Improved Microalgal Biomass Harvesting Using Optimized Environmental Conditions and Bacterial Biofloculants

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By

Derek Conte Manheim

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COMMITTEE MEMBERSHIP

Title: Improved Microalgal Biomass Harvesting Using Optimized Environmental Conditions and Bacterial Bioflocclants

Author: Derek Conte Manheim

Date Submitted: August 10, 2012

Dr. Yarrow Nelson
Committee Chair

Signature

Dr. Tryg Lundquist
Committee Member

Signature

Dr. Chris Kitts
Committee Member

Signature

ABSTRACT

IMPROVED MICROALGAL BIOMASS HARVESTING USING OPTIMIZED ENVIRONMENTAL CONDITIONS AND BACTERIAL BIOFLOCCULANTS

DEREK CONTE MANHEIM

The cost and energy balance of microalgae biofuel production is sensitive to the algae harvesting method, among many other factors. Bioflocculation and settling of suspended microalgae cultures is a harvesting method with potentially low cost and energy input. However, bioflocculation (the spontaneous flocculation of algal cells without chemical addition) has not been a reliable process with cultures grown in ponds. To provide insights to help improve algae settling, factors affecting the settling of algae were investigated in the laboratory using pure cultures of two common microalgae species: *Scenedesmus sp.* and *Chlorella vulgaris*. Bioflocculation of these algae was studied with and without the addition of bioflocculants produced by the bacterium, *Burkholderia cepacia*, to improve settling efficiencies. The bioflocculant produced by this bacterium was used in two different forms: a cell suspension including capsular and dissolved extracellular polymeric substance (EPS) components of *B. cepacia*, and dialyzed filtrate of the bacterial culture (only dissolved EPS fraction). The effects of algal growth phase, mixing time, bioflocculant dose, and environmental conditions such as pH and nutrient deprivation of bacterial bioflocculant cultures on settling of the algae species were studied.

Settling characteristics were different for the two algae cultures, and their settling was affected differently by the many factors studied. *Scenedesmus* settling was best in later growth stages, while *Chlorella* settled much better in early growth phases. Addition of *B. cepacia* cells as a bioflocculant improved settling of *Scenedesmus*, with the greatest effect during mid to late exponential growth of the *Scenedesmus*. In contrast, addition of *B. cepacia* filtrate as a bioflocculant best improved *Chlorella* settling during stationary growth of *Chlorella*. Longer mixing times (contact time between the algae cells and bacterial bioflocculant) improved the settling of *Scenedesmus*, while *Chlorella* settled better with a shorter mixing time. Reducing the pH to 3 (a typical isoelectric point for microalgae) improved the settling of both algae cultures, with and without bioflocculant addition. Increasing the pH to 11 autoflocculated *Scenedesmus* cultures, but not *Chlorella* cultures, at early growth stages.

EPS produced by the algae, bacteria, and wastewater organisms was quantified using dialysis separation followed by total organic carbon (TOC) analysis. Wastewater organisms were included because wastewater is a potential growth medium for biofuel algae. Improved settling of both species of algae depended on both the quantity and type of EPS (dissolved or capsular) produced by both the bacterial bioflocculant, and the algae themselves. *Scenedesmus* settled the best during late growth phases while its own EPS production was high, and combined EPS (capsular and dissolved) from *B. cepacia* improved settling at a higher dosage of bacterial cells to algae (1:2 *B. cepacia* cells to algae cells). Since *Chlorella* settling was not improved at later growth stages when its own EPS production was greatest,

it appears that *Chlorella*'s settling rate was less affected by the production of its own EPS. For *Chlorella*, *B. cepacia* EPS addition (capsular and dissolved) was effective only in low doses (1:6 *B. cepacia* cells to algae cells).

Settling results with the addition of bacterial bioflocculants with the pure algae cultures were compared to settling results of lab experiments with algae pondwater sampled from high-rate algae ponds (HRAPs). These algae samples were used to test the addition of return activated sludge (RAS) to improve settling. RAS addition improved the settling of *Chlorella*, which was the dominant algae species in the HRAP during the time of this study, at two different doses (a ratio of RAS to algae pond water of 1:3 and 1:6).

Nutrient deprivation of *B. cepacia* cells before use as a bioflocculant was found to improve settling for *Scenedesmus*, especially during early phases of growth when EPS production of *Scenedesmus* was low. The EPS produced by the starved bacterial cells was about 30% greater than that produced by cultures which were not nutrient-limited. For the bacterial cultures, EPS production peaked at mid stationary phase for non-starved cultures and during early stationary phase for starved cultures. *Chlorella* settling improved in early growth with starved bacterial cell addition and in later growth with non-starved bacterial cell addition.

These results suggest that the settling of microalgae can vary dramatically by species and that the settling of different species is affected differently by growth phase and environmental conditions. In addition, species of algae respond differently to addition of bacterial bioflocculants. Given the dramatically different

settling behavior of the two species of algae used in this research, more research should be directed to studying settling of other microalgal species. Based on this research, the use of bacterial biofloculants is promising for improving algae settling and may contribute to the development of a reliable, low cost harvesting process for commercial biofuel production from microalgae.

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“ If you keep doing what you have always done, you will keep on being what you have always been. Do the right thing.”-Anonymous

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CHAPTER 1: INTRODUCTION

Section 1.1: Research Importance and Process Goals

The Intergovernmental Panel on Climate Change has reported that to stabilize atmospheric carbon dioxide levels to a “safe” range of 445 to 490 ppm, global emissions must peak before 2015 and a total reduction of 50-85% will be required by 2050 (cited in Schenk et al., 2008). Achieving a carbon dioxide emission reduction of this magnitude is an immense challenge that must be undertaken by humanity to prevent further impairment to the global environment. Second generation biofuels, such as microalgal biofuels, have received attention over the past few years as having the potential to be renewable, carbon dioxide neutral fuels (Schenk et al., 2008). Algae have a theoretical potential of up to forty times more oil extracted per unit land area compared to other feedstocks such as soy or canola (Park et al., 2011). Algal biofuels are projected to be substantially more costly than fossil fuels (Lundquist et al., 2009), as would be expected from any process that requires production instead of extraction of oil. One option for lowering cost is the optimization of large-scale algal biofuel production in conjunction with high rate pond wastewater treatment systems (Benemann and Oswald, 1996).

What contribution to United States transportation fuel consumption can be made by algae biofuel produced using domestic wastewater? The US uses over 378 million gallons of transportation fuel per day (as cited in Christenson et al., 2011), and according to the 2008 Clean Watersheds National Survey, the total wastewater flow (estimated from combined flows of Publicly Owned Treatment Works only) in the U.S. is 32,345 million gallons per day (Christenson et al., 2011). With algae based wastewater treatment, using medium strength domestic wastewater values as an estimate, enough nitrogen and phosphorus exists in wastewater to produce 77.6 million kilograms of algae per day without recycling of fuel extraction residuals (Christenson, 2011). This amount of wastewater produced per day can,

under the right cultivation conditions for algae in wastewater, contribute to 1.7 million gallons per day of biodiesel. Although this is <1% of the total 378 million gallons of transportation fuel consumed in the U.S., this technology could contribute to reduced greenhouse gas emissions as part of the solution to mitigate global climate change.

