increased production of extracellular polymeric substances (EPS) and affect the efficacy of the activated sludge process (Ericsson and Eriksson, 1988; Lee, 1997; Bura et al., 1998; Liu et al., 2006).

Pure culture experiments, especially with rhizosphere bacteria known for EPS production, also show that nutrient limitations cause increased EPS production (Skorupska et al., 2006; Nirmala et al., 2011; Staudt et al., 2011). One such rhizobium, *Bradyrhizobium*, might be of particular interest in drinking-water biofilters. *Bradyrhizobium* are present in drinking-water biofilters (Pang and Liu, 2007; Niemi et al., 2009) and were shown to be a large portion (15%) of the biofilter microbial community under phosphorus-limited conditions (Lauderdale et al., 2011). Pure culture

experiments with *Bradyrhizobium* note increased EPS production under C:N molar ratios of 100:0.1 and 100:0 (Lopez-Garcia et al., 2001; Quelas et al., 2006). As phosphorus-limitations have been shown to affect EPS production in related rhizobia (Skorupska et al., 2006), it is possible that phosphorus-limitations in drinking-water biofilters also are associated with increased EPS.

Further, increased headloss in biofilters, as compared to traditional filters that are not biologically active, might be attributed to the production of EPS (Mauclaire et al., 2004; Lauderdale et al., 2011). In drinking-water pilot biofilters, an increase in EPS and a 15% increase in terminal headloss were observed under phosphorus-depleted conditions as compared to carbon-limited conditions (Lauderdale et al., 2011). Additionally, it has been suggested that the addition of phosphorus may increase biodegradation in drinking-water biofilters (Nishijima and Shoto, 1997).

Thus, nitrogen- and phosphorus-limitation are associated with increased EPS production, which has been suggested to cause increased headloss in biofilters. The drinking-water industry is considering a range of biofilter supplementation options, including the addition of nitrogen, phosphorus, and carbon sources to target a balanced carbon to nitrogen to phosphorus (C:N:P) molar ratio of 100:10:1 (Lauderdale and Brown, n.d.; Lauderdale et al., 2011; Evans et al., 2012). Supplementing drinking-water biofilters with nutrients such phosphorus and nitrogen might be a practical method of decreasing headloss, allowing for longer run times between backwashing, increasing water recovery, and decreasing energy demands. However, further research on the role of nutrients in EPS production by drinking-water microbial communities is necessary.

1.2 Objectives

The primary objective of this research is to investigate the relationship between nutrient limitations and EPS production by drinking-water bacteria. The results of this research will aid in understanding how to optimize drinking-water biofiltration such that headloss and energy demands might be decreased and filter run times and water recovery might be increased. Previous research (Mauclaire et al., 2004; Lauderdale et al., 2011)

has suggested that nutrient limitations might cause increased EPS production and headloss in drinking-water biofilters; however, this research is the first study to investigate the extent of nutrient (mainly phosphorus and nitrogen) limitations required to trigger increased EPS synthesis and the impact of nutrient sources on EPS production in drinking-water microbial communities.

Batch experiments were conducted using pure cultures of *Bradyrhizobium japonicum* USDA 110 and *Bacillus cereus* and a mixed community of drinking-water bacteria. *Bradyrhizobium* was selected because previous research has shown that this genus is present in drinking-water biofilters (Niemi et al., 2009; Lauderdale et al., 2011), and *B. japonicum* USDA 110 has been used in previous studies of EPS (Becker et al., 1998; Quelas et al., 2010). *B. cereus* is another soil-dwelling bacterium found in drinking-water biofilms (Cerrato et al., 2010). In the current work, various C:N:P molar ratios were tested, and EPS (in terms of carbohydrates, proteins, and lipids) and bacteria were quantified over time. These experiments investigated the link between nutrient limitations and EPS production in drinking-water bacteria.

1.3 Thesis Overview

This thesis focuses on the effect of nutrient limitations on the production of EPS by drinking-water microbial communities. Batch experiments were conducted with pure cultures and a mixed community of drinking-water bacteria. The amount of EPS (as carbohydrates, proteins and lipids) produced at various carbon, nitrogen and phosphorus concentrations was measured and normalized to bacterial growth. Chapter 2 reviews published literature on the application of biological drinking-water treatment, the relationship among nutrient limitations, nutrient sources, and EPS production in pure cultures, drinking-water systems, and wastewater systems, and EPS production by rhizobia such as *Bradyrhizobium*. Chapter 3 outlines the materials and methods, and Chapter 4 discusses the results of this study and their implications. Lastly, Chapter 5 summarizes this research study and proposes avenues for future work.

CHAPTER 2: LITERATURE REVIEW

This chapter begins by reviewing the application of biological filtration in drinking-water treatment. The chapter follows by summarizing previous research relating nutrient limitations, biological growth, and extracellular polymeric substance (EPS) production in pure cultures, wastewater systems, and drinking-water systems. Finally, *Bradyrhizobium*, a genus found in drinking-water systems and potentially an important EPS-producer, is discussed.

2.1 Biological Drinking-Water Treatment

Although biological drinking-water treatment has only recently grown in popularity in the United States (US), biological treatment of wastewater has been employed for the past century to remove organics, nutrients and other contaminants (Brown, 2007). Furthermore, biological drinking-water treatment is common in Europe (Bouwer and Crowe, 1988) and Canada (Emelko et al., 2006). Many recent developments have led to the emergence of biologically active filtration (biofiltration) in drinking-water treatment such as new regulations on disinfection by-products (DBPs) and biodegradable contaminants (e.g., perchlorate and bromate) (Brown, 2007). Biofiltration also complements new membrane-based systems which are known for bio-fouling and require costly and complex residual treatment (Brown, 2007). Additionally, the desire for green treatment technologies that remove, rather than concentrate, contaminants and that resolve US water-energy nexus issues has made biofiltration attractive (Brown, 2007).

Biological drinking-water treatment uses indigenous bacteria and is followed by disinfection, thereby minimizing health risks due to bacteria. Both fixed-bed and fluidized-bed processes are employed (Brown, 2007). The influent to biological treatment is commonly pre-ozonated to increase the removal of taste and odor compounds and other organic compounds, thereby reducing distribution regrowth and DBP formation (Cipparone et al., 1997; Nerenberg et al., 2000; Brown, 2007). There are

many advantages to the implementation of biofiltration on granular activated carbon (GAC), especially coal-based GAC, over sand or anthracite media. Microorganisms in biofilters with GAC have been reported to have higher biomass concentrations (LeChevallier et al., 1992; Wang et al., 1995), specific growth rates, and biodegradation rates over other non-adsorbing media (Li and Digiano, 1983). The adsorption ability and porous surface area of GAC allow non-biodegradable contaminants to be adsorbed and poorly biodegradable organics and substrate to be held in the system for a longer contact-time (Li and Digiano, 1983; Scholz and Martin, 1997). GAC provides protection from shear during backwashing and the functional groups enhance biofilm attachment (Fox et al., 1990; Scholz and Martin, 1997). Further, GAC has been shown to have longer filter runs and higher total organic carbon (TOC) and biodegradable organic matter (BOM) removal over anthracite (LeChevallier et al., 1992; Emelko et al., 2006).

Potential advantages of biological treatment of drinking-water include low operating costs, high water-recovery, low sludge production, low or no chemical requirements, and robustness to a wide range of water qualities and operating conditions (Brown, 2007). Biological treatment can remove natural organic matter (NOM) thereby decreasing distribution regrowth (LeChevallier et al., 1992; Cipparone et al., 1997), DBP precursors (Cipparone et al., 1997), membrane foulants (Hu et al., 2005), inorganics such as perchlorate (Brown et al., 2002, 2005; De Long et al., 2010), ammonium (Rice et al., 1982; Yapsakli et al., 2010), nitrate (Herman and Frankenberger, 1999) and bromate (Kirisits and Snoeyink, 1999; Kirisits et al., 2001, 2002; Davidson et al., 2011), and various heavy metals such as iron, manganese, and copper (Srivastava and Majumder, 2008; Lauderdale et al., 2011). Trace organics such as 2-methyl-isoborneol (MIB) and geosmin (Nerenberg et al., 2000), algal toxins (Hall et al., 2000), endocrine disruptors and pharmaceuticals (Ternes et al., 2002; Snyder et al., 2007), and halogenated organics such as perchloroethylene (PCE), trichloroethylene (TCE), dibromochloro-propane (DBCP), and chloroform are amenable to biological degradation (LeChevallier et al., 1992). Biodegradation also can reduce taste, color and odor (Lundgren et al., 1988), residual coagulant requirements (LeChevallier et al., 1992) and disinfection requirements

(Cipparone et al., 1997). Thus, biological treatment may be an effective way of simultaneously removing many contaminants.

2.1.1 Biological Treatment Process Limitations and Optimization

Although biological drinking-water filtration is used at full-scale, there are still concerns and questions regarding biofilter design and performance. Performance variability might be caused by differences in the influent water quality. Changes in dissolved oxygen, the type and concentration of organics, nutrients such as nitrogen and phosphorus, pH, temperature and toxic synthetic organic compounds or heavy metals have the potential to alter the quantity of biomass, microbial community and microbial activity (Bouwer and Crowe, 1988; Scholz and Martin, 1997; Simpson, 2008). Changes in flow could lead to biomass washout or reduction (Gray et al., 1980). Additionally, biological processes generally have a longer startup time than do physical-chemical processes (Bouwer and Crowe, 1988).

There is limited information about byproducts from the growth of microorganisms in drinking-water treatment processes. Further investigation of the health effects of endotoxins, soluble microbial products (SMPs), incompletely degraded organic compounds and the results of the reaction of these compounds with disinfectants is needed (Rice et al., 1982). Microorganism release due to sloughing of biomass is likely. While indigenous bacteria in biological drinking-water treatment processes are thought to be non-pathogenic, additional research of microbial communities in drinking-water processes and causes of biomass sloughing is suggested (Rice et al., 1982; Bouwer and Crowe, 1988). Further, biofilms, especially attached to filter media such as sand or GAC, may be more resistant to disinfection (LeChevallier et al., 1984).

Without optimization, energy requirements for backwashing a biofilter might increase over those for a traditional filter. Biological drinking-water processes commonly involve a fixed biofilm system with a granular support media (Kim and Logan, 2000; Brown, 2007). Over time, biomass builds up on the media, thereby restricting flow and increasing headloss. Thus, systems are periodically taken off line and backwashed (Brown et al., 2005; Lauderdale et al., 2011). It should be noted that

there is limited understanding of the effects of backwashing on microbial activity, biodegradation potential, and biofilm development (Simpson, 2008). A study of pilot GAC filters concluded that although backwashing detaches biofilms, the biomass is able to build up to pre-backwashing concentrations prior to the next backwashing cycle (Gibert et al., 2012). Additionally, there are questions as to whether backwashing should be conducted with or without disinfectant; as expected, higher biomass accumulation on filter media is achieved when no disinfectant is applied to the filter (LeChevallier et al., 1992; Wang et al., 1995).

Process optimization includes bench-scale and pilot-scale reactors in combination with mathematical models and microbial analyses. Microbial analyses allow the identification of conditions to achieve the greatest biological activity and growth and the identification and tracking of bacterial communities (Brown, 2007). Online monitoring tools are available for measuring temperature, headloss, and concentrations of dissolved oxygen, total organic carbon, and pre-oxidants (Evans et al., 2011). Off-line tools are available to measure the concentration of dissolved organic carbon (DOC), assimilable organic carbon (AOC), and carboxylic acids (Evans et al., 2011). Media samples also might be analyzed to determine the bacterial populations and the concentration of adenosine triphosphate (ATP), which is a measure of biological activity (Evans et al., 2011).

Additionally, industry is actively considering supplementation of drinking-water biofilters with nutrients (such as nitrogen and phosphorus) and substrate (electron donor) as a method of optimizing biofilter operation (Lauderdale and Brown, n.d.; Lauderdale et al., 2011; Evans et al., 2012). Further investigation of the role of nutrient limitations in drinking-water biofiltration will be of great value to industry for process optimization.

2.1.2 Nutrients, Extracellular Polymeric Substances (EPS) and Biofilter Headloss

Research has suggested that nutrient limitations might lead to increased production of EPS, a major component of biofilms, and that EPS might be a cause of increased headloss in biofilters compared to traditional filters.

Biofilms are a collection of microorganisms attached to a solid surface and embedded in an EPS matrix. EPS provides structural integrity and stability to microorganisms and promotes adhesion to surfaces. EPS is composed of primarily carbohydrates and proteins, although lipids, humic substances, nucleic acids, uronic acids, and inorganic components might also be present. EPS is both soluble (free) and attached to bacterial cells and/or media (bound). Changes in nutrient conditions, hydrodynamics and substrate concentration have been shown to affect biofilm structure (Wimpenny and Colasanti, 1997; Stoodley et al., 1999). Further, the availability of carbon sources and nutrients, such as nitrogen and phosphorus, are known to affect EPS synthesis in biofilms (Sutherland, 2001).

Mauclaire et al. (2004) examined the pore spaces in a drinking-water biological sand filter. The authors determined that biomass including bacterial cells, carbohydrates and proteins occupied between 6-14% of the top layer pore space and 3-9% of the deep layer pore space. In a highly clogged filter top layer, the study found a maximum ratio of 100:1 v/v of EPS to bacterial cells. Here, bacterial cells were found to occupy 0.01% of pore space whereas total EPS was found to occupy 10% of pore space (approximately 4.2% as proteins and 5.8% as polysaccharides). Considering the top and bottom layers of a heavily clogged and less clogged filter, bacterial cells were found to occupy a maximum of 0.2% of pore space in a highly clogged top layer, and EPS occupied a minimum of 3% of pore space in a less clogged deep layer of the filter. Considering particle deposition, calcite precipitation, and biomass production as possible causes of clogging, the authors concluded that EPS contributed largely to the clogging of the biofilter (Mauclaire et al., 2004).

The role of nutrients in EPS production, and ultimately biofilter clogging, was further investigated using pilot-scale biofilters in Arlington, Texas. This study found a correlation between increased terminal headloss and increased EPS (mg/L of polysaccharides) in the pilot biofilters (Lauderdale et al., 2011). Thus, EPS could be an important parameter to control in biofilters.

2.2 Biological Nutrient Requirements and EPS Production

EPS production has been investigated in lab experiments under environmental stress conditions. The effect of varying nutrient ratios (carbon to nitrogen to phosphorus) and nitrogen and phosphorus sources previously has been investigated in certain pure cultures and wastewater systems. In addition, the role of nutrients in drinking-water distribution system regrowth and biological filter degradation potential has recently been studied. Researchers commonly cite a carbon:nitrogen:phosphorus (C:N:P) molar ratio of 100:10:1 as required for heterotrophic bacteria growth (LeChevallier et al., 1991).

In addition, the quantity of EPS produced has been noted to be influenced by temperature, pH, and calcium, magnesium, iron, and sodium concentrations (Corpe, 1964; LeChevallier et al., 1991; Farrés et al., 1997; Staudt et al., 2011). The presence of calcium has been shown to stimulate EPS production while the presence of iron inhibits it (Corpe, 1964). A study of the photosynthetic bacterium *Rhodopseudomonas acidophila* determined sodium to increase EPS production but calcium to not affect EPS production (Sheng et al., 2006). In a laboratory-scale activated sludge system, calcium and magnesium were shown to increase bound EPS (measured as protein), and high sodium was observed to decrease bound EPS (Higgins et al., 1997). Thus, studies have reached different conclusions about which factors most influence EPS production based on the microorganisms and environmental conditions tested.

2.2.1 An Overview of EPS Production in Planktonic Cells and Biofilms

The composition of EPS varies among microorganisms and environmental conditions but can consist of 40-95% w/w polysaccharides, up to 60% w/w proteins, up to 10% w/w nucleic acids and up to 40% w/w lipids (Flemming and Wingender, 2001). In one study, EPS in activated sludge was composed of 57% w/w proteins, 30% w/w humic substances, 11% w/w carbohydrates, and 1% w/w uronic acids (Frølund et al., 1996). EPS formed by planktonic cells, a biofilm and colonies grown on agar of *Comamonas denitrificans* strain 110 (a common organism studied in wastewater systems) in various nutrient media has been noted to be composed of mainly proteins (3-37%

w/w), nucleic acids (9-25% w/w), and carbohydrates (3-21% w/w). Lipids were noted to be less than 2.5% w/w (Andersson et al., 2009). As a final example, the EPS composition of phenol-fed aerobic granules was determined to be 240, 61 and 51 mg/g volatile suspended solids (VSS) for proteins, carbohydrates and lipids, respectively, which is approximately an EPS protein to carbohydrate ratio of 4 (Adav et al., 2008).

Andersson et al. (2009) investigated EPS production in planktonic versus biofilm cells by *C. denitrificans* strain 110. The study found that biofilm EPS had a protein to carbohydrate ratio of 5:2 while planktonic EPS had a protein to carbohydrate ratio of 1:2. Additionally, planktonic cells of *C. denitrificans* were noted for free EPS production while biofilms were noted for capsular or bound EPS production (Andersson et al., 2009). Compositional differences in EPS from planktonic cells and biofilms also have been identified (Beech et al., 1999; Kives et al., 2006).

The correlation between bacterial growth rates and the amount of EPS produced also varies between biofilm and planktonic cells (Andersson et al., 2009). A study of *Staphylococcus epidermidis* in biofilms showed that increased EPS production correlated with a decreased growth rate; however, a similar relationship was not found in planktonic cells of *S. epidermidis* (Evans et al., 1994). In *Bradyrhizobium japonicum* planktonic cells, an accumulation of EPS during the stationary phase of growth (as compared to exponential growth) was noted under nitrogen-limitation and a decrease in EPS was noted during stationary phase under nitrogen-sufficiency (Lopez-Garcia et al., 2001; Quelas et al., 2006).

2.2.2 The Effect of Nutrient Limitations in Pure Culture Experiments

Numerous pure culture experiments have been conducted to investigate the effects of carbon-, nitrogen- and phosphorus-limitations and carbon and nitrogen sources on EPS production. A summary of the key findings regarding nitrogen limitations and nitrogen sources, phosphorus limitations, and carbon limitations and carbon sources follows. Most studies did not present EPS concentrations normalized to biomass; however, where available, this data is presented.

2.2.2.1 Nitrogen Limitations and Nitrogen Sources

Nitrogen-limitations and various nitrogen sources have been shown to stimulate EPS production and affect the growth and aggregation of cells. The results of studies with *Bradyrhizobium japonicum, Rhizobium tropici* and other *Rhizobium* spp., *Chromobacterium violaceum, Pseudomonas, Rhodopseudomonas acidophila*, and *Azospirillum* are summarized.

A study of *Bradyrhizobium japonicum* under nitrogen-limitation revealed increased EPS production (mg polysaccharide per mg of cell protein) and decreased biomass under nitrogen-limitation or nitrogen-depletion (C:N molar ratios of 100:0.06 and 100:0, respectively) as compared to a C:N molar ratio of 100:0.6 (Lopez-Garcia et al., 2001). Quelas et al. (2006) compared a C:N molar ratio of 100:0.6 to that of 100:0 and noted increased EPS production (mg polysaccharides per mg cell proteins) when no nitrogen was provided. Both studies also showed an accumulation of EPS during the stationary phase of growth under nitrogen-limitation and a decrease in EPS during the stationary phase under nitrogen-sufficiency (comparing young cells at day 5 and old cells at day 14 in mannitol-based medium). Both studies also demonstrated similar exponential growth rates under the different nitrogen conditions (Lopez-Garcia et al., 2001; Quelas et al., 2006).