Greenhouse gas abatement can also result from the savings in energy consumption compared to electromechanical processes used in wastewater treatment (Craggs, et al. 2011). In terms of energy use, Benemann et al. (1981) estimated that, “under suitable climate conditions, a high rate pond system employing bioflocculation-sedimentation for algal removal would require less than one-half the direct energy input of an equivalently sized activated sludge or trickling filter plant. High rate treatment pond systems, as opposed to facultative ponds, are designed as shallow (typically one to three feet deep), raceway single-loop ponds with constant mixing requirements to enhance sunlight penetration, wastewater treatment, and algal biomass production (Park et al., 2011). Moreover, the energy requirement for high rate pond treatment, not biofuel production, could be provided entirely through use of produced biogas from anaerobic digestion of the primary sludge obtained” (Benemann et al., 1981).

Indeed, the U.S. Department of Energy reported that, “Inevitably wastewater treatment and recycling must be incorporated with algae biofuel production” (U.S. DOE, 2010). Revenues from wastewater treatment services might substantially decrease the cost of algae feedstock production (Lundquist et al., 2010). Therefore, scale-up research on algal biofuels in conjunction with wastewater treatment should be a high priority (Lundquist et al., 2010).

A fundamental challenge for scale-up of microalgae technology is improving the cell harvesting process (Christenson et al., 2011). The typically poor settleability of algae means

that more costly and energy intensive methods must be used currently. For example, centrifugation and/or addition of chemical flocculants have proved much too costly and energy intensive (Benemann and Oswald, 1996). Microalgae are characteristically small (5-20 microns), low in cell concentration (less than 500 g/m³ in HRAPS), have densities similar to water, and often form stable suspensions due to a net negative surface charge (Craggs et al., 2011). These characteristics make settling algae from wastewater treatment systems difficult.

Genera of green microalgae commonly seen in wastewater treatment ponds include *Scenedesmus*, *Micractinium*, *Actinastrum*, and *Pediastrum* (Craggs et al., 2011). These genera have been found to settle reasonably well under quiescent conditions, with removals in the range from 50 to 90% (Craggs et al., 2011). However, these settling efficiencies have been reported to be extremely variable and difficult to predict for scale up (Benemann et al., 1981). Natural flocculation or bioflocculation-sedimentation processes are the most cost effective harvesting systems for wastewater treatment (Craggs et al., 2011). Therefore, a focus on the development of natural settling processes, such as bioflocculation, has received increased attention over the past few years.

Bioflocculation relies on the spontaneous aggregation of algal particles due to bridging effects created apparently by extracellular polymers excreted by both the algal and bacterial cultures in a wastewater environment (Pavoni et al., 1974). These extracellular polymers, which are composed of long chain, high molecular weight polysaccharides, nucleic acids, or proteins, are essential to the aggregation of colloidal particles (including algae) to form dense, highly settleable flocs (Tenney et al., 1969; Pavoni et al., 1974). Studies have found the bioflocculant, or dissolved EPS component of bacteria, can be used to improve the settleability of algal cultures (Kim et al., 2011; Lee et al., 2009). Similarly,

effects of return activated sludge conditioning on algal cultures for improved wastewater treatment and settleability have been demonstrated (Valigore et al., 2012; Gutzelt et al., 2005). Related studies have focused on using flocculating cultures of algae as potential sources of bioflocculants, or using activated algae recycle to improve algal species domination and settleability within high rate wastewater ponds (McKinney, 1969; Salim et al., 2011; Park and Craggs, 2011).

Algae are known to exude both a dissolved EPS component as well as a dissolved precursor for the development of a particulate substance known as transparent extracellular particles (Passow et al., 2001). TEP are sticky, like EPS, but larger in size and structure, and formed from the dissolved organic matter excreted naturally from algae (Passow, 2001). However, studies have not yet identified the formation of a TEP-like substance in freshwater environments (Berman, 2005). Dissolved EPS from algae, on the other hand, has been shown to influence the needed dosage of cationic coagulants, as well as to contribute to the widespread aggregation of clay particles (Avnimelech et al., 1982; Tenney et al., 1969). Furthermore, algal biopolymer production has been presented as a patent (two times) for production in nitrogen limited environments (Schenk, 1976; Ramus, 1980).

Environmental factors also play a role in both algal and bacterial exudate formation as well as algal and bacterial bioflocculation to improve settling (Benemann and Oswald, 1996). Generally, in nutrient limited environments, both algae and bacteria have been shown to naturally flocculate (Benemann and Oswald, 1996). Algae, under various light intensities or dark conditions, will produce more EPS to enhance settleability (Shipin et al., 1996). Moreover, differences in pH (at extremes) have shown to positively affect the settling of algae (Lavoie et al., 1987). Growth phase is also another important factor.

Pavoni provided evidence that algae produced EPS during later stages in growth (early stationary phases, similar to bacteria), possibly due to nutrient limitation (Pavoni et al., 1974).

The present research was a wide-ranging screening study to explore ways to improve bioflocculation and settling of microalgal cultures. Within wastewater, a diverse community structure is known to exist of many different types of bacteria and algae. With all of the complex relationships among different microorganisms, it is difficult to pinpoint any individual mechanism or environmental condition that may lead to improved settling or bioflocculation. Thus, studying the effects of one individual species within a given wastewater sample interacting with another species under multiple environmental conditions may provide solutions to improved settling and bioflocculation using more complex communities of microorganisms present in wastewater. With this in mind, the research described here emphasized the use of pure cultures of algae and bacteria to study the effects of bioflocculation.

The two species of algae used in this research were *Scenedesmus sp.* and *Chlorella vulgaris*. The bacterium used in this research was *Burkholderia cepacia*, which is known to produce EPS (Nelson et al., 1996). This bacterium was cultured for use as a potential algal bioflocculant to improve the settleability of both pure cultures mentioned above. The goals of this present research were to achieve faster settling of algae with and without the use of a bioflocculant, under multiple environmental conditions including different ranges of pH, nutrient deprivation, and light/dark conditions of settling. The effects of bacterial EPS and algal exudates on settling, as well as environmental conditions that affect EPS production were other factors studied. The results using pure cultures were also compared to the settling of algae in actual high rate treatment ponds with RAS addition to possibly gain

clearer insight into the improved settleability of mixed algal cultures in treatment ponds.

Specific objectives of this screening study are presented below:

- 1) To investigate the effect of growth phase on settleability of two species of algae without biofloculant addition
- 2) To improve settling with biofloculant addition produced by the bacterium *B. cepacia* (cells or filtered broth)
- 3) To achieve improved settling with biofloculant use by optimizing physical characteristics such as the ratio of biofloculant and algal dose, mixing intensity, and mixing times
- 4) To quantify the dissolved EPS concentrations of both bacteria and algae and to correlate these concentrations with improved settling
- 5) To determine the effects of environmental conditions (nutrient starvation, pH, light/dark intensity, carbon dioxide supplementation) on the production of EPS for both bacteria and algae and to observe effects of varying environmental conditions on the settleability of algae alone
- 6) To compare settling and EPS production by pure cultures and actual pond wastewater treatment cultures treated with RAS

The organization of the experiments created to achieve these objectives is outlined in the figure below. The experiments were conducted in three phases.

Figure 1.1-Outline of experimentation to achieve Process Goals/Objectives

CHAPTER 2: BACKGROUND

Section 2.1 Microalgae in Wastewater Treatment

Microalgae have been used widely for the treatment of both municipal and industrial wastewaters (Benneman et al., 1980). The treatment occurs in a system of engineered ponds typically designed to remove biochemical oxygen demand (BOD) and total suspended solids (TSS), while containing and digesting wastewater sludge. Partial removal of soluble nutrients also often occurs. Such treatment processes involve a symbiotic relationship between both the algal and bacterial communities present (Figure 2.1). To carry out growth and development, along with carbon dioxide in the wastewater and any entering from the atmosphere, the algae rely on the carbon dioxide produced by the bacteria from the consumption of organic constituents in the wastewater (Tchobanoglous, 2003). In turn, some of the oxygen produced by the algae through photosynthesis is used by the bacteria to oxidize organic matter. Algae, aside from consuming carbon dioxide for growth, also require nutrients for growth from the wastewater such as nitrogen and phosphorus, as well as energy from sunlight to complete photosynthesis.

In a typical treatment pond, a portion of both algal and bacterial cells will flocculate and settle to the bottom of the pond, forming an anaerobic layer of sludge along with settled wastewater solids. In this sludge layer, anaerobic bacteria break down the settled biomass, producing methane, carbon dioxide, sulfides and many other reduced compounds (Tchobanoglous, 2003). From these complex microbial interactions occurring within various areas of a pond, a relatively efficient, low cost, and low energy intensity wastewater treatment system can be achieved (Tchnobanoglous, 2003).

Figure 2.1-Interdependence of algae and bacteria in a waste stabilization pond
 (Source: Lundquist, 2010)

Wastewater stabilization pond types used today vary widely in applications and design. Depending on process design options such as pond depth and organic loading, the amount of oxygen received in the wastewater can be controlled (Tchobanoglous, 2003). In this way, based on the desired application, many types of wastewater stabilization ponds have been constructed (Table 2.1). Of the classifications illustrated in the table, it is important to distinguish between both the facultative and low to high rate aerobic ponds. Facultative ponds involve three zones of treatment according to the presence or absence of dissolved oxygen as seen in Figure 2.2. They also have much longer detention times and treat smaller loads of BOD as compared to strictly aerobic ponds. The distribution of the aerobic zone (where dissolved oxygen concentrations are limiting) within this type of pond depends mainly on the loading of BOD, as well as other environmental conditions such as the amount of sunlight (Tchnobanoglous, 2003).

Table 2.1-Comparison of Pond technologies (Source: US EPA, 2011)

Pond	Application	Typical Loading (BOD ₅)*	Typical Detention Time (d)	Typical Depth (m)	Comments
Anaerobic	Industrial wastewater	280-4500 kg / 1000 m ² /d	5-50	2.5-4.5	Subsequent treatment normally required.
Facultative	Raw municipal wastewater. Effluent from primary treatment, trickling filters, aerated ponds, or anaerobic ponds.	22-56 kg/ 1000m ² /d	7-50	0.9-2.4	Most commonly used wastewater treatment pond. May be aerobic through entire depth if lightly loaded.
Aerobic	Generally used to treat effluent from other processes. Produces effluent low in soluble BOD ₅ and high in algal solids.	112-225 kg/ 1000 m ² /d	2-6	0.18-0.3	Maximizes algae production and, if algae are harvested, nutrient removal.

*BOD₅ = Biochemical Oxygen Demand measured over 5 days

Figure 2.2-Schematic of a facultative wastewater stabilization pond (Source: Tchobanoglous, 2003)

High rate algal ponds (HRAPs) produce more algal biomass than conventional deep ponds. Their purported advantage is greater photosynthetic oxygen production than deep ponds, along with some soluble nutrient assimilation by the algae (Craggs et al., 2011).

HRAPs for wastewater treatment are designed to increase algae exposure to sunlight through use of highly mixed, shallow single loop configurations (Figure 2.3 and 2.4). Present day research in HRAPs focuses on CO₂ addition to promote more complete soluble nutrient assimilation.

Figure 2.3-Side view of a typical HRAP with carbon dioxide addition (Source: Park, 2011)

Figure 2.4-New HRAP facility operating under Cal Poly, San Luis Obispo Civil & Environmental Engineering Program (Source: SLO Tribune)

Figure 2.3 shows a schematic of a typical pond and paddlewheel HRAP system. The average depth ranges from 20 to 40 cm to improve cell exposure to sunlight and promote evaporative cooling during especially hot periods. HRAPs are also mixed with paddle wheels and typically cover up to 1 ha (Lundquist et al., 2010). One of the main factors in HRAP operation is mixing, which involves a channel flow velocity of 15 to 30 cm/second (Lundquist et al., 2010). Wastewater HRAPs have been typically part of a system with facultative ponds followed by HRAPs operating in parallel, followed by algal “settling” ponds and maturation ponds (Park et al., 2011).

HRAPs have been observed to produce algal concentrations of about 0.2 tonnes/ML (million liters) of wastewater, which is between two to three-times that of facultative wastewater treatment ponds (Craggs et al., 2011). Given that domestic wastewaters have low C: N ratios compared to algal biomass, carbon limitation in wastewater treatment HRAPs is evidenced by a much higher daytime pond pH, when algae consume carbon dioxide and release hydroxide ions, increasing the pH (Craggs et al., 2011). Increased free ammonia concentrations, as a result of a greater pH, inhibit the growth of both algae and aerobic heterotrophic bacteria, ultimately hindering both biomass productivity as well as wastewater treatment (Craggs et al., 2011). Thus, the addition of carbon dioxide enhances algal biomass production in HRAPs (Craggs et al., 2011).

Wastewater treatment within HRAPs is focused on the oxidation of organic matter (BOD removal) and removal of soluble nutrients (Craggs et al., 2011). Typical organic loadings of HRAPs range from 100 to 150 kg BOD₅ per hectare-day. Nutrient assimilation can reach from 24 kg total nitrogen/hectare-day to 3 kg total phosphorus/hectare-day (Craggs et al., 2011). With these relatively high rates of removals, a smaller capital and

operating cost compared to conventional mechanical treatment technologies is acquired (Craggs et al., 2011).