A study of *Rhizobium tropici* with sucrose as the sole carbon source concluded that a C:N ratio of 20 produced maximum EPS (g/L of polysaccharides and g/L of total ethanol-precipitable material [EPM]) as compared to C:N ratios of 5, 10 and 40 (Staudt et al., 2011). Staudt et al. (2011) determined that the C:N ratio resulting in the greatest production of EPS did not correspond with the ratio allowing for maximum growth.

A study of *Chromobacterium violaceum* grown in glucose noted approximately equal growth and polysaccharide production with ammonia, glutamate, and casamino acids as nitrogen sources. Further considering a variation in ammonium nitrogen concentration, 1-2 mg/mL allowed for maximum growth while 0.1 mg/mL allowed for maximum EPS production. Considering glutamate nitrogen, the highest growth and EPS production were achieved with 0.1 mg/mL of glutamate nitrogen and 10 mg/mL of

glucose. These concentrations correspond to a carbon to nitrogen molar ratio of 100:2 (Corpe, 1964).

The analysis of ten *Rhizobium* strains showed maximum EPS production (μg/mL as glucose) with 2% w/v mannitol and 0.2% w/v potassium nitrate. Experiments comparing 0.1% w/v of various nitrogen sources including potassium nitrate, sodium nitrate, ammonium sulfate, glycine, and glutamic acid demonstrated that potassium nitrate and sodium nitrate resulted in the greatest EPS production. Further experiments then explored the effect of various concentrations (0.05, 0.10, 0.15 and 0.20%) of potassium nitrate when the carbon concentration was held constant. EPS production and growth were greatest at a concentration of 0.2% potassium nitrate (Nirmala et al., 2011).

Studies of other pure cultures have shown a relationship between increased C:N ratio and increased EPS production. A high C:N molar ratio (as high as 100:0) was shown to favor increased EPS production (mg polysaccharides [mg protein]$^{-1}$) in *Pseudomonas* NCIBI 1264. The lowest C:N molar ratio tested (100:7.5) produced approximately 25% less EPS (2 mg [mg protein]$^{-1}$ as compared to 8 mg [mg protein]$^{-1}$) than did the case where no nitrogen was added (Williams and Wimpenny, 1977). A study of the photosynthetic bacterium, *Rhodopseudomonas acidophila,* by Sheng et al. (2006) demonstrated that increased EPS production (but low cell growth) occurred at low concentrations of both carbon and nitrogen. Several C:N molar ratios between 100:0.7 and 100:250 based on carbon concentrations between 0.1 and 1.75 g/L were tested. Generally, total EPS (proteins and carbohydrates, mg g/l^{-1} dry cells) increased with lower absolute carbon concentration and higher C:N ratio. The highest total EPS was observed at a carbon concentration of 0.1 g/L and C:N ratio of 100:13; note that this was the highest C:N ratio tested at this low carbon concentration (Sheng et al., 2006). Finally, a study of rhizobacteria of the genus *Azospirillum* concluded that a high C:N molar ratio (100:1.8) induces aggregation as compared to a low C:N molar ratio (100:8.4) (Burdman et al., 2000).

While the carbon to nitrogen ratio that produced maximum EPS production varied among the studies, it is clear that high C:N ratios increase EPS production. The studies

also show that high C:N ratios result in less biomass growth as compared to lower C:N ratios, and the source of nitrogen affects the quantity of EPS produced.

2.2.2.2 Carbon Sources

Several studies have shown that the available carbon source and concentration greatly affects EPS production. However, the carbon source resulting in the greatest EPS production varies based on the study and microorganism and does not correlate with the carbon source allowing for greatest growth. The study of *C. denitrificans* strain 110 showed a decrease in biomass and increase in EPS over time in a minimal acetate medium as compared to Nutrient Broth or sterile wastewater (Andersson et al., 2009). Staudt et al. (2011) analyzed growth and EPS production of *R. tropici* on arabinose, glucose, sucrose, mannitol, fructose, and glutamate. Maximum EPS production (g/L polysaccharides and total EPM) was determined with glucose and sucrose. EPS was not normalized to biomass in this study. Slowest growth (Colony Forming Units [CFU] [mL-h]$^{-1}$) was obtained using glutamate and fastest growth rates were achieved with fructose and mannitol (Staudt et al., 2011). Williams and Wimpenny (1977) studied *Pseudomonas* NCIBI 1264 and concluded that the composition of EPS was unchanged by variations in carbon and energy source. EPS synthesis was noted with multiple mono-, di-, and tri-saccharide carbon substrates but was generally higher with hexoses (particularly glucose and fructose) than with pentoses (Williams and Wimpenny, 1977). Increasing EPS production of the photosynthetic bacterium, *R. acidophila*, was determined in the following order of carbon sources: malonate, succinate, propionate, butyrate, acetate, and benzoate (Sheng et al., 2006). Lastly, a study of ten *Rhizobium* strains indicated highest EPS production (μg/mL as glucose; EPS normalized to biomass was not presented) with mannitol followed by sucrose, glucose and maltose as compared to galactose, fructose, and mannose (Nirmala et al., 2011). Specific conclusions for why certain carbon sources result in greater EPS production were not offered; however, there was consensus that the carbon source resulting in maximum EPS production varied by species (Nirmala et al., 2011).

The concentration of carbon relative to the concentrations of other medium components (held at constant concentrations) also appears to be important. A study of *C. violaceum* with ammonium sulfate held constant at 0.1 mg/mL noted that a concentration of 10 mg glucose/mL produced maximum EPS polysaccharides (as compared to a range of 0.1-20 mg/mL glucose) and 5-19 mg glucose/mL produced maximum growth (Corpe, 1964). In the study of ten *Rhizobium* strains, the highest EPS production (μg/mL) was noted at a mannitol concentration of 2% added to yeast-extract mannitol (YEM) medium as compared to mannitol concentrations of 1, 3 and 4% (Nirmala et al., 2011).

2.2.2.3 Phosphorus Limitations

While information about the effect of phosphorus-limitations in pure culture experiments is limited, a few studies suggest that phosphorus-limitation causes an increase in EPS production. Phosphorus-limitations in rhizobial bacteria have been noted to increase EPS production (as reviewed by Skorupska et al., 2006). In a study of *Klebsiella* I-714, increased phosphate buffer addition corresponded to decreased EPS production. Maximum EPS production was obtained with no phosphate addition and controlled pH (Farrés et al., 1997). However, in a study of *Pseudomonas* NCIBI 1264 no relationship was found between EPS production and phosphate-limitation; it was hypothesized that a decrease in pH below 5 (due to fermentation at the low buffering capacity) suppressed EPS production (Williams and Wimpenny, 1977). Thus, the effects of extreme pH conditions may confound the effects of phosphorus-limitation on EPS production. However, all studies agree that phosphorus-limitation is expected to increase EPS production.

2.2.3 The Effect of Nutrient Limitations in Drinking-Water

Although there is limited research regarding the role of nutrient limitations in biological drinking-water treatment, there has been substantial research regarding nutrients in drinking-water distribution systems.

2.2.3.1 Carbon as the Limiting Nutrient in Distribution Systems

LeChevallier et al. (1991) showed a correlation between coliform growth and rainfall, temperature, and nutrients (specifically AOC and TOC). AOC was shown to quickly decrease through the distribution system; however, nitrite-N, ammonia-N, ortho-phosphate, total P, and TOC were not shown to decrease through the distribution system. These data suggested that organic carbon was the limiting nutrient in the effluent of the New Jersey American Water Co. – Swimming River Treatment Plant. The study suggested a limit of 50 μg/L of acetate carbon equivalents to prevent regrowth (LeChevallier et al., 1991). The results of the LeChevallier study are close to that of previous results recommending a limit of 10 μg/L AOC (Van der Kooij, 1990).

2.2.3.2 Phosphorus and Nitrogen as the Limiting Nutrients in Distribution Systems

In a study of six waterworks in Finland from surface and groundwater sources, Miettinen and Vartianinen (1997) found phosphorus to be limiting and important to microbial regrowth in distribution systems. Phosphate-phosphorus concentrations were below the detection limit of 2 μg/L (suggested to be caused by upstream treatment processes). The addition of 50 μg/L of phosphorus increased microbial growth. However, other inorganic nutrients including nitrogen, potassium, magnesium, and calcium did not show the same relationship to microbial growth. The study suggested that very minor phosphorus concentration changes have the potential to greatly affect microbial regrowth (Miettinen and Vartiainen, 1997). The sampling of 14 points in the Tokyo drinking-water distribution system (Sathasivan and Ohgaki, 1999) and lab-scale biofilm experiments using water from a Finland waterworks also identified phosphorus as the limiting nutrient (Lehtola et al., 2002). A study in Taiwan found that the addition of nitrate, ammonia, and phosphorus stimulated growth in a water treatment plant effluent and the addition of nitrate and phosphate stimulated growth in water distributed to an urban area. However, the addition of nitrate, ammonia, or phosphorus did not stimulate growth in water distributed to a suburban area; the lack of growth stimulation in the suburban area was suggested to indicate that that water already contained sufficient

nutrients for growth. The study also concluded that biofilm formation was greater with the addition of phosphorus than with an equivalent addition of nitrogen (Chu et al., 2005). Fang et al. (2009) investigated the effect of phosphorus on not only microbial growth but also EPS production and biofilm stability. The addition of 3, 30 and 300 μg/L of phosphorus to de-chlorinated tap water both increased microbial growth and decreased EPS production by as much as 81%; decreased EPS production and increased biofilm structure heterogeneity were hypothesized to indicate reduced tolerance to disinfectants (Fang et al., 2009).

2.2.3.3 Nutrient Limitations in Biological Filters

Nishijima et al. (1997) studied the effect of the addition of phosphorus to biologically active carbon (BAC) on the biodegradation of organic compounds. Using jar tests to coagulate water with various concentrations of polyaluminum chloride (PAC) and alum and a continuously stirred reactor with BAC, the study concluded that coagulation and sedimentation reduced phosphorus concentrations to between 0.01-0.002 mg/L when the influent water contained either 0.213 or 0.063 mg/L phosphorus. Growth and biodegradation was determined to be phosphorus-limited and both were enhanced with the adsorption of phosphorus on BAC or the addition of phosphorus to the influent water (Nishijima and Shoto, 1997). A different study of biofilters found that active biomass increased with increasing EPS near the inlet of the biofilter (Gao et al., 2008).

The role of nutrients in EPS production, and ultimately biofilter clogging, was investigated using pilot-scale biofilters in Arlington, Texas. A phosphorus-depleted biofilter (C:N:P molar ratio of 100:10:0) compared to a control biofilter (100:7:1) produced less free and bound EPS (measured as mg/L carbohydrates). Additionally, the terminal headloss in the control biofilter compared to the phosphorus-depleted biofilter was 15 percent less, which allowed for a 15 percent longer run time and was estimated to save over $17,000 annually. Thus, the supplementation of phosphorus was very practical (Lauderdale et al., 2011).

2.2.4 The Effect of Nutrient Limitations in Wastewater Treatment

The effects of nutrient limitations have been investigated in wastewater treatment processes (Sheng et al., 2010). As with pure cultures and drinking-water experiments, carbon sources have been shown to affect process operations in wastewater treatment. For example, comparing activated sludge reactors fed with acetate and glucose, acetate achieved overall better treatment flocculation, lower effluent suspended solids, and a lower sludge volume index. Glucose-fed sludge was noted to have the greatest amount of EPS (Li and Yang, 2007). In addition, phosphorus- and nitrogen-limitations greatly affect wastewater treatment as described below.

2.2.4.1 Phosphorus Limitation

Phosphorus-limitation is an important consideration in wastewater treatment. Bura et al. (1998) fed a synthetic wastewater into a sequencing batch reactor at chemical oxygen demand to nitrogen to phosphorus (COD:N:P) ratios of 100:5:1 (balanced), 100:5:0 (phosphorus-depleted), 100:5:0.2 (phosphorus-limited), and 100:1:1 (nitrogen-limited). COD:N:P ratios affected the composition and structure of the floc matrix, especially at limited or depleted phosphorus conditions. Total EPS (carbohydrate plus protein, mg/g VSS) was higher under phosphorus-limitation than under balanced conditions; however, differences between the phosphorus-limited and phosphorus-depleted conditions were minimal. Also under both nitrogen- and phosphorus-limitations, proteins consisted of a greater portion of total EPS as compared to the balanced condition. Authors also noted that differences in EPS composition under various nutrient conditions suggest that EPS quality, in addition to quantity, is important to floc structure. The greatest changes in sludge properties (including hydrophobicity, surface charge and the bound water content) were observed under phosphorus-limitation (Bura et al., 1998).

The results of Bura et al. (1998) agree with the results of Liu et al. (2006), Ericsson and Eriksson (1988), and Lee (1997). Under phosphorus-limitation (COD:N:P of 100:5:0.05 and compared to a COD:N:P of 100:5:1), increased phosphatase activity

and increased EPS (total polysaccharides) was observed in sludge. A change in microbial floc structure was also noted (Liu et al., 2006). Sludge swelling under phosphorus-limitation was explained by an increase in EPS at an increased biochemical oxygen demand to phosphorus (BOD:P) ratio (Ericsson and Eriksson, 1988). Lab studies in a sequencing batch reactor with synthetic feed water found phosphorus-depleted conditions (COD:N:P of 100:5:0) to increase carbohydrate, protein and DNA concentrations of EPS, and found phosphorus-limited conditions (COD:N:P of 100:5:0.2) to increase carbohydrates, uronic acids, DNA and proteins as compared to a nutrient-balanced condition (COD:N:P of 100:5:1) (Lee, 1997). Additionally, the best COD:P ratio, in terms of sludge flocculation, dewaterability and settleability, was concluded to be 100:3 to 100:5 (Hoa et al., 2003).

2.2.4.2 Nitrogen Limitation

Nitrogen-limitation is another important consideration in wastewater treatment. Hoa et al. (2003) found a relationship among nitrogen-limitation, EPS (polysaccharides and proteins) and various sludge properties. Flocculation, dewaterability, and settleability were improved at COD:N<100:2 and COD:N>10 (Hoa et al., 2003). However, Durmaz and Sanin (2001) concluded that the mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations increase with increased C:N ratios and that higher C:N ratios (up to 40) produced EPS with more carbohydrates compared to proteins while a low C:N ratio (down to 5) produced protein-rich EPS. Authors noted that at low C:N ratios (under carbon-limitation) carbon is utilized solely for synthesis and energy, while at a high C:N ratios (nitrogen-limitation) excess carbon is used to produce MLVSS or EPS (Durmaz and Sani, 2001). Thus, there is some disagreement as to the C:N ratio allowing for maximum EPS production. However, there is agreement that nitrogen-limitation does affect EPS production. Nitrogen-limited and nitrogen-depleted (COD:N:P of 100:1:1 and 100:0:1) experiments demonstrated a decrease in proteins and DNA concentrations of EPS (Lee, 1997).

In addition, bound and free EPS react differently to nitrogen-limitation. Tightly bound EPS was determined to be independent of C:N ratio while loosely bound EPS

proteins increased and carbohydrates decreased at low C:N ratios. Additionally, floc sizes were shown to increase at high C:N ratios and decrease at low C:N ratios. Only loosely bound proteins were shown to correlate positively with flocculation, settleability, and dewaterability of activated sludge flocs (Ye et al., 2011).

Thus, there is no consistent nitrogen- or phosphorus-limitation that causes maximum EPS production in all wastewater systems; however, it is apparent that both nitrogen- and phosphorus-limitation and the nature of the carbon source greatly affect wastewater treatment operations.

2.3 Rhizobia and EPS Production

As drinking-water biofilter headloss has been linked to EPS production, knowledge of the microbial communities in biofilters and how these communities react to changes in nutrient conditions is essential to optimizing the operation of biofilters.

2.3.1 Bacterial Communities Dominating Biofilters

Prevalent isolates found in drinking-water treatment include *Burkholderiales*, *Sphingomonas*, and *Afipia, Bosea* and *Bradyrhizobium* from the *Bradyrhizobiaceae* family. *Burkholderiales* are noted for mineralizing dissolved organic matter (Niemi et al., 2009). *Bosea* are known for nitrate reduction, and *Bradyrhizobium*, and other *Rhizobiales*, are associated with the production of EPS and the ability to utilize versatile carbon sources. *Rhizobiales* are further noted as consistent foulants in wastewater treatment membranes (Pang and Liu, 2007).

A recent Water Research Foundation study of drinking-water pilot-scale BAC filters found that *Burkholderia* were dominant under carbon-limited conditions, and *Bradyrhizobium* were dominant under phosphorus-depleted conditions; increased EPS production was noted under the phosphorus-depleted condition. The *Bradyrhizobium* population increased from 1.5 to 15 percent of the microbial community under phosphorus-depleted as compared to carbon-limited conditions (Lauderdale et al., 2011).

2.3.2 *BRADYRHIZOBIUM* AS EPS-PRODUCERS UNDER NUTRIENT LIMITATIONS

Bradyrhizobium (a rhizobial genus) are well-known EPS producers (Garrity et al., 2005) that have been noted to be present in drinking-water biofilters (Pang and Liu, 2007; Niemi et al., 2009; Lauderdale et al., 2011). *Bradyrhizobium* is a genus of gram-negative, aerobic, bacilli of the Bradyrhizobiaceae family. *Bradyrhizobium* are soil-dwelling bacteria that nodulate leguminous plants, especially *Glycine* (soybean), initiating a nitrogen-fixing symbiosis (Garrity et al., 2005). They are particularly known for producing EPS composed of glucose, mannose, galacturonic acid, acetate, 4-O-methyl-galactose, and galactose, particularly when grown on glycerol, gluconate or mannitol (Puvanesarajah et al., 1987; Garrity et al., 2005). However, the composition of EPS varies based on the carbon source (Karr et al., 2000).

Colonies are typically opaque, white, and convex. Colonies may become tan with prolonged incubation on carbohydrate-containing solid medium (Garrity et al., 2005). *Bradyrhizobium japonicum* USDA 110 is noted for forming both small, dry (raised to convex) and large, mucoid colonies when grown on yeast-extract-mannitol (YEM) agar. Large colonies exceed 1 mm after 7-10 days at 28°C while small colonies are less than or equal to 1 mm in diameter (Fuhrmann, 1990; Basit et al., 1991).

B. japonicum USDA 110 has been used in several studies of EPS production (Puvanesarajah et al., 1987; Karr et al., 2000; Louch and Miller, 2001; Quelas et al., 2010; Donati et al., 2011), and its genome has been fully sequenced (Kaneko et al., 2002). Limiting nitrogen or phosphorus conditions have been shown to cause an increase in EPS production and a change in the type of EPS produced via several regulatory genes and circuits in related rhizobia (as reviewed by Skorupska et al., 2006). As outlined below, several studies (Parniske et al., 1993; Becker et al., 1998; Quelas et al., 2010) have investigated the relationship between EPS production and symbiotic interactions by *B. japonicum* USDA 110.

2.3.3 EPS-RELATED GENE EXPRESSION

Several genes have been identified in *Bradyrhizobium* and related rhizobia to participate in EPS regulation. The *exo*/*ess* and *pss* gene clusters (e.g., *exoB, exoR, exoS, mucR, expR, syrM, exoD exoX*, *exsB* and *expG* genes) are reported as regulating the synthesis, polymerization, modification, and export of rhizobial EPS (as reviewed by Becker et al., 1998; Skorupska et al., 2006). Deletion of the *exoB* gene has been shown to cause altered EPS composition affecting symbiotic interactions; and increased presence of this gene has been correlated with increased EPS production. Uridine diphosphate (UDP)-galactose is formed from UDP-glucose by UDP-glucose4'-epimerase via the *exoB* gene (Canter Cremers et al., 1990; Buendia et al., 1991; Parniske et al., 1993; Skorupska et al., 2006; Quelas et al., 2010). Quelas et al. (2010) further determined when the *exoB* gene was inactivated in *B. japonicum* the bacterium produced 17-24% of the EPS produced by the wild-type strain. In a mutant with the 5' region of *exoB* and 3' region of *exoP* and *exoT* deleted, EPS production was drastically reduced (Quelas et al., 2006).