Section 2.2: Potential For Production of Algal Biofuels From Wastewater Treatment

High Rate Algal Ponds

Combined wastewater treatment and production of algal biofuel is an old idea that has received renewed attention. In 1960, Oswald and Golueke first proposed large-scale production of microalgae as a biofuel feedstock using HRAPs, with wastewater providing water, nutrient, and carbon for algae production (Oswald and Goluenke, 1960). It was not until the US Department of Energy Aquatic Species Program in the late 1970s that extensive progress was made on the concept (Sheehan et al. 1998). More recently, multiple pilot scale studies have shown that wastewater treatment in HRAPs with carbon dioxide addition could provide nutrient removal with the co-benefit of algal biomass production for biofuel use (Park et al., 2011). Similarly, Pittman et al. (2011) concluded that algae cultivation for biofuels without the use of wastewater is unlikely to be viable or provide a positive energy return. Further, Lundquist et al. (2010) reviewed different scenarios of algae-based wastewater and reported that cases that emphasized wastewater treatment were able to produce cost-competitive biofuels.

Algae, compared to existing biofuel feedstocks such as soy or canola, show the promise in that they have demonstrated up to forty times more oil extracted per unit land area (Park et al., 2011). In terms of biomass, algae can exhibit annual average high productivities of about 60 tonnes/ha, nearly 10-fold higher than other terrestrial biomass crops (Craggs et al., 2011). Moreover, a 25% to 50% oil (triglycerides) content is thought to be achievable, with the lower value obtainable by current standards of technology and the higher value not consistently possible until breakthroughs in new research methods in

molecular biology and mass cultivation of microalgae (Lundquist et al., 2010). However, with photoautotrophs, extremes in oil content and productivity are not possible simultaneously due to thermodynamic limits (Lundquist et. al., 2010).

Currently, wastewater treatment HRAPS are considered the only economical way to produce algal biomass for conversion to biofuels with minimum environmental impact (Park et al., 2010). Commercial algal production in HRAPs requires many resources in which wastewater provides naturally such as nitrogen and phosphorus. For example, commercial farm's resources such as water, nutrients, and carbon dioxide, contribute to about 10-30% of total production costs (Park et al., 2011). To reduce these costs, commercial farms may recycle the growth medium for following algal harvest (Kim et al., 2011). This reuse of growth medium, however, leads to a decline in overall algal productivity and an increased risk of algal pathogens or the accumulation of inhibitory secondary metabolites (Park et al., 2011). In addition, the wastewater contains substantial amounts of carbon and is necessary in large quantities necessary for extensive algal growth (Lundquist et al., 2010). Thus, as compared to commercial production (dedicated production for biofuel only) , wastewater proves a more economical option for biofuel production. Still, creating and operating HRAPs dedicated to produce algal biomass for conversion to biofuels has been considered as a large environmental impact in its own right (Park et al., 2011).

Several significant issues exist for successful operation of algal biomass production in wastewater HRAPs. A major challenge is economical harvesting of algae (Christenson et al., 2011). Due to their small size, colloidal nature, and similar density to water, algae are generally difficult to remove or separate from a dilute suspension of water (Park et al., 2011). Traditional chemical and physical flocculation, such as synthetic polymer addition or

filtration techniques have proved too expensive and complicated for affordable biofuel production (Park et al, 2011). Thus, natural aggregation or bioflocculation of algal cells grown in wastewater treatment HRAPs is important to pursue for cost-effective harvesting (Park et al., 2011).

Other challenges of algal biomass production include nutrient supply, gas transfer and exchange, culture integrity, environmental control, as well as land and water availability (Christenson et al., 2011). Algal biomass production is primarily limited by environmental factors (light and temperature), operating parameters (pH, carbon dioxide supply, dissolved oxygen and nutrients) and biological conditions (zooplankton grazing, viral infection, etc.) (Park et al., 2011). Environmental factors such as temperature and light vary daily and seasonally. Adjusting hydraulic residence time and/or pond depth are common means to maintain the cultures over the seasons (Park, et al., 2011). In terms of biological operating parameters, a topic of study is maintaining a desirable colonial algal species to attain reliable harvest within a HRAP wastewater treatment system (Park et al., 2011). This species dominance can be achieved by recycling algae from settling basins to develop an activated algae process (Park and Craggs, 2011).

Challenges such as obtaining a reliable source of carbon dioxide and optimizing carbon dioxide mass transfer (CO_2 delivery) between the gas-liquid interfaces further pose a hindrance to algae production (Lundquist et al., 2010; Christenson et al., 2011). Removing oxygen to prevent the inhibition of photosynthesis, especially for closed reactor systems, at the same time of elevating the transfer efficiency of carbon dioxide, also remains an engineering problem (Christenson et al., 2011). Obtaining reliable sources of carbon dioxide, such as flue gas from power plants, also are not always available (Lundquist et al.,

2010). Lastly, large-scale production involves a large expanse of land with an available water or wastewater resource.

Section 2.3: Methods for Harvesting of Microalgal Biomass

The harvesting of microalgae is one of the most difficult, and thus far least satisfactorily resolved, problems in algal mass culture (U.S. DOE, 2010). Microalgal cultures are dilute, typically less than 500 mg/L (0.05%) w/w on a dry organic weight basis and cells are small (sometimes less than five microns) (Benemann and Oswald, 1996). In addition, extraction of one ton of dried biomass requires the extraction of these small cells from over 2,000 tons of medium (Benemann and Oswald, 1996). Here, a short comparison is made on harvesting processes found in the literature.

Section 2.3.1 Physical Methods

Physical harvesting methods include simple settling, lamella settling, centrifugation, filtration, micro straining, membrane filtration, and dissolved air flotation, as well as electrical based (electrophoresis) methods. Of all types of physical methods of separation, centrifugation is the most rapid and reliable method of recovering suspended algae (Christenson et al., 2011). However, high investment and operating costs must be considered. The U.S. Department of Energy concludes that the current level of centrifugation technology is cost-prohibitive for any large-scale use involving algae in the U.S. (U.S. DOE, 2010).

Filtration and microscreening of algae has also been considered as a potential harvesting technique (Benemann and Oswald, 1996). There are three major drawbacks to filtrations methods of unicellular algae: small size of the cells, spherical shape, and other extracellular material that continually clogs filter pores (Benemann and Oswald, 1996). In

addition, the relatively high concentration of cells in algae cultures required frequent filter cleaning. Thus, filtration is best carried out with filamentous or large colonial algae, readily handled by mesh screens (Benemann and Oswald, 1996). Further, the non-colonial algae, including *Scenedesmus* and *Chlorella*, may remain in suspension even when the filtration is applied due to their small size and non-colonial nature (Benemann et al., 1980). Thus, filtration methods are not practical for separation of dense algae cultures from the liquid stream on a large-scale basis.

Membrane filtration is a harvesting technology that includes a high cross-flow of wastewater across the surface to prevent excess accumulation of algae and solids (Shelef et al., 1984). Even though the effluent quality is superior compared to other filtration processes (up to twenty-fold concentration of the algae had been reached with very high quality effluent), the high power consumption required to provide a trans-membrane pressure differential proved much too costly, at least with earlier technology (Shelef et al., 1984).

Lamella settlers, as used in wastewater treatment, have also been investigated (Shelef et al., 1984). This type of clarifier provided more efficient clarification as compared to normal gravity settling (Shelef et al., 1984). In one study by Mohn (reported in Shelef et al., 1984), algae separation was concentrated to 1.6% TSS and addition of flocculent was required when algae were fed to the lamella settler. This method of separation was determined to have a fair operational reliability and additional concentration of the alga slurry was usually performed, requiring more expense for dosages of flocculants/polymers alike (Shelef et al., 1984).

Dissolved air flotation (DAF), another prominent physical method of separation used in wastewater treatment, has also been explored for algae harvesting. DAF uses

saturation of air at a pressure higher than atmospheric followed by subsequent flotation of algal solids under atmospheric conditions (Shelef et al, 1984). Algae separation by DAF should be operated in conjunction with chemical treatment in order to increase the flocculation efficiency (Shelef et al., 1984). Algal oxidation pond effluents, which contain a wide variety of microalgal species, may be successfully clarified by DAF and thickened to create an algal slurry of up to 6%. However, power and energy consumption costs as well as costs for chemical pre-treatment may be too costly for mass separation of algae on the wastewater scale (Benemann and Oswald, 1996).

Separation methods based on electrical methods such as electrophoresis of algal cells have also been considered. Typically, movement in an electric field concentrates algal cells, where negatively charged algae move towards a positively charged cathode (Christenson et al., 2011). The major advantage of this electrical approach is that no chemical addition is required (Christenson et al., 2011). However, major drawbacks to electrical separation include the high power requirements and electrode costs (maintenance/replacement) that do not make electrical harvesting methods economically feasible for micro algae harvesting (Christenson et al., 2011).

Section 2.3.2 Chemical Methods of Harvesting

Chemical-based methods for harvesting are usually methods of pretreatment or conditioning to allow the cells to chemically flocculate and increase in particle size before another method of harvesting is performed (i.e. sedimentation) (Benemann et al., 1980). Both metal salts and synthetic polymers are added as a chemical treatment method to coagulate (neutralize the charge) and to flocculate the cells (Christenson et al., 2011). The various chemicals used can be classified into two groups: 1) inorganic agents 2) polymeric organic flocculants, which may be cationic, non-ionic or anionic (Shelef et al., 1984). The

term polyelectrolyte is used to describe the polymeric flocculants including the nonionic species and synthetic/natural polymers (Shelef et al., 1984). Costs for chemical coagulation/flocculation can be 40% less than centrifugation (Benamnn and Oswald, 1996). However, certain disadvantages exist that will be discussed in more detail below.

Both aluminum and ferric cations are often used for coagulation processes and compose the inorganic side of chemical coagulants (Christenson et al., 2011). However, the use of aluminum sulfate has been shown to inhibit anaerobic digestion of algae biomass, which is a possible biofuel pathway (Christenson et al., 2011). In addition, the major problem with alum and lime treatment is the very high amounts of chemicals required (Benemann and Oswald, 1996). Dosages of over 100 mg/L of alum were required to achieve over a 90% clarification after initial gravity sedimentation, and attempts to recover the alum proved unsuccessful (Benemann and Oswald, 1996). Also, the algae-chemical sludge produced is a disposal problem and should be discouraged for fuel production (Benemann and Oswald, 1996).

Polyelectrolyte addition has been studied and demonstrates several advantages over inorganic chemical coagulants (Benamann et al., 1980). Of the polyelectrolytes tested, synthetic cationic polymers proved to be the most efficient flocculants (Shelef et al., 1984). The major advantage of cationic polymers is the relatively small dosages required, typically less than 10 mg/L as well as the ability to use the biomass for additional applications (Benemann and Oswald, 1996). The major drawback to polyelectrolytes is the high chemical costs as compared to inorganic coagulants and flocculants (Benemann and Oswald, 1996). Improvements in the costs of polyelectrolyte flocculants, suggest that the economics of polyelectrolytes have improved somewhat (Benemann and Oswald, 1996). Thus, the application of synthetic polyelectrolytes has expanded to aid in both

bioflocculation processes and sludge conditioning for dewatering aspects used throughout municipal wastewater treatment (Benemann and Oswald, 1996).

Lastly, natural polymers (not synthetic polyelectrolytes) are another source of chemical flocculating agents (Christenson et al., 2011). These polymers such as alginate, chitosan, and cationic starches have been shown to be effective flocculating agents (Christenson et al., 2011). However, as these polymers are from natural, organic sources, the cost of purchase is much higher compared to synthetic polyelectrolytes and inorganic flocculants. For this reason, natural polymers are not prevalent in harvesting methods today (Christenson et al., 2011).

Section 2.3.3 Biological Methods of Harvesting

Biological methods of harvesting encompass techniques such as autoflocculation or self-induced bioflocculation of algal cells, and bioflocculation through the addition of bioflocculants (both microbial, algal and floc-forming) (Benemann and Oswald, 1996). Harvesting costs could be greatly reduced with bioflocculation because no chemical costs are incurred as with chemical addition, and little to no energy consumption is necessary (Christenson et al., 2011).

Defined acceptable settling rates are in the range of about 10 cm/hr or higher to produce a biomass concentrate typically 1.5 to 4 % solids (Beneman, 1982). Natural settling rates can be in fact either positive or negative, where the negative term is defined as natural flotation (Benemann and Oswald, 1996). The characteristic of some species of algae to exhibit natural flotation could be caused by gas vacuoles (blue-green algae), simple cellular respiration, or high lipid contents (Benemann and Oswald, 1996). Therefore, unlike colloidal particles, algal cells tend to show characteristics of re-suspension during natural

sedimentation. However, natural flotation usually occurs with individual cells, where bioflocculation covers the behavior of aggregated or floc forming organisms.

Autoflocculation relies on the ionic composition of the culture medium, as well as photosynthetic pH increase to cause precipitation of calcium phosphate minerals leading to sweep flocculation (Benemann et al., 1980). This process will be further evaluated in later sections.

Bioflocculation usually refers to the naturally induced flocculation due to secreted biopolymers by either the algal or bacterial cells (Christenson et al., 2011). The presence of high molecular weight EPS and exudates of both algae and bacteria, like polyelectrolytes in chemical addition, have been proven to bridge and flocculate algae and bacterial cells in solution (Pavoni et al., 1972; Pavoni et al., 1974). Therefore, optimizing the conditions for EPS production can aid in enhancing bioflocculation and enhance sedimentation/harvesting efficiencies of microalgal species in the long run (Benemann and Oswald, 1996). Many studies suggest EPS production of both bacteria and algae are maximal at the end of the growth phase (early stationary phase). EPS production is additionally controlled by environmental conditions such as light, pH and temperature (Christenson et al., 2011).