2.4 Summary

Previous research has shown that carbon-, nitrogen-, and phosphorus-limitations affect the production of EPS, and might be linked with increased headloss in drinking-water biofilters. *Bradyrhizobium* species might be important to EPS production in drinking-water biofilters, especially under nitrogen- and phosphorus-limitations. Studies suggest that nitrogen and phosphorus supplementation may be a practical method to optimize biofilter operation, thereby decreasing headloss, increasing run time between backwashes, increasing water recovery, and decreasing energy requirements. However, additional investigation of the role of EPS production by drinking-water microbial communities under drinking-water conditions is essential.

CHAPTER 3: MATERIALS AND METHODS

This chapter details pure culture batch experiments, including a description of the growth and storage conditions of *Bradyrhizobium japonicum* and *Bacillus cereus,* and mixed community batch experiments. It explains the methods used for extracellular polymeric substance (EPS) quantification and the water quality analyses performed. Appendix B contains step-by-step lab protocols for the methods described in this chapter, and Appendix C contains a chemical inventory list.

3.1 Chemicals

Chemicals used were generally ACS, analytical, or reagent grade, unless otherwise noted in Appendix C. Appendix C provides a detailed list of chemical grades and manufacturers.

3.2 Batch Experiments

Pure culture (*B. japonicum* and *B. cereus*) and mixed community batch experiments were conducted with several carbon to nitrogen to phosphorus (C:N:P) molar ratios and compared against a nutrient-balanced molar ratio of 100:10:1.

3.2.1 SYNTHETIC GROUNDWATER

A synthetic groundwater (based on the composition determined for the groundwater in Rialto, CA) used previously in this research group (Li et al., 2010) was selected to control the concentration and bioavailability of nutrients. The original recipe was modified to exclude perchlorate, include ammonia-nitrogen in place of sodium nitrate, and add a bioavailable organic carbon (acetic acid) and phosphorus source (phosphoric acid). The carbon source and concentration were varied, and the nitrogen and phosphorus concentrations were based on the C:N:P target molar ratio for each experiment. Carbon sources used include mannitol as the sole carbon source for *B. japonicum* experiments and a multi-carbon source (for 2 g/L as carbon: 0.6 g/L as carbon

of mannitol, 0.7 g/L as carbon of sucrose, and 0.7 g/L as carbon of acetate) for mixed community and *B. cereus* experiments. In addition, one set of experiments replaced the sole nitrogen source (ammonium chloride) with 40% liquid ammonium sulfate (LAS). Vitamins (Staley, 1968) and minerals (London et al., 2011) were added based on stock solutions used previously in this research group. In mixed community and *B. cereus* experiments, an organic buffer, MOPS, was added. Lastly, the pH was adjusted to 7.2-7.5 with sulfuric acid or sodium hydroxide to encourage the growth of *B. japonicum*, which grows best between pH 6.0-7.0 (Garrity et al., 2005). Final concentrations of the synthetic groundwater components are provided in Table 3.1.

Table 3.1: Synthetic Groundwater Composition.

Components	**Concentration (mg/L)**	**Components**	**Concentration (mg/L)**
Salts:		**Nutrients:**	
Na_2SO_4	17.8	Carbon Source	Varied
K_2CO_3	6.9	NH_4Cl or LAS	Varied
$NaHCO_3$	289.2	H_3PO_4	Varied
NaCl	13.7	**Trace Metals:**	
$CaCl_2$	2.8	$CuSO_4*5H_20$	0.0574
$MgCl_2$	3.9	$ZnSO_4*7H_2O$	0.2880
Vitamins:		$NiCl_2*6H_2O$	0.0216
biotin	0.020	$FeCl_2*4H_2O$	0.7016
folic acid	0.020	$AlCl_3*6H_2O$	0.2000
pyridoxine-HCl	0.100	$MnCl_2*4H_2O$	0.2807
riboflavin	0.050	$CoCl_2*6H2O$	0.0382
thiamine-HCl	0.050	$Na_2MoO_4*2H_2O$	0.0254
nicotinamide	0.050	H_3BO_3	0.0303
calcium pantothenate	0.050	Na_2SO_4	0.1420
B12	0.001	**Buffer:**	
p-aminobenzoic acid	0.050	MOPS	Varied

A 20X stock solution of all components, except for nutrients (carbon, nitrogen and phosphorus), vitamins and minerals, and MOPS buffer, was composed, filter-sterilized (0.22 μm), and stored at room temperature. Stock solutions of phosphoric acid, ammonium chloride, and mannitol were made separately, autoclaved, and stored at room temperature. Stock solutions of liquid ammonium sulfate (LAS) and carbon sources including glucose and sucrose were filter-sterilized and stored at 4°C. Stock solutions of vitamins and trace metals were made separately, filter-sterilized (0.22 μm), and stored at 4°C.

3.2.2 *B. JAPONICUM*

B. japonicum USDA 110 (NRRL # B-4361) was obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection (Peoria, Illinois). The culture obtained was contaminated, and thus, *B. japonicum* was isolated from the culture based on morphology.

The isolate was sequenced to confirm that the purified culture was *Bradyrhizobium* sp. Universal bacterial primers 8F and 926R were used to amplify extracted DNA to target the 16S rRNA genes. The Qiagen Gel extraction kit was used to further purify the specific target bands (1000 base pairs). The resulting amplified DNA was sent to the Institute for Cellular and Molecular Biology (The University of Texas, Austin, TX) for sequencing (Applied Biosystems 3730 DNA analyzer, Life Technologies Corporation, Grand Island, NY). The Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD) was used to compare the sequence data and confirm the isolate was *Bradyrhizobium* sp.

3.2.2.1 *B. japonicum* Growth

B. japonicum was grown in yeast extract mannitol (YEM) medium (modified from Danso and Alexander, 1974) containing 5 g/L mannitol, 0.5 g/L yeast extract, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.33 g/L $K_2HPO_4 \cdot 3H_2O$, 5 g/L Na gluconate, and 1 mL of 16.6% $CaCl_2$ (sterilized by autoclaving separately and added to cool medium after

autoclaving). Cultures were grown in 250-mL baffled culture flasks in YEM medium at 30°C and 150 rpm; the medium was inoculated with a single *B. japonicum* colony grown on a YEM agar plate (15 g/L of agar were added to YEM medium) for 2 days. Cultures were grown for two to three days to mid-log phase before washing cells and inoculating into experiments. Figure 3.1 shows a photograph of *B. japonicum* colonies on YEM agar plates.

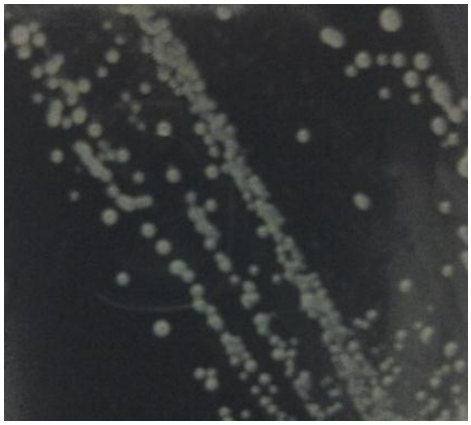

Figure 3.1: *B. japonicum* Colonies on YEM Agar Plates.

Cells were washed by centrifuging cells grown in YEM medium at 10,976 x g for 20 minutes (min), resuspending in sterile synthetic groundwater (with a C:N:P molar ratio matching the current experiment), vortexing, and repeating two more times before transferring cells into synthetic groundwater experiments.

3.2.2.2 *B. japonicum* Storage

B. japonicum was stored long-term at either -80°C or in a liquid nitrogen dewar. Stocks were created by growing *B. japonicum* into mid-log phase (approximately 3 days) in YEM medium and mixing with an equal volume of 50% (v/v) glycerol solution.

3.2.2.3 *B. japonicum* Enumeration

Colonies were enumerated by spot plating on YEM agar plates (15 g/L of agar were added to YEM medium) and incubating at 30°C for two days. Dilutions (1:10) were

performed in the synthetic groundwater used for each experiment, and three dilutions were plated in triplicate 10-µL spots.

3.2.3 *B. CEREUS*

B. cereus was obtained from the American Type Culture Collection (ATCC 13061). *B. cereus* is another soil-dwelling bacterium found in drinking-water biofilms (Cerrato et al., 2010). *B. cereus* was stored in 25% glycerol at -80°C and grown for 24 hours to an optical density at 600 nm (OD_{600}) of 0.578 in Luria Broth (LB) medium before inoculating batch experiments.

3.2.4 PURE CULTURE BATCH EXPERIMENT DESIGN

Pure culture batch experiments were conducted using *B. japonicum* USDA 110 or *B. cereus ATTC 13061*.

3.2.4.1 *B. japonicum* Experiments

Sterile synthetic groundwater (50 mL) with the appropriate C:N:P molar ratio was added to a sterile 250-mL baffled culture flask. Vitamins, minerals and washed bacteria were added. Experiments were incubated for 2 weeks at 150 rpm and 30°C. The *B. japonicum* population was monitored via triplicate spot plates at 1:10 dilutions in the same synthetic groundwater. Population concentrations were recorded at the start of each experiment and for each measurement of EPS. EPS was measured via the concentrations of carbohydrates, proteins and lipids in triplicate.

Due to the unexpected results of the *B. japonicum* experiments in synthetic groundwater medium (no differences in total extracellular proteins and carbohydrates [including medium components] were apparent among balanced-nutrient, nitrogen-limited or –depleted and phosphorus-limited or –depleted experiments), a set of batch experiments was conducted using Götz Minimal Medium to replicate the results of previous research (Lopez-Garcia et al., 2001; Quelas et al., 2006) finding that nitrogen-limitation increased EPS production in *B. japonicum*. *B. japonicum* was grown to mid-log phase in YEM medium from a single colony grown on YEM agar or directly from

frozen stock in YEM medium. *B. japonicum*, grown both ways, was then inoculated into 50 mL of nitrogen-sufficient (1 mM $(NH_4)_2SO_4$) or nitrogen-depleted (no addition of $(NH_4)_2SO_4$) MOPS-Götz medium consisting of 27 mM mannitol as the sole C-source, 1.0 mM $(NH_4)_2SO_4$; 1.0 mM $MgSO_4$; 6.1 mM K_2HPO_4; 3.9 mM KH_2PO_4; 0.1 mM $CaCl_2$; 0.1 mM NaCl; 0.01 mM Na_2MoO_4; 0.001 mM $FeSO_4$ 0.001, the following vitamins at 0.02 mg l^{-1} each: biotin, thiamine–HCl, riboflavin, p-aminobenzoic acid, pyridoxine, and the pH adjusted to 7.0 with 40 mM MOPS buffer (Quelas et al., 2006). At five days after inoculation, the batch experiments were measured for their total extracellular carbohydrate and protein content (including medium components) and their free carbohydrate and protein concentration.

3.2.4.3 *B. cereus* Experiments

Batch experiments were conducted in test tubes. Sterile synthetic groundwater (10 mL) with the appropriate nutrient concentrations, vitamins, minerals and MOPS buffer was added to sterile 17-mm test tubes. The *B. cereus* inoculum (0.2 mL) was added. Experiments were incubated for 5 days at 150 rpm and 30°C. The bacterial population was monitored via OD_{600}. EPS was measured via the concentrations of free and bound carbohydrates and proteins in triplicate.

3.2.5 Mixed Community Batch Experiment Design

Mixed community batch experiments were conducted using biologically active carbon (BAC) received from the City of Arlington's full-scale biologically active filter. Bacteria were released from the filter media by shaking 15 g BAC in synthetic groundwater (50 mL) for one hour. The supernatant was then used to inoculate batch experiments.

Batch experiments were conducted in test tubes. Sterile synthetic groundwater (10 mL) with the appropriate nutrient concentrations, vitamins, minerals and MOPS buffer was added to sterile 17-mm test tubes. The mixed community inoculum (0.2 mL) was then added. Experiments were incubated for 5 days at 150 rpm and 30°C. The bacterial population was monitored (OD_{600}). EPS was measured via the concentrations of

total extracellular carbohydrates and proteins (including medium components) and free and bound carbohydrates and proteins in triplicate.

3.3 EPS Analyses

EPS was extracted from planktonic cultures or wet BAC and analyzed for carbohydrates, proteins, and lipids. When using microtiter plates to measure EPS components or optical density, a fresh well was used for each sample.

3.3.1 PHOSPHATE BUFFERED SALINE SOLUTION (PBS)

A stock of 1M PBS was prepared by dissolving 4 g NaCl, 0.1 g KCl, 0.5755 g $Na_2HPO_4 \cdot 7H_2O$, and 0.1 g KH_2PO_4 in deionized, distilled (DDI) water to a final volume of 500 mL (Ausubel et al., 2002). A solution of 0.01M PBS was prepared by diluting 5 mL of the 1M PBS to 500 mL DDI water. The pH was adjusted to 7. Each solution was autoclaved and stored at room temperature.

3.3.2 EPS EXTRACTION

As described below, different EPS extraction protocols were used for planktonic cultures versus wet BAC.

3.3.2.1 EPS Extraction from Planktonic Batch Experiments

For the batch experiments (containing only planktonic cells), sequential processing steps were used to isolate different EPS fractions prior to carbohydrate and protein analysis as illustrated in Figure 3.2. First, cells were separated from the sample by centrifugation (Avanti J-E Centrifuge, Beckman Coulter, Brea, CA) for 20 min at 4°C and 10,976 x g (Xu and Chellam, 2005), and the supernatant (spent culture media) was retained for analysis of total extracellular carbohydrates and proteins (including medium components) and further extraction of free EPS. The cell pellet was retained for bound EPS extraction. Next, free EPS was precipitated from the supernatant with three volumes

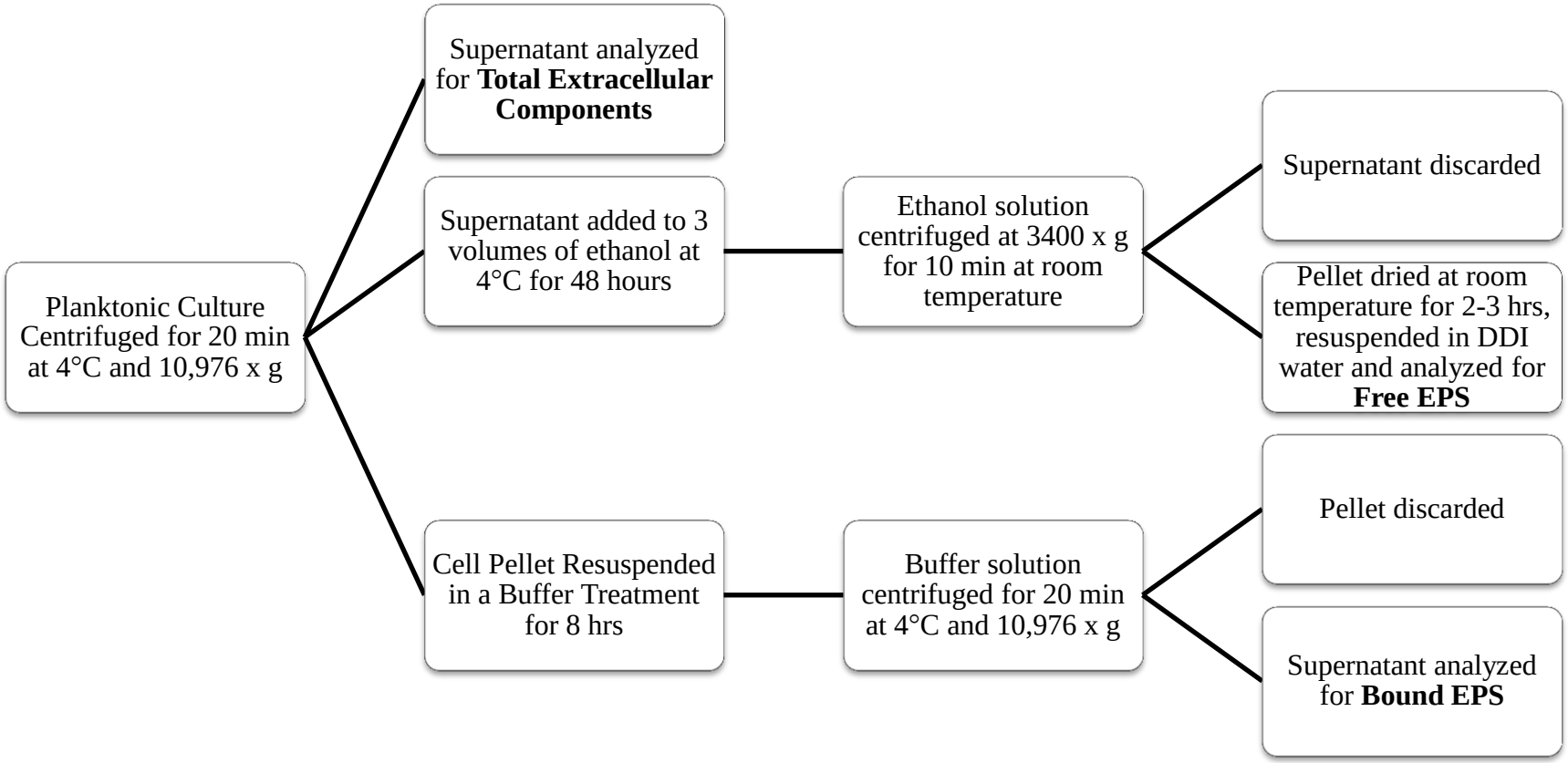

Figure 3.2: Processing Steps for Planktonic Cultures Prior to Measurement of EPS Fractions

of chilled ethanol and incubated at 4°C for 48 hours. As ethanol does not precipitate mono- and disaccharides, ethanol precipitation would not precipitate carbon sources from the media. After the incubation period, the sample and ethanol solution was centrifuged (Centrific™ Centrifuge Model 225, Fisher Scientific, Pittsburgh, PA) at 3400 x g for 10 min at room temperature. The supernatant was discarded and the ethanol precipitable material was dried at room temperature for 2-3 hrs and resuspended in DDI water for analysis (Quelas et al., 2006; Badireddy et al., 2008; Staudt et al., 2011). Lastly, bound EPS was extracted by re-suspending the cell pellet for 8 h at room temperature in a buffer (10 mM Tris/HCl, pH 8, 10 mM EDTA, 2.5 percent NaCl). After the 8-h incubation period, the solution was centrifuged (Avanti J-E Centrifuge, Beckman Coulter, Brea, CA) for 20 min at 4°C and 10,976 x g to separate the cell pellet from the supernatant containing bound EPS . The cell pellet was discarded and the supernatant was retained for analysis (Badireddy et al., 2008; Lauderdale et al., 2011).

Due to low concentrations of EPS found in drinking-water samples with planktonic bacteria, it is suggested that future analyses adjust this method to concentrate the samples by resuspending the precipitated free EPS in a smaller volume of DDI water and resuspending the cell pellet in a smaller volume of buffer.

3.3.2.2 EPS Extraction from Wet BAC

For wet BAC samples, EPS was extracted using an optimized version of the formaldehyde + heat method (Fang and Jia, 1996). Tzu-Hsin Chiao and Ameet Pinto (The University of Michigan, personal communication, November 20, 2011) optimized EPS extraction for drinking-water BAC following the methods outlined by Fang and Jia (1996). Fang and Jia considered five extraction methods and concluded that the EDTA method was best for sludge. Chiao and Pinto concluded that a formaldehyde + heat extraction provided the most consistent carbohydrate (via the anthrone method) and protein (via the modified Bradford assay) results while minimizing cell lysis for drinking-water wet BAC samples. However, as a 600RPM shaker (used in the Chiao and Pinto method) was unavailable in our lab, the method was further modified. EPS extraction was carried out with three shaker options for comparison: (1) a horizontal platform

shaker (New Brunswick Scientific Model M1058-0002, Enfield, CT) operated at approximately 500 rpm, (2) a 360° rotating Labquake® rotating shaker (Barnstead-Thermolyne Model 4002110, Thermo Fischer Scientific, Inc., Waltham, MA) operated at 9 rpm, and (3) an orbital shaker (Model 980001, VWR International, Radnor, PA) operated at approximately 400 rpm. Additionally, measurement of carbohydrates was performed with both the phenol-sulfuric (Dubois et al., 1956) and anthrone methods (Raunkjaer et al., 1994; Frølund et al., 1996).

Experiments were carried out using wet BAC from pilot filters at the John Kubala Water Treatment Plant in Arlington, Texas. Water was decanted from wet BAC samples. Then, 2 g of wet BAC were transferred into a 15-mL sterile centrifuge tube, and 10 mL of 0.01M PBS at pH 7 and 60 μL of 36.5% (v/v) formaldehyde were added. Samples were incubated at 4°C for 1 h on each shaker described previously and then transferred to an 80°C water bath for 10 min. Samples were centrifuged (Centrific™ Centrifuge Model 225, Fisher Scientific, Pittsburgh, PA) for 10 min at 5000xg and room temperature. The supernatant containing the extracted total (bound and free) EPS was analyzed to determine the carbohydrate and protein content. The remaining BAC was dried at 105°C for 1 hour to obtain its dry weight.

3.3.3 CARBOHYDRATE CONCENTRATION

Carbohydrates were measured by either a modified version of the anthrone method (Raunkjaer et al., 1994; Frølund et al., 1996) or the phenol-sulfuric method (Dubois et al., 1956). The anthrone method has previously been shown to yield similar results as the phenol-sulfuric method in an activated sludge sample, and the anthrone method has shown a lower coefficient of variation (Frølund et al., 1996).

3.3.3.1 Anthrone Method

The anthrone method was used to measure carbohydrates in batch experiments. Anthrone reagent was made fresh daily (2 h prior to use) by adding 25 mg of anthrone to 20 mL concentrated H_2SO_4. Glucose standards (1.25 - 100 mg/L) were prepared. To measure carbohydrates, 0.8 mL of sample followed by 1.6 mL of anthrone reagent was

added to a 15-mm glass test tube. The test tubes were placed in a 100°C heat block for 14 min, allowed to cool for 5 min in a 4°C water bath, and vortexed. Carbohydrates were measured using a standard polystyrene 96-well microplate (Costar EIA/RIA plates) with 200 µL of sample in triplicate by reading the absorbance at 625 nm in a microtiter plate reader (Bio-Tek Synergy HT, BioTek, Winooski, VT). Absorbances were measured with a 1-cm pathlength correction. The coefficient of determination of standard curves measured in a microplate ($y=0.0062x+0.0646$, $R^2=0.998$) either matched or exceeded the coefficient of determination measured using a 1-cm quartz cuvette ($y=0.0167x+0.1857$, $R^2=0.979$) on an Agilent 8453 UV-Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA). Figure 3.3 displays a typical standard curve using the microplate. A suggestion for improvement on this method would be to acid-wash and bake the glass test tubes to remove residual polysaccharides and to use a 5-cm cuvette for low concentrations.

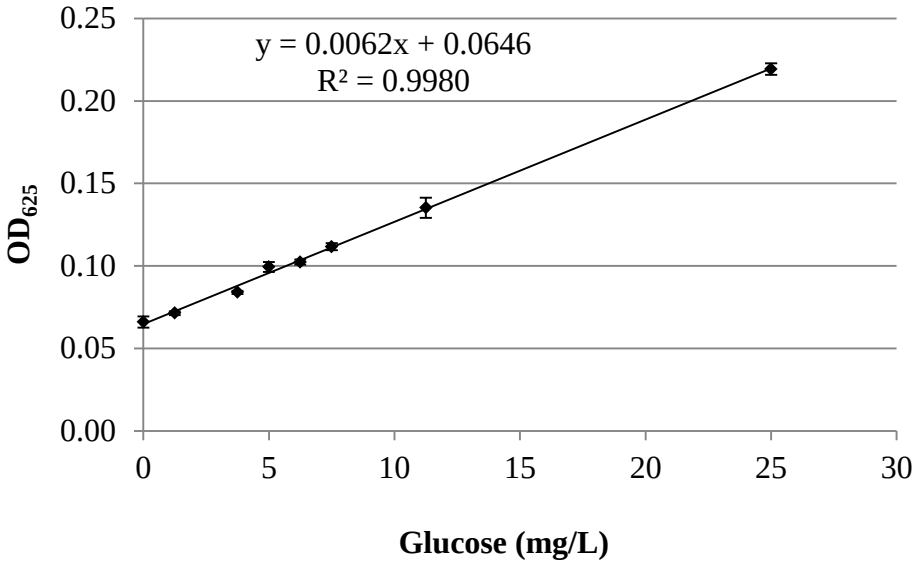

Figure 3.3: Typical Anthrone Method Standard Curve Using a Microplate. Error bars represent standard deviation among triplicate absorbance measurements.

3.3.3.2 Phenol-Sulfuric Method

The phenol-sulfuric method (Dubois et al., 1956) was used to quantify carbohydrates in early experiments aimed at optimizing the extraction of EPS from

drinking-water BAC. Glucose standards were prepared at 5, 7, 11 and 25 mg/L. To measure carbohydrates, 2 mL of sample were added to a 17-mm glass test tube. Phenol (0.05 mL of 80%) was added, and 5 mL of 95% sulfuric acid was added rapidly. After 10 min, samples were vortexed and placed in a 27.5°C water bath for 15 min. Carbohydrates were measured using a standard polystyrene 96-well plate (Costar EIA/RIA plates) with 200 µL of sample in triplicate by reading the absorbance at 490 nm on a microtiter plate reader (Bio-Tek Synergy HT, BioTek, Winooski, VT). Absorbances were measured with a 1-cm pathlength correction. Figure 3.4 displays a typical standard curve.

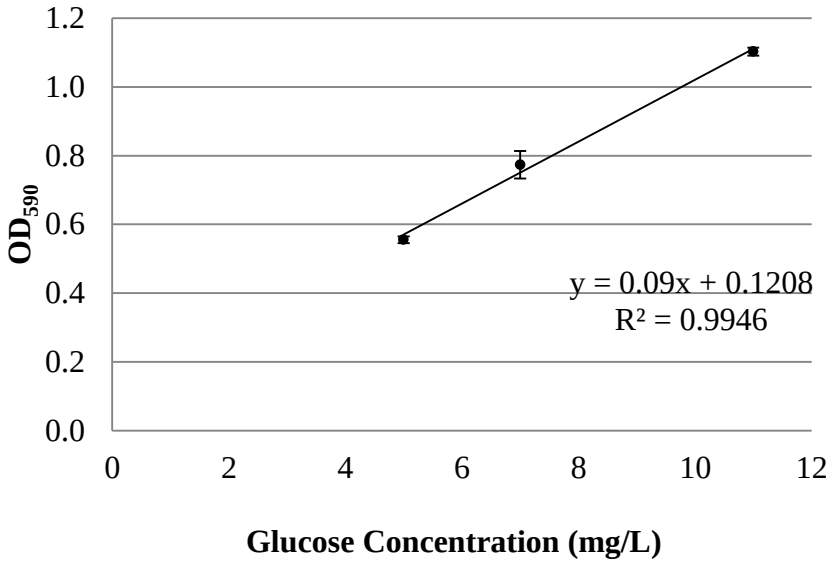

Figure 3.4: Typical Phenol-Sulfuric Method Standard Curve. Error bars represent standard deviation among triplicate absorbance measurements.

3.3.4 PROTEIN CONCENTRATION

Proteins were measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) based on the Bradford method (Bradford, 1976). The microassay procedure for a linear range of 1.25 to 10.00 µg/mL was used with bovine serum albumin (BSA) as the standard. Proteins were measured using a polystyrene 96-well plate (Costar EIA/RIA plates) with 200 µL of sample in triplicate by reading the absorbance at 595 nm on a microtiter plate reader (Bio-Tek Synergy HT, BioTek,

Winooski, VT). Absorbances were measured with a 1-cm pathlength correction. Figure 3.5 displays a typical standard curve. Using a 5-cm cuvette for low concentrations of proteins may increase the accuracy of measurements.

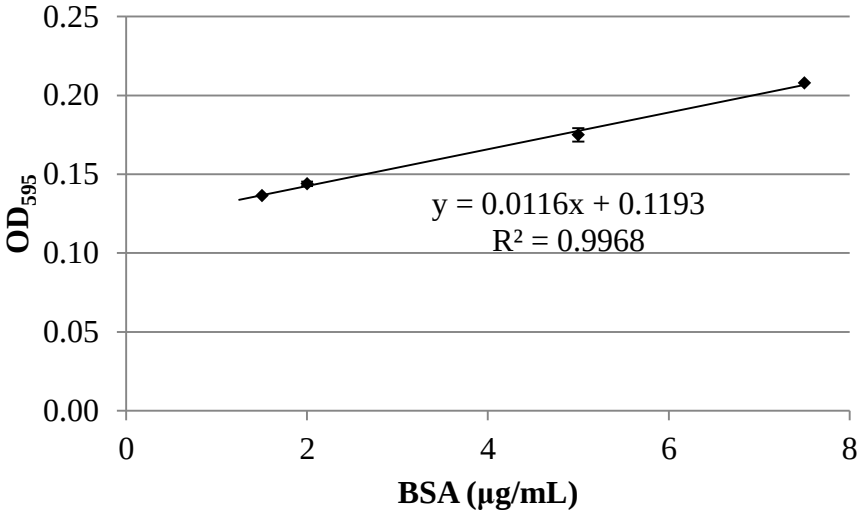

Figure 3.5: Typical Protein Standard Curve. Error bars represent standard deviation among triplicate absorbance measurements.

3.3.5 LIPID CONCENTRATION

Lipids were measured using the sulfo-phospho-vanillin method (Frings et al., 1972). Olive oil (0.9135 g/mL, Acros Organics) standards were constructed in ethanol for final concentrations of 639.45, 767.34, 1023.12, and 1278.9 mg/dL. Frings et al. (1972) demonstrated a linear range of 600-1250 mg/dL. Vanillin reagent was made by adding 1.05 g of vanillin (99%, Acros Organics) to 200 mL DDI and used immediately to make phospho-vanillin reagent. Phospho-vanillin reagent was made by adding 300 mL of concentrated (85%) H_3PO_4 to 200 mL of vanillin reagent and was stored in a brown bottle at room temperature for up to 2 months.

To measure lipids, 20 µL of sample and 0.2 mL of concentrated (95%) H_2SO_4 were added to a 15-mm test tube and vortexed. Samples were heated for 10 min in a 100°C heat block and cooled in a 4°C water bath for 5 min. 10 mL of phospho-vanillin reagent was added to the test tubes, which were then vortexed, incubated for 15 min in a 37°C water bath, and cooled for 5 min at room temperature. Lipids were measured using

a polystyrene 96-well plate (Costar EIA/RIA plates) with 200 µL of sample in triplicate by reading the absorbance at 540 nm on a microtiter plate reader (Bio-Tek Synergy HT, BioTek, Winooski, VT). Absorbances were measured with a 1-cm pathlength correction. Figure 3.6 displays a typical standard curve.

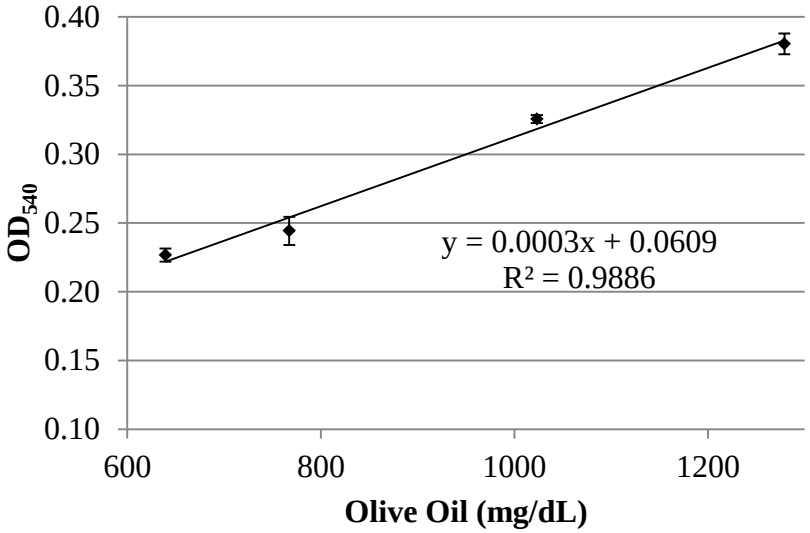

Figure 3.6: Typical Lipid Standard Curve. Error bars represent standard deviation among triplicate absorbance measurements.

3.3.6 Optical Density

Optical density was monitored as a measure of bacterial growth. The optical density of samples was measured using a standard polystyrene 96-well plate (Costar EIA/RIA plates) with 200 µL of sample in triplicate by reading the absorbance at 600 nm on a microtiter plate reader (Bio-Tek Synergy HT, BioTek, Winooski, VT). Absorbances were measured with a 1-cm pathlength correction.

3.4 Water Quality Analyses

pH, dissolved organic carbon (DOC), ortho-phosphate, and ammonia-nitrogen were measured in the synthetic groundwater.

3.4.1 pH

pH was measured in triplicate with stirring using a combination electrode and meter (Model 720A, Orion Research Inc., Boston, MA).

3.4.2 Ortho-Phosphate

The ascorbic acid method (Standard Method 4500-P E) was followed to measure dissolved reactive phosphorus (APHA et al., 2005). KH_2PO_4 was dried for 1 h at 105°C, and a 1000 mg/L as phosphorus standard stock solution was prepared in DDI. The stock solution was stored in a glass bottle at 4°C. A 40 mg/L solution of ammonium molybdate tetrahydrate and a 3.4285 mg/L solution of antimony potassium tartrate were made in DDI water and stored in glass-stoppered bottles. A 0.1 M ascorbic acid solution was made fresh daily by adding 0.1056 g of ascorbic acid to 6 mL of DDI water. The combined reagent (used within 4 hours) was composed of 10 mL 5N H_2SO_4, 1 mL antimony potassium tartrate solution, 3 mL ammonium molybdate solution and 6 mL 0.1 M ascorbic acid solution.

To measure ortho-phosphate (dissolved phosphorus), 5 mL of sample or standard were added to an acid-washed 17-mm glass test tube. One drop of phenolphthalein indicator was added; sulfuric acid (5N) was added drop wise to dissipate the color, if necessary. After adding 0.8 mL of combined reagent, samples were vortexed and incubated at room temperature for 10 min. Ortho-phosphate was measured using a polystyrene 96-well plate (Costar EIA/RIA plates) with 200 μL of sample in triplicate by reading the absorbance at 880 nm on a microtiter plate reader (Bio-Tek Synergy HT, BioTek, Winooski, VT). Absorbances were measured with a 1-cm pathlength correction. Figure 3.7 displays a typical standard curve.

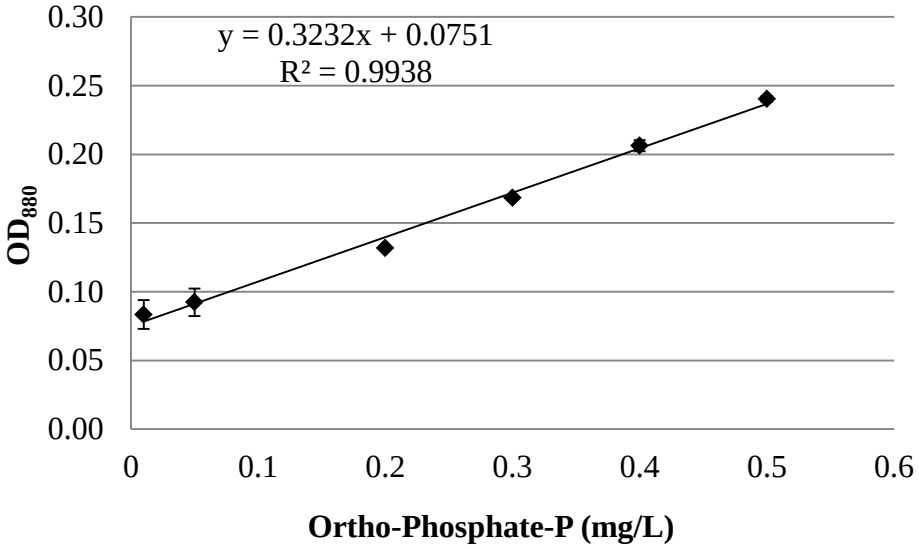

Figure 3.7: Typical Ortho-Phosphate Standard Curve. Error bars represent standard deviation among triplicate absorbance measurements.

3.4.3 AMMONIA-NITROGEN

Ammonia-nitrogen was measured with a Model 95-12 Orion® ion-selective electrode probe and Model 920A meter (Thermo Fischer Scientific, Inc., Waltham, MA). Ionic strength adjuster ("Ammonia Gas-Sensing Electrode Instruction Manual," n.d.) was prepared by adding 100 g NaOH to 250 mL DDI water and stirring until pellets dissolved. Then, 9.3 g of disodium EDTA was added. The solution was again stirred and allowed to cool. Separately, 15 mg of thymol blue were added to 50 mL methanol and dissolved by stirring. The methanol solution was added to the solution with NaOH and EDTA, stirred, raised to a final volume of 500 mL and stirred.

To create a standard curve, 100 mL of DDI water and 2 mL of ionic strength adjuster were added to a 150-mL flask and stirred on a stirplate. Incremental amounts (20 µL, 80 µL, 100 µL, 300 µL and 500 µL) of 500 mg/L ammonia-nitrogen stock solution (made with ammonium sulfate) were added to the solution, and the mV were measured after each addition. The mV values were plotted against the log of the concentrations (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.5 mg/L, and 5.0 mg/L of NH_3-N, respectively), and a best fit line was plotted.

To measure the ammonia-nitrogen concentration of a sample, the pH was raised by adding 200 μL of ionic strength adjuster to a 10-mL sample in a 20-mL vial with continuous stirring. The mV response was recorded and used to calculate ammonia-nitrogen from the standard curve. Note that this method measures total ammonia since the ionic strength adjuster converts ammonium to ammonia. A typical ammonia-nitrogen standard curve is shown in Figure 3.8. To prevent loss of ammonia gas, all samples and standards were covered during measurements as recommended by the manual (*Orion 95-12 Ammonia Electrode Instruction Manual*, n.d.).

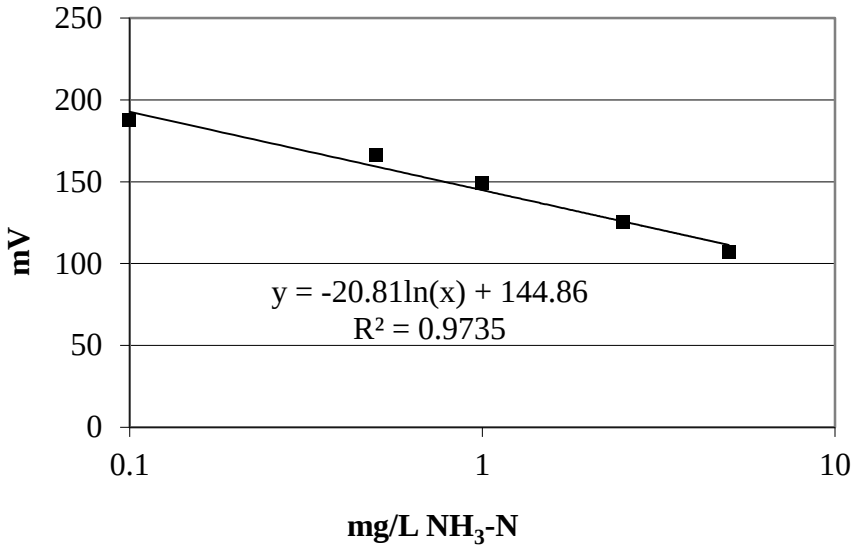

Figure 3.8: Typical Ammonia-Nitrogen Standard Curve.