Finally, the addition of bioflocculants, or bacterial microorganisms that naturally produce flocculants, to a culture of algae has been shown to enhance bioflocculation processes and to ultimately improve the harvesting efficiency of multiple microalgal species (Christenson et al., 2011). Microbial flocculants can include any type of bacteria or floc forming microorganism that is a known high EPS producer (Benemann et al., 1981). In addition, bioflocculants can include diatoms and microalgal species themselves that are also known exudate producers under certain environmental conditions or stresses (Salim et al.,

2011). Multiple studies are reviewed in the upcoming sections on the effectiveness of biofloculant addition to enhance the harvesting efficiency and settleability of microalgae.

Section 2.4 Characterization of Algal Exudates

The topic of microalgal exudates was reviewed to better understand improved algal flocculation and harvesting methods. Algae produce polysaccharides and other polymers (Avnimelech et al., 1982). The amounts released may represent 15 to 60 percent of the total photo-assimilated carbon (Avnimelech et al., 1982). Literature suggests that algae exude two main types of extracellular products: transparent extracellular particulates (TEP) and extracellular polymers (EPS) (Passow, 2002; Lewin, 1956).

Section 2.4.1 Algal EPS (Extracellular Polymeric Substances)

Initial studies in the late 1950s by both Lewin and Moore suggest that algae, like bacteria, are producers of extracellular polymeric substances. Lewin observed that eighteen unicellular or colonial green microalgal cultures characteristically formed both mucilaginous capsular EPS and dissolved, soluble EPS (Lewin, 1956). Lewin observed their EPS production, both capsular and dissolved, from the eighteen *Chlamydomonas* species (Lewin 1956). About fourteen of the *Chlamydomonas* species isolated were seen to produce a capsular EPS slime layer, while the other species did not (Lewin, 1956). To date, this study provides one of the more detailed analysis of algal polymeric exudates found in the literature. The results of the yield of polymers are presented in the table below (Table 2.1) Different total polysaccharide levels were observed for both non-mucilaginous and mucilaginous species, suggesting that different species of algae produce a very broad (order of magnitude) range of EPS.

Chlamydomonas mexicana produced the largest amount of soluble polymer ranging as high as 1 g/L in a culture of 500 mg/L algae, which represented 20 to 25% of the total organic matter produced (Lewin, 1956). In addition the mucilage around *C. parvula* and *C. pterfi* represented 40 to 60% of the total organic matter produced (Lewin, 1956). The chemical composition of the polysaccharide components of EPS for the *Chlamydomonas* species included galactose and arabinose (Lewin, 1956). Both cellular and capsular EPS productions were shown to increase following the growth curve of the algae. The capsular EPS was also observed as being comparatively lower to the soluble fraction (Lewin, 1956).

Table 2.2-Production and quantification of EPS for different species of green microalgae (Source: Lewin, 1956)(S, C-1, C-2, C-3b refer to different amounts of polysaccharide for a specified series of precipitations)

	Genus and Species	Polysaccharide in extract (mg/L)				Total Polysaccharide (mg/L)	Cell Residue (mg/L)	Total Organic Matter (mg/L)	% Polysaccharide
		S	C-1	C-2	C-3b				
Non-mucilaginous species	<i>Chlamydomonas dysosmos</i> Moewtus	24	0	0	0	24	533	557	4
	" " <i>moewusii</i> Gerloff	43	0	0	0	43	514	557	8
	" " <i>philotes</i> nom. Prov.	14	17	6	0	37	451	488	8
Mucilaginous Species	<i>Chlamydomonas acidophile</i> Negoro	55	0	0	0	55	305	360	15
	" " <i>applanata</i> Pringsheim	106	0	89	0	195	310	505	39
	" " <i>callosa</i> Gerloff	4	16	73	176	269	497	766	35
	" " <i>debaryana</i> Goroschankin	36	37	85	0	158	629	787	20
	" " <i>inflexa</i> Prinsheim	61	14	46	0	121	514	635	19
	" " <i>off. infleza</i> (TT. 1/19)	27	0	54	181	262	706	968	27
	" " <i>mexicana</i> nom. Prov.	141	14	86	0	241	471	712	34
	" " "	170	0	47	0	217	438	655	33
	" " <i>parvula</i> Gerloff	51	15	155	0	221	318	539	41
	" " <i>pelerfi</i> Gerloff	53	0	391	0	444	335	779	57
	" " <i>sphagnophila</i> Pascher	27	19	22	255	323	358	681	47
	" " <i>ulvaensis</i> nom. Prov.	73	120	143	158	494	650	1144	43
	" " <i>sp.</i> (B.5)	124	0	20	0	144	262	406	35

In a similar study, Moore measured the yields of both capsular and dissolved EPS from eight species of green and blue-green algae (Moore, 1964). The range of extracellular polymeric substance observed was from 174 mg/L to 557 mg/L (Moore, 1964). The dry weight of cells, as seen in the Table 2.3 below, ranged from about 1-3 g/L for all of the species (Moore, 1964). This result demonstrates that the EPS production may have a

correlation with density of algal cultures as well as increased carbon dioxide input. Across algal cultures of a similar density, however, different EPS productions were observed (Figure 2.5). Overall, a yield of 344 mg/L of EPS for *Chlorella vulgaris*, 224 mg/L of EPS from *Chlamydomonas*, and 174 mg/L of EPS from *Chlorella* was achieved (Moore, 1964). For all three species, capsular polymer was about one third less than the soluble polymer produced, agreeing with Lewin's previous results on capsular polymers. Again, with all cultures, the production of the extracellular polymer was dependent on the age of the culture (Moore, 1964). The most significant production was observed for all cultures during the late exponential to early stationary phase (8-10 days). This differed from Lewin's results, where the most significant increase occurred during mid to late stationary phase of growth (Moore, 1964). Therefore, the effect of algal growth stage and maximum production or increase in EPS varies across different species of algae. A typical data set for EPS production over a growth curve is seen in Table 2.4 for this same study using a culture of *Anabaena flos-aquae* (Moore, 1964).