3.5 Microscopy

The Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY) was used for phase contrast microscopy of bacterial cells at a 60X objective and 10X ocular (600X total) magnification.

CHAPTER 4: RESULTS AND DISCUSSION

In this chapter, the results of the current research are presented. The first section summarizes experiments aimed at optimizing the extracellular polymeric substances (EPS) extraction procedure from wet biologically active carbon (BAC). The second section summarizes observations relating to the growth of *Bradyrhizobium japonicum* and batch experiments conducted with *B. japonicum.* In the final two sections, experiments with a mixed community of drinking-water bacteria and a pure culture of *B. cereus* are described. Batch experiments were conducted with various carbon sources and with varied carbon to nitrogen to phosphorus (C:N:P) molar ratios. All C:N:P ratios noted are the targeted ratios.

4.1 BAC EPS Extraction and Carbohydrate Measurement Methods

Experiments were run to select a shaker to use in the EPS extraction protocol for wet BAC and to compare the anthrone and phenol-sulfuric methods for carbohydrate determination. Table 4.1 shows the percent difference among the mean carbohydrate concentrations calculated among all three shakers and the carbohydrate concentration measured using each shaker (|mean-shaker measurement|/mean). The orbital shaker produced the concentration closest to the mean of the three methods using the phenol-sulfuric acid method, and the horizontal shaker produced the concentration closest to the mean of the three methods using the Anthrone method. However, as the rotating shaker was the most convenient to use due to reliability, availability, cost, and ease of moving it to and from the 4°C cold room, the 9 rpm shaker was selected.

Figure 4.1 displays the carbohydrate concentration normalized to the dry weight of granular activated carbon (GAC). The anthrone method provided more consistent results across the various shaking options and produced higher EPS concentrations using the rotating shaker. While the standard deviation among absorbance measurements appears greater with the anthrone method than with the phenol-sulfuric method for carbohydrate determination in this set of experiments, this was later corrected by

vortexing each sample immediately before removing triplicate 200-µL aliquots for absorbance measurement.

Table 4.1: Percent Difference Among Mean Carbohydrate Concentrations Among all Three Shakers and the Carbohydrate Concentration Extracted Using Each Shaker.

	Dubois Method		
	Horizontal Shaker	**Orbital Shaker**	**Rotating Shaker**
Filter 2, Sampled at Top	13%	45%	32%
Filter 2, Homogenized	116%	71%	45%
Filter 16, Sampled at Top	60%	23%	38%
Filter 16, Homogenized	29%	34%	64%
Average:	55%	43%	45%
	Anthrone Method		
	Horizontal Shaker	**Orbital Shaker**	**Rotating Shaker**
Filter 2, Sampled at Top	16%	2%	15%
Filter 2, Homogenized	18%	36%	18%
Filter 16, Sampled at Top	5%	27%	22%
Filter 16, Homogenized	44%	91%	47%
Average:	21%	39%	25%

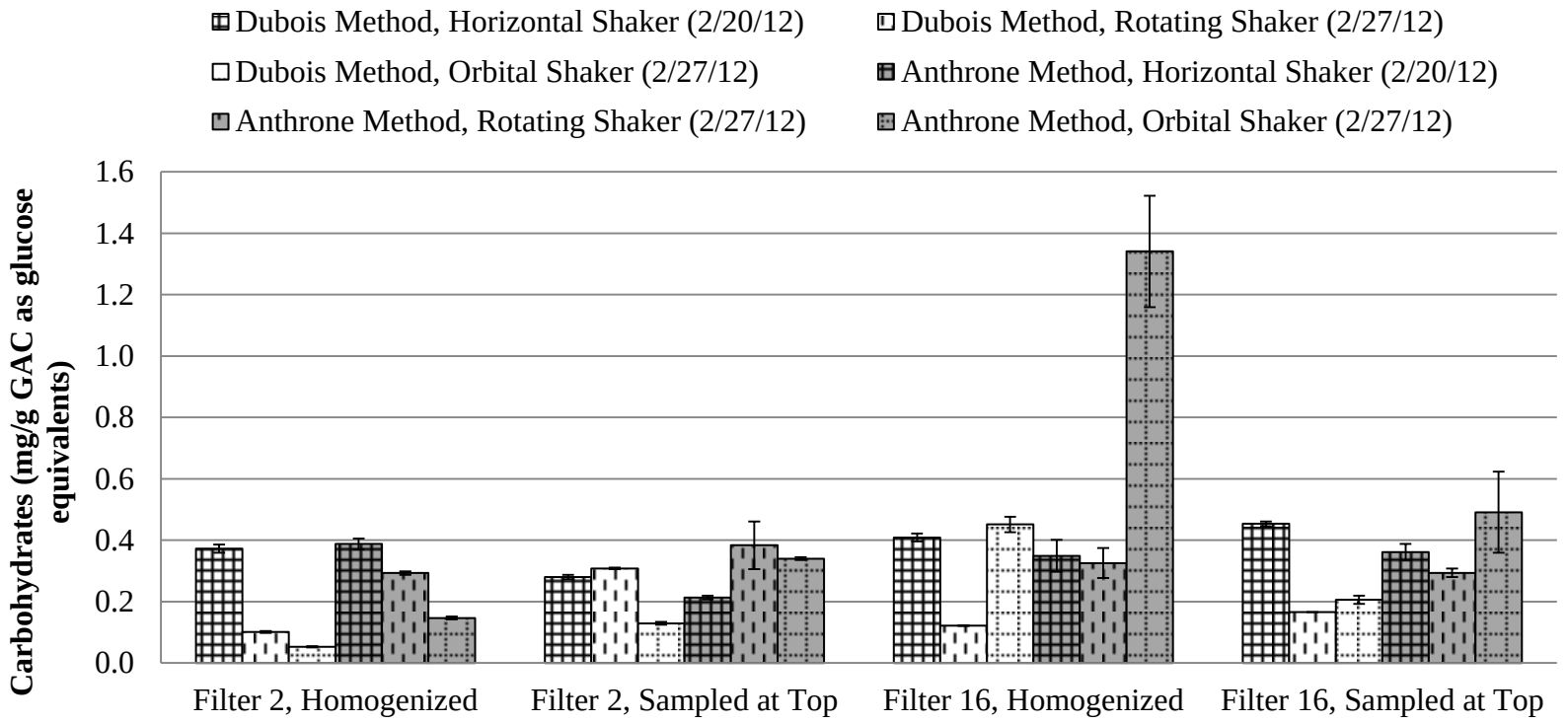

Figure 4.1: Carbohydrate Content per Gram of BAC of Extracted EPS from Pilot-Scale Filters in Arlington, Texas Using Three Shaker Variations in the EPS Extraction Procedure and Two Different Methods of Carbohydrate Determination. Error bars represent standard deviation among triplicate absorbance measurements.

4.2 *Bradyrhizobium japonicum* USDA 110 Experiments

4.2.1 *B. japonicum* Batch Experiments

The results of batch experiments with *B. japonicum* are provided in Appendix A. Additional work is required to optimize *B. japonicum* batch experiments.

4.2.2 *B. japonicum* Morphology and Growth Characteristics

Several important observations and modifications were made in response to morphology and growth changes in *B. japonicum* over time.

- Three colony morphologies were observed when *B. japonicum* was grown on yeast extract mannitol (YEM) agar plates. Most commonly, a wild-type (WT) morphology was observed as described in literature (Figure 4.2 and 4.3). The single colonies either matched the classification of large, mucoid (LM) colonies exceeding 1 mm in diameter after 10 days or small, defined (SD) colonies ***not*** exceeding 1 mm in diameter after 10 days (Fuhrmann, 1990). However, a change to an extreme mucoid morphology (Figure 4.4) was observed when cells were grown in YEM medium where the calcium had precipitated (due to adding calcium stock solution to YEM medium while the medium was still too hot from autoclaving). The extreme mucoid morphology produced a lawn of bacterial cells (did not produce single colonies). When viewed by phase contrast microscopy, the LM and mucoid cells clearly looked like rods; however, the SD cell morphology looked different (Figures 4.2Figure -4.4).

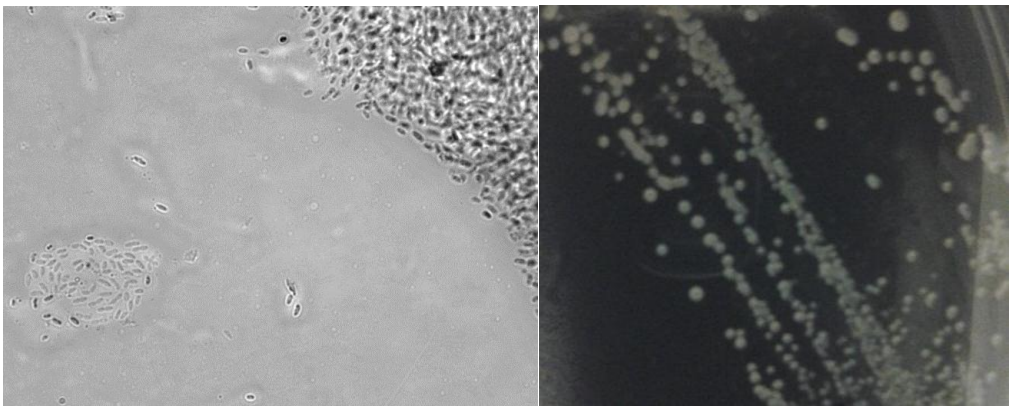

Figure 4.2: Large, Mucoid (LM) Colonies: Left: LM Cells Under a Microscope (600X Total Magnification). Right: LM Colonies on YEM Agar.

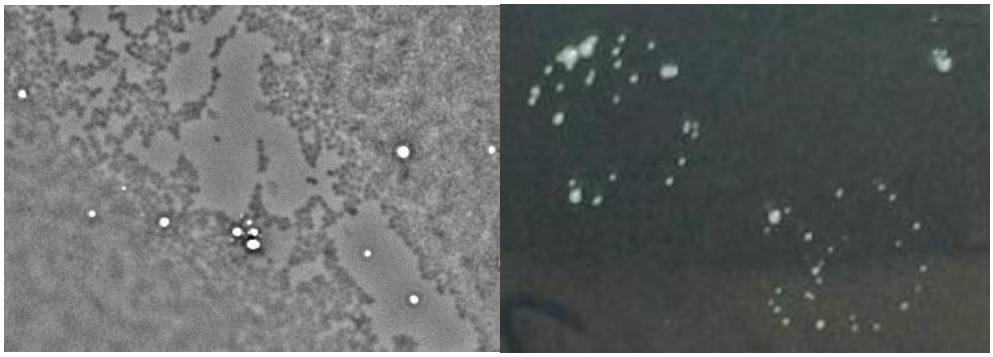

Figure 4.3: Small, Defined (SD) Colonies. Left: SD Cells Under a Microscope (600X Total Magnification). Right: SD Colonies on YEM Agar.

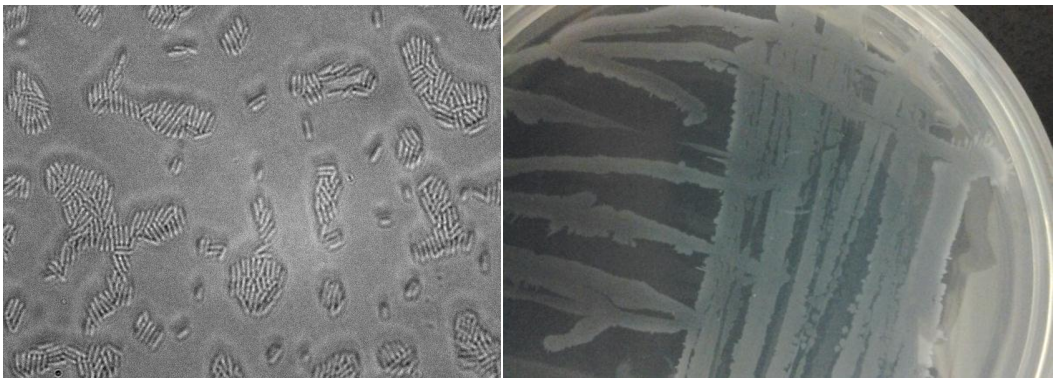

Figure 4.4: Mucoid Colony Morphology. Left: Rafts of Mucoid Cells Under a Microscope (600X Total Magnification). Right: Mucoid Colonies on YEM Agar.

- A decrease in bacterial density was observed in freezer stocks stored at -80°C over a period of about four months. Liquid nitrogen was selected as a better long-term storage option for these bacteria (Allievi and Salardi, 1993; Safronova and Novikova, 1996). No decreases in colony counts were observed over time under liquid nitrogen storage.
- During growth of *B. japonicum* in YEM medium, various degrees of flocculation were observed. Dual experiments conducted with the same medium and colonies grown on the same plates resulted in very different amounts of flocculation; thus the trigger for flocculation is unclear. Additionally, due to this flocculation, centrifugation of a cell pellet for washing before transferring into synthetic groundwater experiments was difficult. Centrifugation resulted in more of a floating cloud of cells than a typical pellet of cells (see Figure 4.5). The extent of this clouding varied among the colonies selected for inoculation; at times, a pelleted cloud could be formed but was still much larger and fluffier than a typical cell pellet. To accommodate this flocculation, stir bars were added to flasks to break up the flocs and increase homogeneity during shaking.

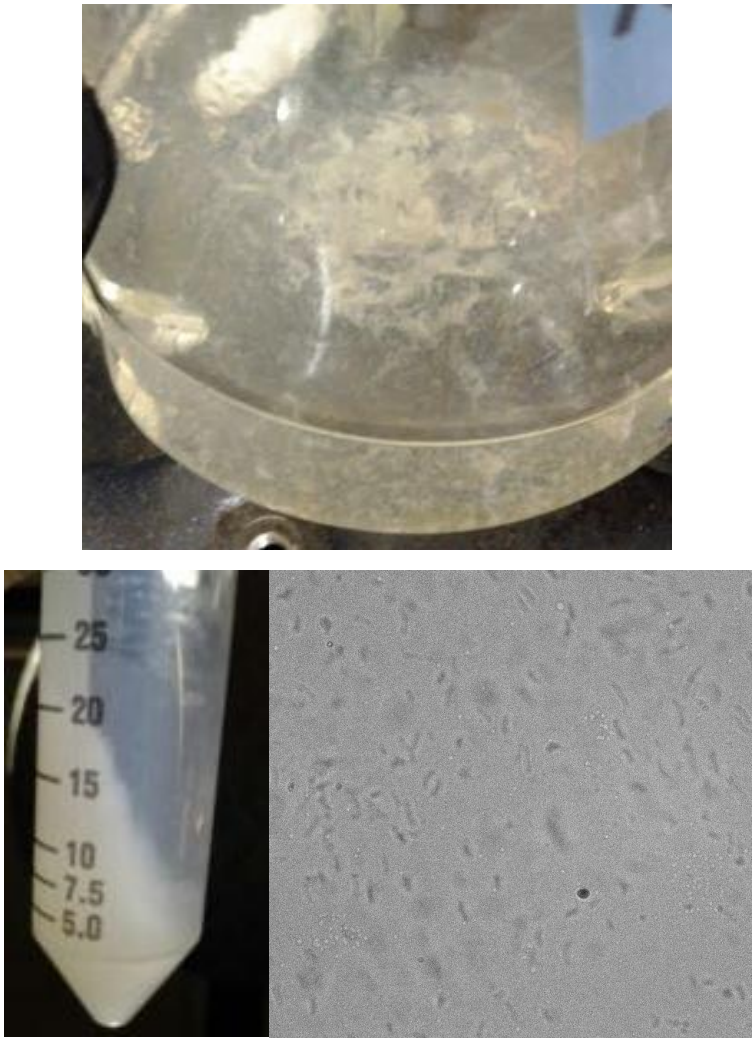

Figure 4.5: Flocculation of *B. japonicum* in YEM Medium. Top: Flocs in 250-mL Baffled Flask. Bottom Left: Centrifuged "Cloud" of Flocculated Cells. Bottom Right: Flocculation Viewed Under a Microscope (600X Total Magnification).

- Traditional dilutions of *B. japonicum* in 1.5-mL centrifuge tubes followed by spot or spread plating were very inconsistent, and cell numbers often did not decrease with increasing dilution. Several methods of spread plating and spot plating using various diluents including phosphate buffered saline (PBS), synthetic groundwater, and YEM medium and various dilution flasks (e.g., glass test tubes, plastic 1.5-mL tubes, and tissue-culture-treated sterile microplates) were tested. Sterile tissue-culture-treated microplates were selected. The treating on these

plates was suspected to minimize cell adhesion to walls, and allowed for the shortest amount of time between preparing dilutions and plating. Spot plating was selected as it produced consistent results with the well plates. Dilutions were performed using the type of medium in which the bacteria were grown to minimize shock to bacteria due to sudden environmental changes.

- When grown in YEM medium, *B. japonicum* flocculated and the growth medium became very turbid; centrifugation at 10.976 x g for 20 min produced a fluffy cloud. However, when grown in groundwater medium or Götz Minimal Medium, no turbidity was produced; centrifugation at 10.976 x g for 20 min did not produce a cell pellet. Additional investigation is required to determine why cells did not grow as expected in synthetic groundwater or Götz Minimal Medium.

4.3 Mixed Community Batch Experiments

Batch experiments were run under several C:N:P molar ratios to examine the relationship between C:N:P molar ratio and EPS production. To quantify changes in EPS, the concentrations of protein, carbohydrate and lipids were monitored. However, lipid concentrations were immeasurable in all batch experiments. A mixed community of drinking-water bacteria extracted from BAC in full-scale biological filters in Arlington, Texas was used to inoculate the batch experiments.

4.3.1 Mixed Community Experiments at 2g/L of Carbon

Triplicate batch experiments were conducted at a carbon concentration of 2 g/L composed of a mixture of mannitol, acetic acid and sucrose. Nutrient-balanced (C:N:P molar ratio of 100:10:1), nitrogen-depleted (C:N:P molar ratio of 100:0:1) and phosphorus-depleted (C:N:P molar ratio of 100:10:0) batch experiments with a mixed community of drinking-water bacteria were conducted.

At five days after inoculation, the batch experiments were measured for the optical density (OD_{600}) and carbohydrates and proteins, the two main components of this EPS. Figure 4.6 displays the OD_{600} for each sample at Day 5. Growth was limited under

the nitrogen- and phosphorus-depleted conditions as compared to the nutrient-balanced condition. EPS was analyzed following three different processing methods (described in section 3.3.2): (1) cells were removed from the supernatant and then total extracellular proteins and carbohydrates were quantified in the supernatant (including medium components), (2) cells were removed and ethanol precipitation was performed on the supernatant to obtain free EPS, and (3) the removed cells (pelleted) were processed to obtain bound EPS.

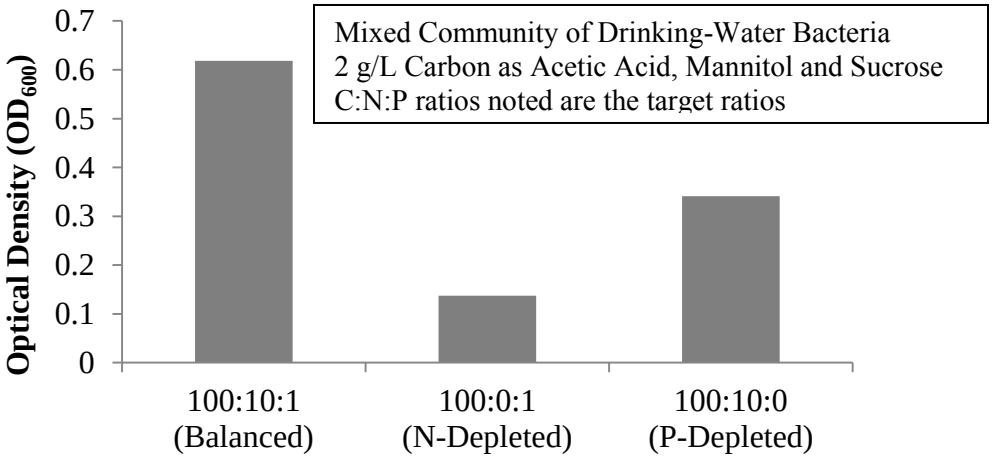

Figure 4.6: Mixed Community Batch Experiment
Optical Density at Day 5.

Total extracellular carbohydrates and proteins (including medium components) were monitored at day 5 and compared to carbohydrates and proteins in sterile media. All sterile media (100:10:1, 100:0:1, and 100:10:0) contained carbohydrate concentrations greater than 100 mg/L (the upper method detection limit). The spent culture medium following the first processing method (analysis of the supernatant after centrifugation) under a balanced (100:10:1) nutrient condition contained 21.25 ± 1.84 mg/L as glucose (mean ± standard deviation of triplicate absorbance measurements). Both the nitrogen- and phosphorus-depleted spent culture media, following the first processing method, contained carbohydrate concentrations greater than 100 mg/L. Comparing the data for total extracellular carbohydrates to the OD_{600} data for the three nutrient conditions, the greatest growth (highest OD_{600}) corresponded to the lowest

remaining carbohydrate concentration in the culture (including medium components). The decrease in carbohydrate concentration in the spent culture medium as compared to sterile medium is likely a reflection of growth as bacteria are consuming carbohydrates to produce energy and biomass. Thus, the decrease in carbohydrates in the nutrient-balanced culture medium is likely a reflection of cell growth rather than EPS production. These data illustrate the importance of extracting EPS from the media. Total extracellular proteins were higher in the spent culture media (approximately 10 mg/L as BSA) than in the sterile culture media (below the 1.25 mg/L detection limit) under each nutrient condition. As proteins in sterile media were so low, it is likely these increases in protein concentrations demonstrate EPS production.

Figure 4.7 displays the carbohydrate and protein concentrations following ethanol precipitation to obtain free EPS and processing of the cell pellet to obtain bound EPS. The processing of free and bound EPS is a common means of EPS extraction in literature (Badireddy et al., 2008; Lauderdale et al., 2011). Bound polysaccharides were found to be the greatest component of EPS measured (by mass). The concentration of bound polysaccharides was higher under nitrogen- and phosphorus-depleted conditions as compared to the nutrient-balanced condition. Free polysaccharides normalized to OD_{600} followed the same trend. However, free and bound proteins normalized to OD_{600} appeared similar under the three nutrient conditions.

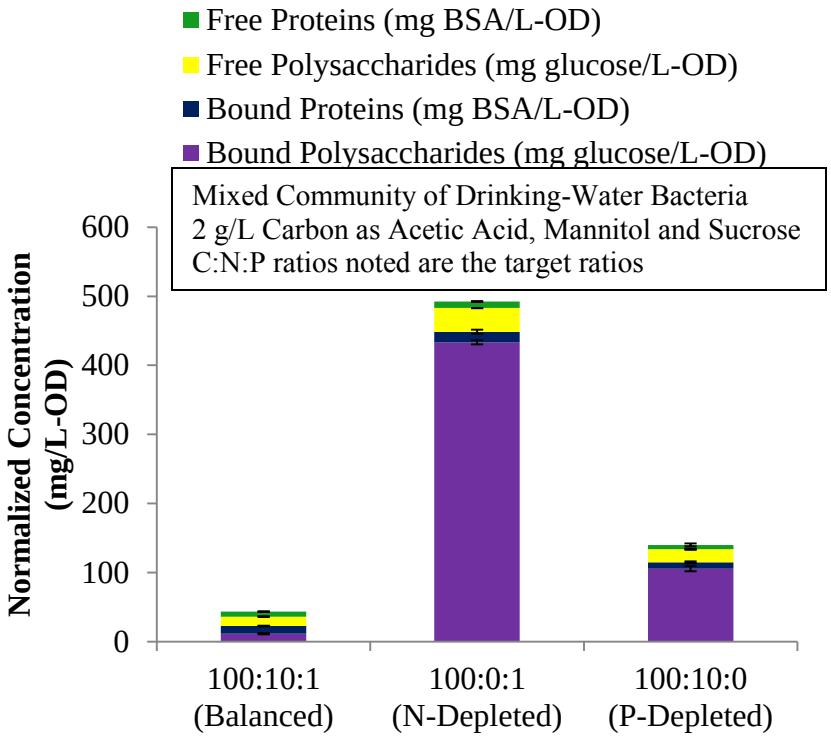

Figure 4.7: Mixed Community Batch Experiment Normalized Free and Bound Carbohydrate and Protein Measurements at Day 5. Error bars represent standard deviation among triplicate absorbance measurements.

4.3.2 Mixed Community Batch Experiments with 200, 20, and 2 mg/L of Carbon

Using the same experimental set-up, batch experiments were run at lower carbon concentrations (200, 20 and 2 mg/L). The results of the experiments at 200 mg/L are shown in Figure 4.8 (OD_{600}) and Figure 4.9 (free and bound EPS measurements). Note that a third nutrient limitation was added where some phosphorus (approximately 0.5 mg/L) was added to reach a C:N:P molar ratio of 100:10:0.1 (phosphorus-limited). The nutrient-balanced condition allowed for greater growth (Figure 4.8) than did the nutrient-depleted or nutrient-limited conditions. The overall growth under all nutrient conditions was less than that at 2 g/L carbon (Figure 4.6), as expected. Bound polysaccharides normalized to OD_{600} were again higher under nitrogen- and phosphorus-depleted conditions as compared to the nutrient-balanced condition. Considering the phosphorus-limited condition (100:10:0.1), Figure 4.9 shows that the addition of only about 0.5 mg/L

of phosphorus reduced the overall EPS produced as compared to the phosphorus-depleted condition. However, cell growth was still limited (Figure 4.8) as compared to the nutrient-balanced (C:N:P molar ratio of 100:10:1) condition.

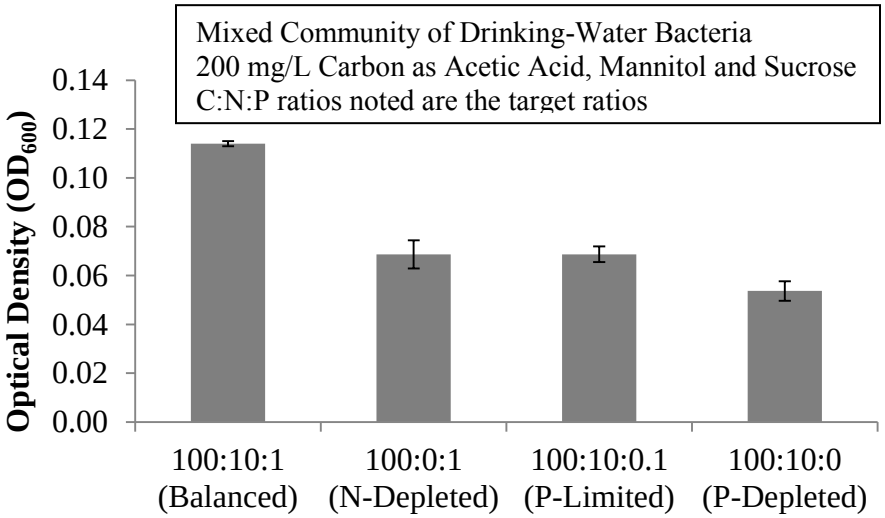

Figure 4.8: Mixed Community Batch Experiment Optical Density at Day 5. Error bars represent standard deviation among triplicate absorbance measurements.

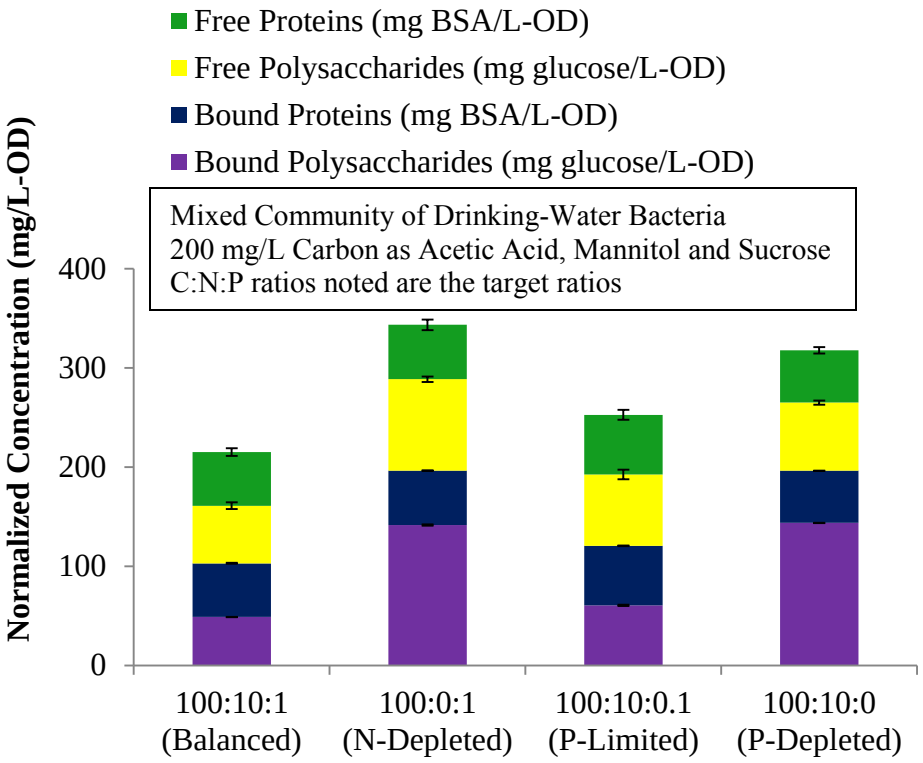

Figure 4.9: Mixed Community Batch Experiment Normalized Free and Bound EPS Carbohydrate and Protein Measurements at Day 5. Error bars represent standard deviation among triplicate absorbance measurements.

Batch experiments run with 2 or 20 mg/L showed very low growth and EPS after 5 days. The measured free and bound carbohydrates and proteins (data not shown) were at or below the method detection limits. Future experiments at low carbon concentrations should concentrate the samples prior to EPS measurements. Additionally, trace amounts of nitrogen and phosphorus in the chemicals and the DDI water used to prepare the synthetic groundwater may have been significant enough to alter the C:N:P ratios targeted. Measuring the background nitrogen and phosphorus concentrations would provide a more accurate calculation of C:N:P ratios.

4.3.2 Mixed Community Batch Experiments Comparing Ammonium Chloride to Liquid Ammonium Sulfate as the Sole Nitrogen Source.

Recent Water Research Foundation tailored collaboration 4346 sponsored by the Dallas Water Utilities implemented a nutrient (phosphorus and nitrogen) enhancement strategy (targeting a C:N:P ratio of 100:20:>2) as a method of controlling headloss in

pilot-scale biofilters. When supplementing the pilot-scale biofilter with liquid ammonium sulfate (LAS) as a nitrogen source, an increase in headloss was noted as compared to a different season where background ammonia concentrations in the influent were sufficient to reach the target carbon to nitrogen ratio. Collaborators on the project questioned whether LAS could have triggered increased EPS production resulting in greater headloss in the pilot-scale biofilter (Chance Lauderdale, Carollo Engineers, personal communication, April 5, 2013).

Batch experiments with a mixed community were run to determine whether ammonium chloride versus LAS as the sole nitrogen source would differentially impact EPS production. At a carbon concentration of 2 g/L composed of a mixture of mannitol, acetic acid and sucrose, a nutrient-balanced condition (C:N:P molar ratio of 100:10:1) with ammonium chloride as the sole nitrogen source, a nutrient-balanced condition with LAS as the sole nitrogen source, and nitrogen-depleted (C:N:P molar ratio of 100:0:1) batch experiments were conducted.

At five days after inoculation, the batch experiments were measured for free and bound EPS. Total free and bound proteins and carbohydrates with ammonium chloride as the sole nitrogen source were 32 mg/L and with LAS as the sole nitrogen source were 46 mg/L. The EPS concentrations corresponded with OD_{600} measurements of approximately 0.65 (ammonium chloride) and 0.80 (LAS). Thus, an increase in total EPS concentration and cell density was measured when LAS as compared to ammonium chloride was dosed in batch experiments as the sole nitrogen source. Total EPS concentrations normalized to OD_{600} are shown in Figure 4.10. Slight increases in normalized free EPS were observed in the experiment with LAS over that with ammonium chloride. These measurements correspond with greater precipitation of EPS observed visually during the ethanol precipitation step. However, there were not apparent differences between total EPS production normalized to OD_{600} in the batch experiments with LAS and ammonium chloride.

A difference in cell pellet color between the two samples was observed; the LAS batch experiment produced a pink cell pellet while the ammonium chloride batch

experiment produced a cream colored cell pellet. As one possible explanation for the cell pellet color difference might be a shift in the microbial community or the composition of the polysaccharides or proteins, further investigation is required.

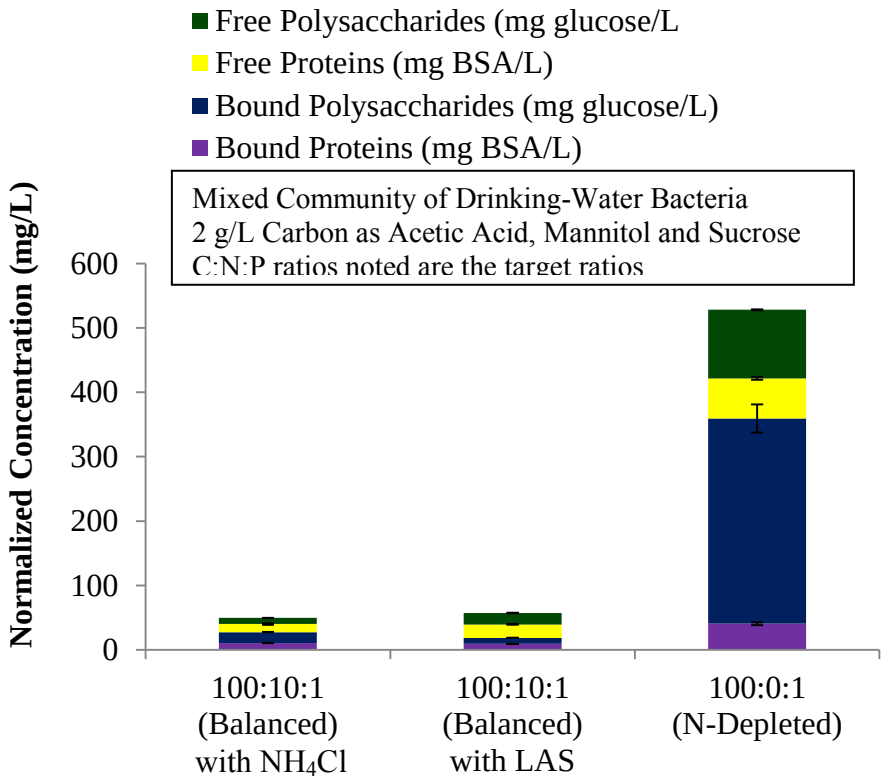

Figure 4.10: Mixed Community Batch Experiment Normalized Free and Bound EPS Carbohydrate and Protein Measurements at Day 5. Error bars represent standard deviation among triplicate absorbance measurements.

4.3.3 SUMMARY

Based on the batch experiments conducted with a mixed community of planktonic drinking-water bacteria, an increase in EPS production under nitrogen- and phosphorus-depleted conditions was observed at the bench-scale. Batch experiments with very high carbon concentrations (based on 2 or 0.2 g/L carbon) of a carbon source mixture (acetic acid, mannitol, and sucrose) showed an increase in EPS normalized to OD_{600} under nitrogen- and phosphorus-depleted conditions as compared to a nutrient-balanced condition. While these carbon concentrations are not relevant to drinking-water, they allowed us to confidently assess differences in growth and EPS production among the

various C:N:P molar ratios tested. Experiments at lower concentrations of carbon should be conducted, with concentration of the extracted EPS prior to EPS measurement. Additionally, background concentrations of nitrogen and phosphorus in the media should be measured so that actual C:N:P molar ratios (as opposed to dosed C:N:P molar ratios) can be calculated. Batch experiments comparing the effect of ammonium chloride versus LAS as the sole nitrogen source did not show any apparent differences between total (free plus bound normalized to OD_{600}) EPS production by a mixed community of drinking-water bacteria.

In addition, these batch experiments allowed for the evaluation of the EPS extraction methods that have been used in this research. Without performing ethanol precipitation, the carbohydrate concentrations measured reflect EPS production and remaining carbohydrates dosed in the medium. Thus, increases in EPS production might be confounded by decreases in the concentrations of carbohydrates in the medium. Further, extracting bound EPS from these experiments made it apparent that the majority of EPS in these planktonic cultures was bound to cells. Overall, these experiments have provided a baseline upon which flow-through chemostat with biofilm supports and bench-scale column experiments will be run and monitored.

4.4 *B. cereus* Batch Experiments

Batch experiments with *B. cereus* were conducted at a carbon concentration of 2 g/L composed of a mixture of mannitol, acetic acid and sucrose. Nutrient-balanced (C:N:P molar ratio of 100:10:1), nitrogen-depleted (C:N:P molar ratio of 100:0:1) and phosphorus-depleted (C:N:P molar ratio of 100:10:0) nutrient conditions were tested.

At five days after inoculation, the batch experiments were measured for the OD_{600}, carbohydrates, and proteins. Figure 4.11 displays the OD_{600} for each nutrient condition. Growth was limited under the nitrogen- and phosphorus-depletion as compared to the nutrient-balanced condition, as expected. EPS was analyzed following three different processing methods (described in section 3.3.2): (1) cells were removed from the supernatant and then total extracellular proteins and carbohydrates were

quantified in the supernatant (including medium components), (2) cells were removed and ethanol precipitation was performed on the supernatant to obtain free EPS, and (3) the removed cells (pelleted) were processed to obtain bound EPS.

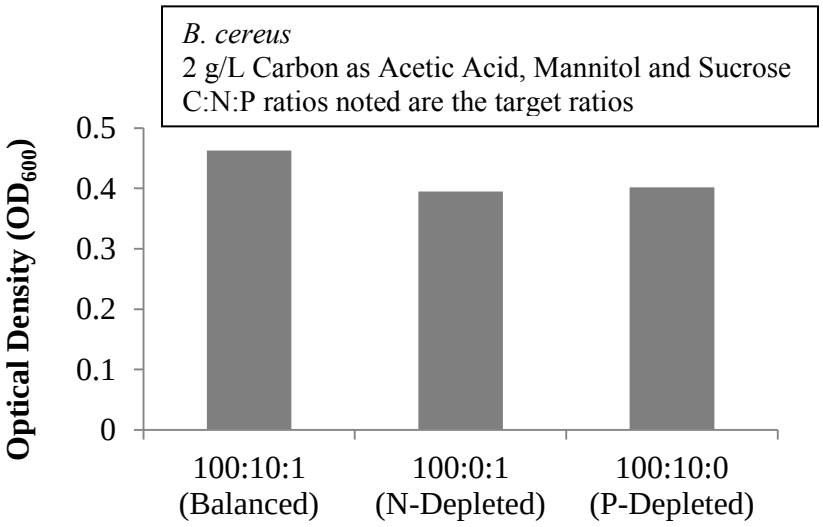

Figure 4.11: *B. cereus* Batch Experiment Optical Density at Day 5.