Table 2.3-Yields of polysaccharides and dry weight for 8 species of green microalgae (Source: Moore, 1964) EP=extracellular polysaccharide, CP=capsular polysaccharide, IP=water soluble intra-cellular polysaccharide, TP=total polysaccharide

Algal Culture	Incubation Temperature (Deg. Celsius)	EP (mg/L)	CP (mg/L)	IP (mg/L)	TP (mg/L)	Cells (mg/L)
<i>Anabaena flos-aquae</i>	40	557	13	126	696	1379
<i>Nostoc sp.</i>	40	415	15	23	453	1315
<i>Palmella muscosa</i>	25	271	36	196	503	2133
<i>Chlorella Vulgaris</i>	25	235	34	75	344	3203
<i>C. ellipsoidea</i>	25	234	15	26	275	1959
<i>Chlamydomonas sp.</i>	25	234	19	62	305	1391
<i>Oocystis sp.</i>	25	197	22	48	267	2206
<i>Chlorella sp.</i>	25	174	15	26	215	1959

Figure 2.5-Yields of extracellular polysaccharides versus dry weight for 8 species of green microalgae (Source: Moore, 1964)

Table 2.4-Increasing polysaccharides produced over the growth curve for *Anabaena* cultures (Source: Moore, 1964)

Time (days)	Extracellular Polysaccharide (mg/L)	Cells (mg/L)
2	25	268
4	44	323
6	50	1128
8	77	1203
10	138	1748
12	205	2068

Moore's study indicated that the composition of extracellular polymer produced by two species of *Chlorella* differed significantly (Moore, 1964). The monosaccharides of *Chlorella vulgaris* were composed of galactose, xylose, glucuronic acid, and rhamnose, while the composition of *Chlorella sp.* was glucose, arabinose, glucuronic acid, and rhamnose (Moore, 1964). This varied chemical composition across species might induce different effects in terms of settling and flocculating abilities.

In a more pertinent study to wastewater treatment, Allen (1956) observed that the growth of *Chlamydomonas* species in wastewater treatment ponds increased the dissolved oxidizable organic material present (Allen, 1956). Allen measured the oxidizable organic material of the exudates by dichromate oxidation similar to the method used in chemical oxygen demand testing (COD) for wastewater treatment. The excretion of oxidizable organic matter from 6 *Chlamydomonas* species studied paralleled the growth of the alga (Allen, 1956). Similar to the observations of Lewin (1956), again about 10 to 45 % of the organic material present was soluble (Allen, 1956). The organic oxidizable matter filtrate was determined to have polysaccharide but little nitrogen content (Allen, 1956), assuring the presence of high molecular weight polymers, and specifically not proteins.

The studies reviewed above involved some error of disrupting the cell wall of the microalgal species during the EPS extraction processes. Some physical and chemical extraction processes used in the procedures above have been known to lyse the cell walls of algal species, releasing intracellular products, and interfering with extracellular byproduct analysis. Inadequate separation of these cellular byproducts between low and high molecular weight polymeric components was another problem not addressed. A study by Whitton in 1965 examined the soluble extracellular materials produced by *Anabaena cylindrica*, focusing on polypeptide production. Of the species analyzed, 15 species produced extracellular material (polypeptides) detectible by dialysis (Whitton, 1965). About 14 to 50% of the EPS produced by the algae in this species was dialyzable, indicating that the other half consisted of high molecular weight compounds (Whitton, 1965). In addition, prolonged dialysis of microalgal samples showed that the culture filtrate had more precipitable EPS or more polypeptides accumulated (Whitton, 1965). This study reveals further that all above results reviewed are valid based on the procedures used, but may be subject to error based on non-elimination of lower molecular weight polysaccharides as

dialysis achieves. If algal EPS is indeed composed of higher molecular weight polymers greater than 6,000 to 8,000, as these multiple studies suggests (arabinose, rhamnose, xylose, etc.), a fraction of low molecular weight polymers may be included but not detected in the earlier studies. These results may explain why the relatively high extracted polymers were attained (higher EPS measurements). Thus, the requirement for dialysis of algal exudates is an issue investigated by this research.

Section 2.4.2 Algal Exudates: Transparent Exopolymeric Particulates (TEP)

TEP are large, discrete, transparent particles that form from the polysaccharides released by many microalgal and some bacterial microorganisms (Passow et al., 2001). In marine and some freshwater environments, diatoms release TEP precursors abundantly. These precursors are responsible for TEP formation and the aggregation of diatoms after an algal bloom (Passow et al., 2001). In addition, TEP play a role in formation of biofilms on water treatment membranes (Berman, 2005; Berman, 2007). TEP have been found to facilitate the formation and subsequent sedimentation of fast sinking aggregates (called “lake snow”) from individual TEP particles too small to sink to a great depth individually (Passow et al., 2001; Berman, 2005). TEP range in size from about 2 μm to 100-200 μm and appear in many forms: amorphous blobs, clouds, sheets, filaments, or clumps (Berman, 2007). TEP are also composed of deformable, gel-like particles suspended in the water (as they sometimes do not adhere to surfaces as EPS does), thereby considered a “planktonic” subgroup of EPS (Berman, 2007).

The photomicrograph in Figure 2.6 below illustrates the particulate, gel-like nature of TEP, which was found in freshwater and marine environments (Berman, 2007). In marine environments, TEP concentrations range from 3,000 to 4,000 TEP particles/mL (Berman, 2005). In freshwater environments, TEP concentrations range up to 20,000 TEP

particles/mL. Although TEP is comprised mostly of polysaccharides, other organic compounds such as proteins are incorporated into these particles (Berman, 2005). Some TEP is also created from the mucous envelopes that surround bacterial and algal cells (Berman, 2005). Bacteria also colonize TEP, using the TEP as a source of nutrition to grow and develop (Berman, 2005).

Figure 2.6-Photomicrograph depicting TEP particles (from a freshwater source) and string like arrangements stained with Alcian Blue dye (Source: Berman, 2005)

Quantification of TEP relies on either a colorimetric method or enumeration by a microscopic technique (Passow et al., 2001). The colorimetric method relies on staining the negatively charged groups on the surface of polysaccharides with an Alcian blue dye (Passow et al., 2001) and comparing the samples colorimetrically to stained, known concentration standards of xanthan gum. However, both of these methods for quantification proved too extensive for TEP analysis; therefore, TEP production and quantification were not done in the current study of this thesis.

In aquatic environments, the formation of TEP involves bridging the colloidal and dissolved organic substances, as well as larger particulate organic materials (Passow, 2002). The production involves two separate steps: one abiotic and one biotic (Passow, 2002). The biotic step involves dissolved precursor substances and is affected by various environmental conditions such as turbulence, ion density, and concentration of inorganic

colloids (Passow, 2002). The abiotic step consists of the coagulation of colloids to form sub-micron aggregates and also to form submicron gels by gelation and annealing processes (Passow, 2002).

The environmental factors affecting the release of TEP precursors and the formation of TEP aggregates parallel the factors affecting the release of EPS from both algae and bacteria (Passow, 2002). The release of TEP precursors depends on the species, as well as the individual physiological state and environmental growth conditions, as was observed for EPS (Passow, 2002). TEP are generated during exponential growth, stationary phase, and senescence of most algal species (Passow, 2002). However, it has been observed in freshwater algal cultures that maximum TEP production is reached during senescence or late stationary phase of growth (Passow, 2002). In addition, TEP production can vary according to light intensity and duration, carbon dioxide concentration, growth rate, and nutrient (nitrogen-limiting) or trace metal depletion (Passow, 2002; Mari, 1999).