Total extracellular carbohydrates and proteins (including medium components) were monitored at day 5 and compared to carbohydrates and proteins in sterile media. All sterile media (100:10:1, 100:0:1, and 100:10:0) contained carbohydrate concentrations greater than 100 mg/L (the upper method detection limit). The spent culture medium following the first processing method (analysis of the supernatant after centrifugation) under a nutrient-balanced (100:10:1) condition contained 34.07 ± 0.62 mg/L as glucose (mean ± standard deviation of triplicate absorbance measurements). Note that this carbohydrate concentration in the spent nutrient-balanced medium is the only difference from similar batch experiments with a mixed community of drinking-water bacteria (see section 4.3.1). Both the nitrogen- and phosphorus-depleted spent culture media, following this first processing method, contained carbohydrate concentrations greater than 100 mg/L. Comparing the data for total extracellular carbohydrates to the optical density data for the three nutrient conditions, the greatest growth (highest optical density) corresponded to the lowest remaining carbohydrate

concentration in the culture (including medium components). The decrease in carbohydrates in the spent culture media as compared to sterile medium is likely a reflection of growth as bacteria are consuming carbohydrates to produce energy and biomass. Thus, the decrease in carbohydrates in the nutrient-balanced spent culture medium is likely a reflection of cell growth rather than EPS production. These data illustrate the importance of extracting EPS from the media. Total extracellular proteins were higher in the spent culture media (approximately 10 mg/L as BSA) than in the sterile media (below the 1.25 mg/L detection limit) under each nutrient condition. As proteins in sterile media were so low, it is likely that these increases in protein concentrations demonstrate EPS production.

Figure 4.12 displays the carbohydrate and protein concentrations following ethanol precipitation to obtain free EPS and processing of the cell pellet to obtain bound EPS. A similar trend was seen in the *B. cereus* experiments as in the mixed community of drinking-water bacteria in terms of bound carbohydrates normalized to OD_{600}. Bound carbohydrates normalized to OD_{600} increased under nitrogen- and phosphorus-depleted conditions, but free normalized carbohydrates and both free and bound normalized proteins decreased under nitrogen- and phosphorus-depleted conditions as compared to a nutrient-balanced condition. Overall free and bound EPS production normalized to OD_{600} and growth was less than that of the mixed community of drinking-water bacteria under the same nutrient conditions. Additionally, these experiments confirmed the importance of free and bound EPS extraction protocols to separate carbohydrate and protein due to EPS from that in the medium as was also illustrated by the mixed community experiments.

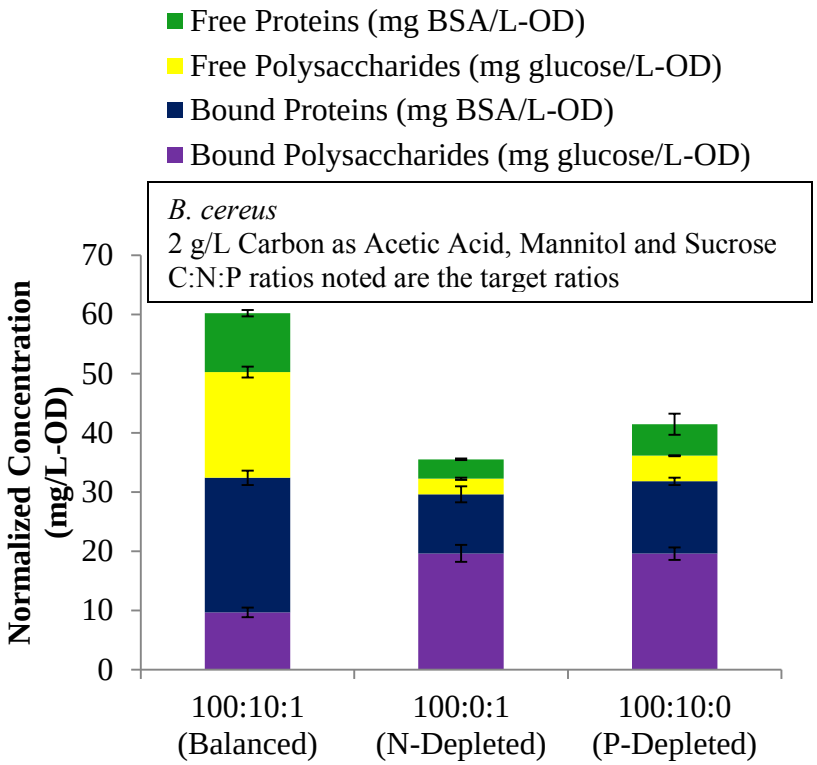

Figure 4.12: *B. cereus* Batch Experiment Normalized Free and Bound EPS Carbohydrate and Protein Measurements at Day 5. Error bars represent standard deviation among triplicate absorbance measurements.

CHAPTER 5: CONCLUSIONS

In this chapter, a summary of the conclusions reached through this research is presented and recommendations for future work are outlined.

5.1 Summary of Findings

This research studied the effect of nitrogen- and phosphorus-limitations on the production of extracellular polymeric substances (EPS) in pure cultures of *Bradyrhizobium japonicum* and *Bacillus cereus* and a mixed community of drinking-water bacteria obtained from a full-scale biologically active carbon (BAC) filter. The main objective of this project (the first phase of an on-going research project) was to investigate and optimize the methods that will be used in future research and obtain a baseline to aid in designing flow-through, bench-scale experiments. Batch experiments were conducted at multiple carbon to nitrogen to phosphorus molar ratios (C:N:P ratios of 100:10:1, 100:0:1, 100:10:0.1 and 100:10:0) and several carbon concentrations (2 g/L, 200 mg/L, 20 mg/L and 2 mg/L). EPS, measured as carbohydrates and proteins, and bacterial population density, measured as the optical density at 600 nm (OD_{600}), were monitored. EPS was analyzed following three different processing methods: (1) cells were removed from the supernatant and then total extracellular proteins and carbohydrates were quantified in the supernatant (including medium components), (2) cells were removed and ethanol precipitation was performed on the supernatant to obtain free EPS, and (3) the removed cells (pelleted) were processed to obtain bound EPS. The main findings of these experiments are summarized as follows:

- *B. japonicum* was identified through a literature review to be an interesting organism for studying the role of nutrient limitations on EPS production under drinking-water conditions. However, while *B. japonicum* grew well in YEM medium used prepare the inoculum for subsequent experiments, no visible biomass formed in batch experiments with synthetic groundwater or Götz Minimal Medium where the impact of nutrient limitations on EPS production

was to be tested. Neither could cells be pelleted from the batch experiments with groundwater or Götz Minimal Medium. Additional work is required to investigate why *B. japonicum* did not grow as expected.

- Batch experiments with a mixed community of drinking-water bacteria resulted in increased EPS production under nitrogen- and phosphorus-depleted conditions as compared to a nutrient-balanced condition. Under high carbon conditions (2.0 or 0.2 g/L carbon concentrations dosed as a mixture of acetic acid, mannitol and sucrose), bound polysaccharides were found to increase under nitrogen-depleted and phosphorus-depleted conditions as compared to a nutrient-balanced condition. A phosphorus-limited C:N:P ratio of 100:10:0.1 decreased the normalized EPS concentration to near that of the nutrient-balanced condition. Even though compared to the nutrient-balanced condition (100:10:1) there was still a phosphorus-limitation, the addition of a small amount of phosphorus (0.5 mg/L) reduced the amount of EPS produced. Thus, the minimum carbon to phosphorus ratio required to prevent excessive headloss in biological filters may be less than 100:1.
- Similar results, although to a lesser extent, were also obtained with a pure culture of *B. cereus* grown in these same media. Under nitrogen- and phosphorus-depleted conditions as compared to a nutrient-balanced condition, batch experiments with *B. cereus* showed increased bound carbohydrates.
- In batch experiments with a mixed community of drinking-water bacteria, use of ammonium chloride versus liquid ammonium sulfate (LAS) as the sole nitrogen source did not result in apparent differences in total EPS production normalized to cell growth.
- The importance of extracting EPS from groundwater medium prior to analysis of carbohydrates and proteins was illustrated in both the mixed community and *B. cereus* experiments. Without EPS extraction, the decrease in carbohydrate concentration in the medium due to bacterial consumption could confound the increase in polysaccharide concentration due to EPS production.

Thus, the application of an extraction procedure to remove EPS from both cells and medium is very important.

These batch experiments have allowed for the investigation of EPS production of pure cultures and a mixed community of drinking-water microorganisms under various nutrient conditions. The largest increases in EPS production under nitrogen- and phosphorus-depleted conditions were observed in the carbohydrates bound to cells of *B. cereus* and a mixed community of drinking-water microorganisms under carbon concentrations of 2.0 and 0.2 g/L (mixture of acetic acid, mannitol and sucrose). While these concentrations might not be relevant to drinking-water biofilters, these experiments are a baseline upon which flow-through reactors will be designed. Flow-through reactors including chemostats (with biofilm supports) and bench-scale columns will provide biofilms that will be interrogated for changes in EPS concentrations.

5.2 Recommendations for Future Work

Future work should include flow-through chemostat (with biofilm supports) and bench-scale column experiments, which better replicate conditions in drinking-water biofilters. The use of plastic media to grow biofilms in flow-through chemostats will allow biofilm growth without the complications of sampling a BAC filter. The use of BAC columns will allow for confirming that the same factors are still apparent in a bench-scale reactor replicating actual drinking-water biofilter conditions; bench-scale columns will allow EPS production to be related back to biofilter headloss increases. Research objectives for these systems should include:

- Investigating various carbon concentrations ranging from 2 g/L to 2 mg/L;
- Determining how stringent a nitrogen- or phosphorus-limitation is required to induce the production of excessive EPS;
- Examining the effect of multiple carbon sources, such as acetic acid, mannitol, and sucrose, nitrogen sources, such as ammonium chloride, ammonium sulfate and liquid ammonium sulfate, and phosphorus sources,

such as phosphoric acid, potassium phosphate and sodium hexametaphosphate on EPS production;

- Rectifying the lack of growth of *B. japonicum* in synthetic groundwater or Götz Minimal Medium, such that nutrient/EPS experiments can be conducted with that isolate;
- Assessing the effects of low concentrations of trace nutrients (e.g., calcium, molybdenum, magnesium, iron) on EPS production.

Future work should include the use of both a pure culture, such as *B. japonicum*, to investigate the effects of increases in EPS production by individual populations and a mixed community of drinking-water bacteria to investigate shifts in the microbial community caused by changes in nutrient conditions.

APPENDIX A: PURE CULTURE BATCH EXPERIMENTS

As previously discussed, *B. japonicum* is a well-known EPS-producer found in drinking-water filters (e.g., Lauderdale et al., 2011), which has been shown to increase EPS production under nitrogen-limitation (Lopez-Garcia et al., 2001; Quelas et al., 2006). Thus, to study the effects of nutrient limitations on a pure culture under drinking-water conditions, batch experiments were conducted with *B. japonicum* in a synthetic groundwater and Götz Minimal Medium.

A.1 Batch Experiments with Groundwater

Triplicate batch experiments were inoculated with *B. japonicum* grown to mid-log phase. Three groundwater media with varying target C:N:P molar ratios of 100:10:1 (nutrient-balanced), 100:0:1 (nitrogen-depleted) and 100:10:0 (phosphorus-depleted) based on a carbon (as mannitol) concentration of 2 g/L were analyzed. The carbon source and concentration selected were based on that used in previous experiments with *B. japonicum*, which resulted in increased EPS production under nitrogen-limitation (Lopez-Garcia et al., 2001; Quelas et al., 2006). Total extracellular proteins and carbohydrates (including media components) and bacterial densities (as colony forming units, CFU) were monitored over time and are displayed in Figure A.1. Note that these plots represent the average of three triplicate batch experiments run at each C:N:P molar ratio; each replicate showed similar trends (as demonstrated by the low standard deviations among measurements).

No trends among total extracellular proteins and carbohydrates (including media components) and nutrient limitations were observed. Additionally, no trends among nutrient limitations and total extracellular proteins and carbohydrates (including media components) were observed when proteins and carbohydrates were normalized to the bacteria population (mg/CFU, data not shown). As it appears the bacterial population was dying throughout the experiment, it is possible that starting with a lower concentration of cells may allow for net cell growth.

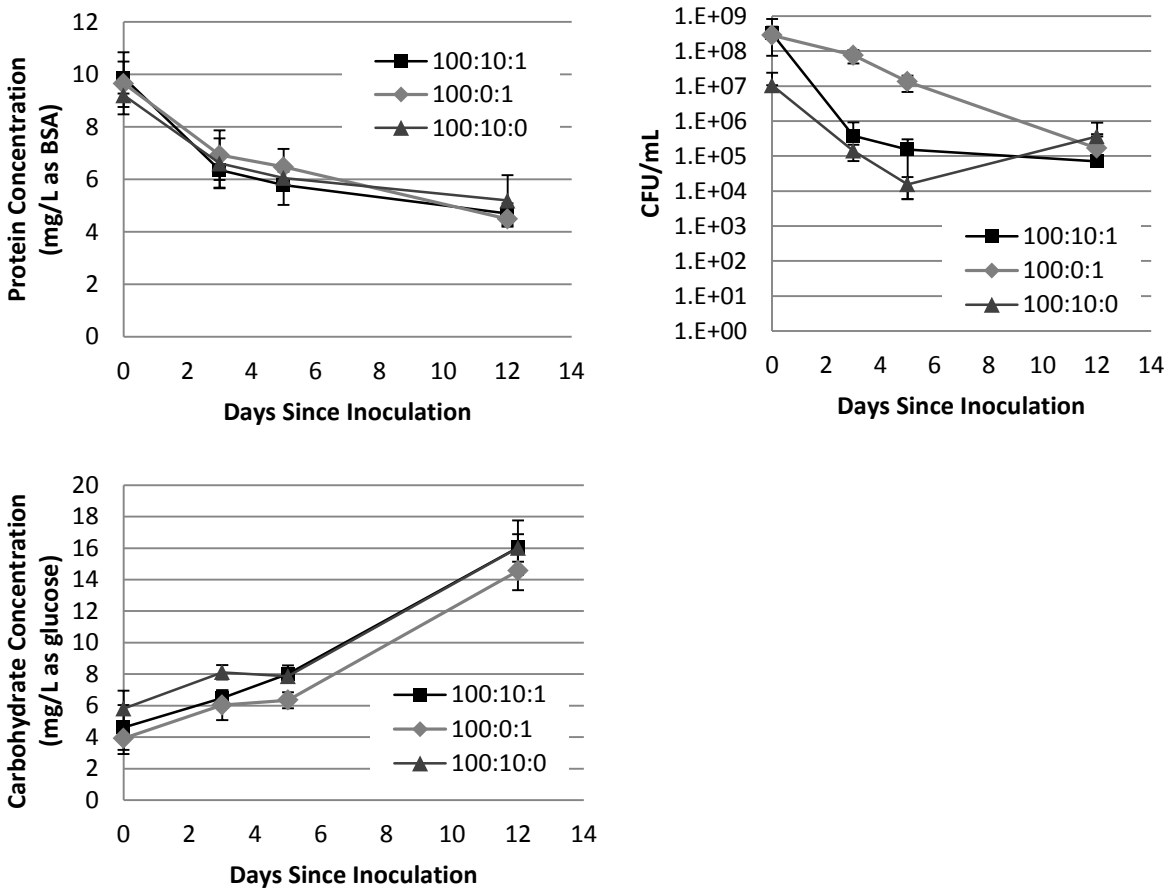

Figure A.1: *B. japonicum* Pure Culture Batch Experiment Total Extracellular Protein (Top Left), Total Extracellular Carbohydrate (Bottom Left), and CFU (Top Right) Measurements over Time. Error bars represent standard deviation among triplicate absorbance measurements. C:N:P ratios shown are target ratios.

A.2 Experiments with Götz Minimal Medium

Due to the unexpected results of the *B. japonicum* experiments in synthetic groundwater medium, a set of batch experiments was conducted using Götz Minimal Medium to replicate the results of previous research (Lopez-Garcia et al., 2001; Quelas et al., 2006). *B. japonicum* was grown to mid-log phase in YEM medium from a single colony grown on YEM agar and directly from frozen stock. *B. japonicum*, grown both ways, was then inoculated into 50 mL of nitrogen-sufficient (1 mM $(NH_4)_2SO_4$) or nitrogen-depleted (no addition of $(NH_4)_2SO_4$) MOPS-Götz medium.

At five days after inoculation, the batch experiments were measured for EPS following two different sample processing methods: (1) cells were removed from the supernatant (0.2 μm filter) and then total extracellular proteins and carbohydrates were quantified in the supernatant (including medium components), and (2) cells were removed by 0.2 μm filtration and ethanol precipitation was performed on the supernatant to obtain free EPS. As no cell pellet was formed from the centrifugation of these cultures, bound EPS could not be determined. This was the only set of batch experiments with *B. japonicum* that was subjected to ethanol precipitation before analysis of carbohydrates and proteins.

Total extracellular carbohydrates and proteins (including medium components) and ethanol precipitated free carbohydrates and proteins (data not shown) were below the carbohydrate and protein method detection limits. Thus, the results of other research studies were not replicated (Lopez-Garcia et al., 2001; Quelas et al., 2006).

A.3 Summary

Although *B. japonicum* appears to be an interesting organism upon which to study the effects of nutrient limitations on EPS production under drinking-water conditions based on previous research, no relationship among nutrient limitations and increased EPS production was demonstrated in these batch experiments. Additional experimentation is required to determine why *B. japonicum* did not grow in synthetic groundwater and Götz Minimal Medium.

APPENDIX B: LAB PROTOCOLS

B.1 Polysaccharides/Carbohydrates

Method: Anthrone Method Modified from
Raunkjaer K., Hvitved-Jacobsen T. & Nielsen P. H. (1994). WATER RES, 28, 251-262.
Linear Range: ~5-100 mg/L.
Location: UNDER FUME HOOD!!
Reagent – anthrone (2 hrs prior to use): Measure 25 mg anthrone and add to 40 mL vial. Measure 20 mL 95% H_2SO_4 using a 25 mL volumetric flask and add to anthrone. Must be remade each day.
Standards: Store at room temperature in glass.

Stock solution 1, S1	0.2	g glucose/200mL
	0.001	g/mL

Stock solution 2, S2	**1**	**mL S1/10mL**
Use a 1 mL pipet	0.0001	g/mL

To make 0.8 mL of standard:

Volume S2 (mL) *(use the 200 µL pipet)*	Volume DDI (mL) *(use the 1 mL pipet)*	Final Concentration
0	0.8	0 ppm or µg/mL or mg/L
0.04	0.76	5 ppm or µg/mL or mg/L
0.06	0.74	7.5 ppm or µg/mL or mg/L
0.09	0.71	11.25 ppm or µg/mL or mg/L
0.2	0.6	25 ppm or µg/mL or mg/L

Procedure:
Turn on the heat block to 6.2 high (100°C).

1. Add 0.8 mL of sample in 15 mm glass test tube using the 1 mL pipet.
2. Add 1.6 mL reagent under the fume hood using the 1 mL acid pipet. Vortex (~2 seconds).
3. Place samples in 100 C heat block for 14 min under the fume hood. Vortex.
4. Cool at 4 C for 5 min in a water bath.
5. Vortex. Transfer into an unsterile microplate in triplicate using the acid pipet. Read at **625** nm in triplicate.

Method: Dubois Method (Modified from Dubois et al., 1956)
Linear Range: ~5-100 mg/L.
Location: UNDER FUME HOOD!!
Standards: Store at room temperature in glass.

Stock solution 1, S1	0.2	g glucose/200mL
	0.001	g/mL

Stock solution 2, S2	**5**	**mL S1/50mL**
	0.0001	g/mL

To make 2 mL of standard:

Volume S2 (mL) per 1 mL	Volume (mL) S2 per 2 mL	Volume (mL) DDI per 2 mL	Final concentration	Final Concentration
0	0	2	0 g/mL	0 ppm or µg/mL or mg/L
0.05	0.1	1.9	0.000005 g/mL	5 ppm or µg/mL or mg/L
0.07	0.14	1.86	0.000007 g/mL	7 ppm or µg/mL or mg/L
0.09	0.18	1.82	0.000009 g/mL	9 ppm or µg/mL or mg/L
0.11	0.22	1.78	0.000011 g/mL	11 ppm or µg/mL or mg/L
0.25	0.5	1.5	0.000025 g/mL	25 ppm or µg/mL or mg/L
0.5	1	1	0.00005 g/mL	50 ppm or µg/mL or mg/L
0.75	1.5	0.5	0.000075 g/mL	75 ppm or µg/mL or mg/L

Procedure:

Turn on water bath to 27.5°C

1. 2 mL sample added to 17 mm glass test tube.
2. 0.05 mL 80% phenol added.
3. 5 mL concentrated H_2SO_4 (95%) added rapidly against liquid. (*standards yellow, samples pink*)
4. Let stand 10 min. Vortex.
5. Place in 27.5 C water bath for 15 min.
6. Vortex. Read at 490 nm in triplicate.

B.2 Protein Microassay

Method: Bio-Rad Protein Assay Kit I, Bio-Rad Laboratories. Modified from: Bradford, M. (1976). ANAL BIOCHEM, 72, 248-254.
Linear range: 1.25 µg/ml to 10 µg/ml (edited from manual).
Dye Reagent: Filter 2 mL dye (stored in flammable fridge in Kinney Lab) with 8 mL DI through at Whatman #1 Filter. Store at room temperature in a 15 mL tube for up to 2 weeks.
Standards:
Bovine Serum Albumin: Stored in the freezer in the Kirisits Lab – S2 divided into 1 mL aliquots. ***Must be used within 60 days and kept at 4°C or frozen.***

Stock 1, S1:	1.42	mg BSA/mL

Stock 2, S2:	0.352 mL	mL S1/10 mL (9.648mL DI)
	0.05	mg/mL
	50	µg/mL

To make 1 mL in 1.5 mL tubes:

Volume S2 (**µL**) *(use 200 µL pipet)*	Volume DI (**mL**) *(use 1 mL pipet)*	Final concentration
200	0.8	10.00 µg/mL
150	0.85	7.50 µg/mL
100	0.9	5.00 µg/mL
40	0.96	2.00 µg/mL
30	0.97	1.50 µg/mL

Procedure:
1. Pipet 160 µl of each solution into separate microtiter plate wells using the 200 µL pipet.
2. Add 40 µl of dye reagent concentrate to each well using the 200 µL pipet.
3. Mix the sample and reagent thoroughly. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tip and add reagent to the next set of wells.
4. Incubate at room temperature for 5 min. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
5. Measure absorbance at **595** nm.

B.3 Lipids

Method: Sulpho-phospho-vanillin Method. Modified from: Frings, C., Fendley, T., Dunn, R., & Queen, C. (1972). CLIN CHEM, 18(7), 673-4.
Linear range: 6-12.5 g/L
Location: UNDER FUME HOOD!!
Reagents:

Vanillin Reagent:	Add 1.05 g of vanillin (stored in Kirisits fridge) to a 200 mL volumetric flask and dilute to volume with DI.
Phospho-Vanillin Reagent: **EXP: 2 months**	Add 200 mL of vanillin reagent to a 500 mL Erlenmeyer flask. Add 300 mL concentrated phosphoric acid with constant stirring. Store in a brown bottle at room temperature.

Standards: Store pure olive oil at room temperature. Store standards in Kirisits 4 C fridge. Used anhydrous ethanol solution.

Pure Olive Oil (S1):	0.9135	g/mL
	91350	mg/dL

Stock 2: *(use 1 mL pipet)*	0.14	mL S1/10 mL
EXP. 1 month	1278.9	mg/dL

To make 1 mL in 1.5 mL tubes:

Volume S2 (mL) *(use 1 mL pipet)*	Volume Ethanol (mL) *(use 1 mL pipet)*	Final concentration	
0.5	0.5	639.45	mg/dL
0.6	0.4	767.34	mg/dL
0.8	0.2	1023.12	mg/dL
S2		1278.9	mg/dL

Procedure:
Turn on water bath to 37°C.
Turn on heat block to 6.2 high.

1. Add 20 µL of sample to 15 mm glass test tubes using the 20 µL pipet.
2. Add 0.2 mL of H_2SO_4 (95%) using the 1 mL acid pipet – *yellow/orange.*
3. Vortex (~ 2 seconds).
4. Place samples in 100 C heat block for 10 min under fume hood.
5. Cool at 4 C for 5 min in a water bath.
6. Add 10 mL phospho-vanillin reagent under fume hood using the 25 mL glass graduated cylinder – *pinkish.*
7. Vortex (~ 2 seconds).
8. Incubate in 37 C water bath for 15 min.
9. Allow samples to cool for 5 min.
10. Vortex. Transfer into an **unsterile** microplate in triplicate. Read at **540** nm in triplicate.

B.4 Ortho-phosphate

Method: Ascorbic Acid Method (modified from APHA et al., 2005)
Range: 0.15-1.3 mg/L P at 1 cm light path. OR 0.01-0.25 mg/L P at 5 cm light path.
Reagents:

0.13% (5 N) Sulfuric Acid:	Add 7 mL 95% (35.6 N) sulfuric acid to 50 mL DI.
Ammonium molybdate:	Dissolve 8 g $(NH_4)_6Mo_7O_{24}$*$4H_2O$ in 200 mL. Store in glass-stoppered bottle.
Antimony potassium tartrate:	Dissolve 0.6857 g $K(SbO)C_4H_4O_6$*$0.5H_2O$ in 200 mL DI. Store in glass-stoppered bottle.
0.1 M Ascorbic Acid **(EXP. 1 week):**	Dissolve 0.1056 g/6mL ascorbic acid in DDI. Use most accurate scale.
20 mL Combined Reagent **(EXP. 4 hrs):**	1. Add 10 mL 5N H_2SO_4. Mix 2. Add 1 mL antimony potassium tartrate. Mix 3. Add 3 mL ammonium molybdate. Mix 4. Add 6 mL 0.1 M ascorbic acid. Mix

Standards:
To make stock solution:

1. Dry approximately 1 g of KH_2PO_4 for 1 hr at 105 C. Allow to cool in desiccator.
2. Measure out 0.8789 g dried KH_2PO_4 and add to 200 mL DI. Stored at 4 C.

Stock solution **1, S1**	**0.2**	**g of P/200mL**
	1000	mg/L

Stock solution **2, S2**	**0.05**	**mL S1/10mL**	Stock solution **3, S3**	**20**	**µL S1/100mL**
	5	µg/mL or mg/L		0.2	µg/mL or mg/L

To make 5 mL of standard in 17 mm test tubes:

Volume of S2 (mL)	Volume of DDI (mL) *(use 1 mL pipet)*	Final concentration
0.01 *(20 µL pipet)*	4.99	0.01 mg/L P
0.05 *(200 µL pipet)*	4.95	0.05 mg/L P
0.2 *(200 µL pipet)*	4.8	0.2 mg/L P
0.3 *(1 mL pipet)*	4.7	0.3 mg/L P
0.4 *(1 mL pipet)*	4.6	0.4 mg/L P
0.5 *(1 mL pipet)*	4.5	0.5 mg/L P

Procedure:

1. Add 5 mL sample in ***acid-washed*** 17 mm glass test tube.
2. Add one drop phenolphthalein indicator. If red color develops add sulfuric acid dropwise.
3. Add 0.8 mL combine reagent. Vortex (~ 2 seconds).
4. Let stand 10 min.
5. Read at 880 nm (no more than 20 min later) in triplicate in unsterile (acid) well plate. Must use 5 cm quartz cuvette for <0.15 mg/L P.

B.5 Synthetic Groundwater

(modified from Li et al., 2010)

- Composition determined for a real groundwater (Rialto, CA).
- ClO_4^- omitted.
- Sodium Nitrate replaced with Ammonium Chloride.
- Carbon source modified.

Groundwater Salts Stock Solution

Begin by making a solution of all components (except the C, N and P Sources):

Total Volume:	**0.5**	Liters at 500 mL 1X:	**20** **25**	Times Concentrate mL of 20X

Component:	Final Concentration (mg/L)	20X Concentrate (mg/L)	Mass to add to 500 mL to make a 20X Concentrate (mg)
Na_2SO_4	17.75	355.00	177.5
K_2CO_3	6.9	138.00	69
$NaHCO_3$	289.18	5783.60	2891.8
NaCl	13.68	273.60	136.8
$CaCl_2$	2.81	56.20	28.1
$MgCl_2$	3.88	77.60	38.8

Dissolve to volume with DI.

Dilute to 500 mL of 1X.

To make stock solution with C:N:P ratios:
Add 25 mL groundwater stock and the following.
Fill to 500 mL with DDI and autoclave.

Stock solutions for nutrients varied among experiments. MOPS buffer was added.

Add Vitamins and Minerals:

100X Vitamin Stock Solution (10 mL for 1000 mL) (Staley, 1968)

DDI Water	1	L	
biotin	2	mg	In desiccator in Kirisits refrigerator
folic acid	2	mg	
pyridoxine-HCl	10	mg	In desiccator on shelf
riboflavin	5	mg	
thiamine-HCl	5	mg	In desiccator in Kirisits refrigerator
nicotinamide	5	mg	Nicotinic acid
calcium pantothenate	5	mg	
B12	0.1	mg	In desiccator in Kirisits refrigerator (cyanocobalamin)
p-aminobenzoic acid	5	mg	4-aminobenzoic acid

Mineral Stock Solution (1 mL per 1000 mL) (London et al., 2011)

DDI Water	1	L		
$CuSO_4*5H_20$	0.0574	g	5.74177E-05	$CuSO_4*5H_20$
$ZnSO_4*7H_2O$	0.2880	g	0.000288	
$NiCl_2*6H_2O$	0.0216	g	2.15653E-05	$NiCl_2*6H_2O$
$FeCl_2*4H_2O$	0.7016	g	0.0007016	
$AlCl_3*6H_2O$	0.2000	g	0.0002	In Speitel Lab
$MnCl_2*4H_2O$	0.2807	g	0.0002807	In Kirisits Fridge
$CoCl_2*6H2O$	0.0382	g	0.0000382	
$Na_2MoO_4*2H_2O$	0.0254	g	0.0000254	
H_3BO_3	0.0303	g	3.0348E-05	H_3BO_3
Na_2SO_4	0.1420	g	0.000142	

B.6 Ammonia-Nitrogen

Method: Ammonia Probe. This measures **total ammonia** (including ammonium converted to ammonia).
Location: Speitel Lab pH meter.
Range: Accurate to ~.05mg/l.
Calibrate:

1. 100ml DDI water + 2ml ammonia adjusting solution in a 150 mL flask with a stirbar. Keep covered with parafilm to keep ammonia gas from escaping.
2. Add 20µl of 500 mg/l ammonia [made with ammonium sulfate]
3. Check that it is on ammonia probe; if not, press 2nd: channel (look for pH 2 at the bottom left of the window)
4. Check that it is set to units of MV not pH (press mode to change)
5. Wait for it to get stable; record 0.1 mg/L and the MV value
6. Add 80µl ammonia
7. Wait for it to get stable; record 0.5 mg/L and the MV value
8. Add 100µl ammonia
9. Wait for it to get stable; record 1.0 mg/L and the MV value
10. Add 300µl ammonia
11. Wait for it to get stable; record 2.5 mg/L and the MV value
12. Add 500µl ammonia
13. Wait for it to get stable; record 5 mg/L and the MV value

Plot MV versus mg/L to get a semi-logarithmic regression equation.
To Use:

1. Raise pH of solution by adding 200µl of ammonia adjusting solution per 10ml solution into a 20 mL vial and stir. Keep covered with parafilm to help with stability.
2. Insert detector and read when solution becomes stable.

If solutions are low, make more.
Waste can be neutralized by adding acid until the blue color turns clear.

B.7 EPS Extraction – Wet BAC

Method: Modified from: Fang, H., & Jia, X. S. (1996, November 10). BIOTECHNOL TECH, 803-808. Optimized by: Tzu-Hsin and Ameet Pinto, University of Michigan.

Application: Drinking-Water BAC Samples

Procedure:

1. From each sample, measure 2g of GAC into 15 ml centrifuge tubes. Decant water from top 2 mL of GAC sample and allow water to settle before sampling.
2. Add 10 ml of 0.01M PBS buffer (**aseptically**) at pH=7 into each 15ml centrifuge tube.
3. Add 60µl of 36.5% (v/v) formaldehyde solution (stored below hood) into each sample tube. UNDER FUME HOOD!
4. Let the sample incubate in the 4C walk-in fridge (Katz Lab) for 1 hour on the 9 rpm rotating Labquake shaker.
5. Turn on the water bath and set to 80°C.
6. At the end of the 1 hour incubation, transfer the tubes from the fridge into the 80°C and incubate for 10 min.
7. Centrifuge all tubes at 5000xg for 10 min. The extracted EPS components should now be in the supernatant.
8. From each tube, transfer supernatant into 15 mL tube and vortex.
9. Measure the dry weight of GAC samples by drying at 105°C for 1 hr.

B.8 YEM Medium

Yeast Extract Mannitol (YEM) Medium (modified from Danso and Alexander, 1974)
Reagent – 16.6% $CaCl_2$: Add 1.66 g $CaCl_2$ to 10 mL DI. Autoclave.
Procedure:

For Liquid Medium:	Prepare as shown below for 1 L of medium in a glass 1 L bottle.
For Plate Agar:	Prepare as shown below for 500 mL (1 sleeve of plates) or 1 L (2 sleeves of plates) in an Erlenmeyer flask. Dissolve completely, then add 15 g Agar (large white bucket) per L of medium and mix thoroughly. Cover with foil. Allow leftover medium to cool and dump in trash. Do not pour agar down the drain!

	1 liter	**500 mL**
Mannitol	5 g	2.5 g
Yeast extract	0.5 g	0.25 g
$MgSO_4$ x 7 H_20	0.2 g	0.1 g
NaCl	0.1 g	0.05 g
K_2HPO_4 x $3H_2O$	0.66 g	0.33 g
Na Gluconate (AKA gluconic acid sodium salt)	5 g	2.5 g
add DI using a graduated cylinder	1 L	500 mL

Autoclave in Speitel Lab on cycle 10 (approx. 2.5 hrs to complete) or on "slow" for 15 min in the small autoclave.
Add 1 ml of **the 16.6% $CaCl_2$ solution** to 1 liter YEM medium (1:1000).
Store at room temperature or 4 °C.

APPENDIX C: CHEMICAL LIST

Polysaccharides:

- **Anthrone**, ACS, Acros Organics, AC10496-0250
- **Sulfuric Acid**, 95% solution in water, ACS, Acros Organics, AC42452-0025
- **D-Glucose**, ACS, Sigma-Aldrich, G5767-500G

Proteins:

- **Bio-Rad Protein Assay**, Kit I, Bio-Rad Laboratories

Lipids:

- **Vanillin**, pure, 99%, Acros Organics, AC14082-1000
- **Phosphoric Acid**, 85%, Analytical Reagent, Mallinckrodt, 2796-8
- **Olive Oil**, pure, refined, Acros Organics 0.9135g/mL, AC41653-0250
- **Ethanol**, anhydrous, Histological Grade, Fisher, M-4702
- **Sulfuric Acid**, 95% solution in water, ACS, Acros Organics, AC42452-0025

Ortho-phosphate:

- **Sulfuric Acid**, 95% solution in water, ACS, Acros Organics, AC42452-0025
- **Ammonium Molybdate Tetrahydrate,** ACS reagent, Sigma-Aldrich, A7302-100G
- **Antimony Potassium Tartrate**, Certified, Fisher Scientific Company, A-865
- **Ascorbic Acid**, Analytical Reagent, Mallinckrodt, 4407
- **Potassium Phosphate Dibasic**, ACS, >98%, Sigma-Aldrich, P3786

Synthetic Ground Water:

- **Sodium Sulfate**, 99%, extra pure, anhydrous, Acros Organics, 7757-82-6
- **Potassium Carbonate**, ACS, Sigma-Aldrich, 548-08-7
- **Sodium Bicarbonate**, GR, powder, EM Science, 144-55-8
- **Sodium Chloride**, ACS, Fisher Scientific, 1065
- **Calcium Chloride**, Anhydrous, Spectrum Chemical Mfg. Corp., C1075
- **Magnesium Chloride**, ACS, Fisher Scientific, M33-500
- **Phosphoric Acid**, 85%, Analytical Reagent, Mallinckrodt, 2796-8
- **Acetic Acid**, glacial, certified ACS plus, Fisher, UN2789
- **Ammonium Chloride**, ACS, Fisher Scientific, A661-500
- **Liquid Ammonium Sulfate**, 40%, Martin Resources, Plainview, Texas
- **D-Mannitol**, 98+%, Acros Organics, 69-65-8
- **MOPS**, ≥99.5%, Certified, Sigma, M3183
- **D-Glucose**, ACS, Sigma-Aldrich, G5767-500G
- **Sucrose**, ACS, Sigma-Aldrich, S5016

Ammonia-Nitrogen:

- **Ammonium Sulfate**, ACS, MCB Reagents, AX1385-3
- **NaOH**, Certified ACS pellets, Fisher Scientific, L-12647
- **Disodium EDTA Dihydrate**, 99+%, for molecular biology, Sigma, E-5734
- **Thymol Blue**, powder, ACS,MC/B, TX620, NB172
- **Methanol**, optima, Fisher, A454-4

YEM:

- **D-Mannitol**, 98+%, Acros Organics, 69-65-8
- **Gluconic Acid**, Sodium Salt, 98%, Acros, 18139-0010
- **Yeast Extract**, Fisher Scientific, BP1422-500
- **Sodium Chloride**, Certified ACS, Fisher, S271-3
- **Potassium Phosphate Dibasic Trihydrate**, min 99%, Sigma, P5504
- **Magnesium Sulfate Heptahydrate**, USP/FCC/EP, Fisher, FL-01-0100
- **Agar**, granulated molecular genetics, Fisher, BP1423-2

PBS:

- **Sodium Chloride**, Certified ACS, Fisher, S271-3
- **Potassium Chloride**, GR crystals, EM Science, PX1405-1
- **Sodium Phosphate Dibasic Heptahydrate**, ACS, 98.0-102%, Sigma-Aldrich, S9390
- **Potassium Phosphate Monobasic**, ACS, ≥99%, Sigma, P0662-500G

Misc.:

- **Glycerol**, biotechnology grade, Amresco, 0854
- **R2A**, HIMEDIA, M1687-500G
- **LB**, Difco LB Broth, Lennox, 240210
- **Formaldehyde**, 37% wt, ACS, Sigma, Aldrich, 252549
- **Tris/HCl**, ultra pure grade, Amresco, 0234
- **Phenol**, 99.5%, extra pure, loose crystals, Acros Organics, 108-95-2

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