TEP formation depends on physical factors such as turbulence, where a turbulent environment provides a large amount of shear forces that serve to enhance coagulation of TEP precursors (Passow, 2002). The surface charge of the algae and bacteria, as well as the ionic strength of the water affects the formation of TEP (Passow, 2002). Aquatic environments with higher ionic strengths decrease the electric double layer of the colloids and allow more rapid formation of TEP (Passow, 2002).

During a study in the Santa Barbara channel between May 1995 and June 1997, the flux of TEP in a marine environment was measured to investigate whether TEP is necessary for the aggregation and sedimentation of particles (Passow, 2001). During the testing period, phytoplankton appeared to be the most significant source of TEP, showing that some species of marine algae will produce this sticky, particulate substance (Passow, 2001).

In addition, bacteria, along with phytoplankton were observed to add an appreciable amount of TEP to the aquatic environment and this allowed the diatoms to aggregate. This result suggests that the presence of bacterioplankton in conjunction with algae (phytoplankton) may be necessary for TEP formation and aggregation to occur (Passow, 2001).

A study detailing TEP quantification in freshwater environments and in a controlled laboratory setting was also reviewed (Grossart et al., 1998). This study discovered TEP aggregates from the filamentous cyanobacteria *Aphanizomenon ovalisporium* and from green algae *Scenedesmus quadricada* present in detectable amounts ranging from 3 to 30 mm in diameter in Lake Kinneret, Israel. The dry weight of the TEP aggregates was found to be between 2 and 297 micrograms per aggregate, and the abundance of TEP aggregates was found to range between 1 and 100 aggregates per liter of freshwater depending on the time of year, phytoplankton standing stock, and wind conditions (Grossart et al., 1998). The corresponding number of TEP particles (individual, not aggregated) reached up to 6,915 particles per milliliter of freshwater (Grossart et al., 1998). Pure cultures of the cyanobacteria *Aphanizomenon ovalisporium* studied in the laboratory showed that these algae released individual TEP upon reaching stationary phase (Grossart et al., 1998). A large fraction of the TEP was capsular (attached to the surface), and only a smaller fraction detached as individual TEP (Grossart et al., 1998). Also, rapid flocculation of the cultures of filamentous algae occurred in less than 2 hours in the laboratory setting used. This result showed TEP from pure cultures have been observed to possess flocculating capabilities (Grossart et al., 1998).

Section 2.4.3 Effects of Growth Conditions on Algal EPS Production

The environmental growth conditions can induce algal exudate production of many algal species (Benemann and Oswald, 1996; Salim et al., 2011). These conditions include applying extreme pH, nutrient depletion in the growth media, temperature changes and the concentrations of dissolved oxygen and carbon dioxide (Salim et al., 2011).

Autoflocculation, or self-induced flocculation of algal cells due to pH changes and mineral composition of the media, allows improved flocculation efficiency (Benemann et al., 1980) and may have an effect on algal exudate production. The effect of light intensity and dark or light culture conditions has been seen to have an effect on algal exudate production in some species of algae (Lavoie, 1987). Therefore, investigation into algal exudate production through changes in environmental growth conditions would be beneficial to understand the mechanisms behind improved algal flocculation and harvesting efficiencies.

Section 2.4.3.1 Effect of Nutrient Limitation on Algal EPS Production

Nutrient limitation has been shown to improve algal exudate production and flocculation processes (Benemann et al., 1980). Benemann et al. (1980) observed that certain nitrogen-limited algal cultures often clump and settle, although this phenomenon has neither been widely studied nor reported. This result was observed by Benemann and Weissman in laboratory experiments with several fresh-water algal species including *Porphyridium* and *Dunaliella* cultures, where flocculation was studied over the various growth conditions (Benemann, 1980). The most important growth factor observed was nitrogen limitation, which induced the cells to flocculate and settle (Benemann, 1980).

Other studies also indicate nitrogen limitation improves algal flocculation as well as exudate production (Fogg, 1965). Schenck and Ramus developed patents for nitrogen starvation of algal cultures followed by subsequent harvesting of biopolymers (Ramus,

1980; Schenck, 1976). Lastly, algal flocculation has been observed during phosphorus limited algal growth (Schenck, 1976). These studies will be further reviewed in upcoming sections.

Section 2.4.3.2 Effect of Autoflocculation on Algal EPS Production

Autoflocculation is defined as a naturally induced pH increase in culture medium and subsequent carbon limitation resulting in co-precipitation of magnesium, calcium, phosphate, and carbonate salts with microalgae (Benemann, 1980). An increase in culture pH due to carbon dioxide consumption by algal photosynthesis or by direct addition of alkali substances leads the culture medium to a supersaturation state with respect to calcium and phosphate ions (Lavoie, 1987). This supersaturation allows calcium phosphate ions to precipitate. The algal cells, serving as a solid negatively charged surface, adsorb to the calcium phosphate precipitate, which results in a dense, settleable floc (Lavoie, 1987). Oswald and Golueke first observed this phenomenon for microalgal cultures photosynthetically active in a shallow oxidation pond during warm and sunny days (Oswald, 1960). Several studies to further prove this observation by Oswald and Golueke soon followed and will be discussed below to address the significance of algal flocculation as a result of different culture conditions.

One of the first studies reviewed to scientifically prove the autoflocculation phenomenon involved the analysis of the microalgal species *Scenedesmus obliquus* (Lavoie, 1987). This strain of algae was isolated from a wastewater treatment plant and grown with the addition of secondary effluent wastewater for investigation of both improved bioflocculation and autoflocculation (Lavoie, 1987). Autoflocculation experiments were focused on algal culture growth conditions such as pH, culture age, and the influence of calcium and orthophosphate addition to the culture medium (Lavoie, 1987). The growth of

Scenedesmus obliquus was characterized over a 22-day period. Ammonium was observed to be exhausted from the culture medium by day 6, and phosphate uptake continued until day 9. The declining growth phase and beginning of stationary growth phase was attained around day 11 (Lavoie, 1987). Algal biomass continued growth up until day 22 (Lavoie, 1987).

Effective flocculation of around 50% was observed at pH 11 and 12 during the earlier days of growth (Lavoie, 1987). However, after Day 6 there was no flocculation observed between a pH range of 7 and 11, and the flocculation efficiency at a pH of 12 dropped to around 25% (Lavoie, 1987). This result was explained through analysis of residual phosphate concentrations within the culture medium. A better flocculation efficiency at pH 12 was observed at the stationary phase of growth for cultures showing high residual phosphate concentrations, demonstrating that phosphate concentrations in the culture medium have an effect on the autoflocculation of microalgal species (Lavoie, 1987).

Next, the addition of extra phosphate and calcium to the culture medium at various pH levels and culture ages was investigated. The addition of calcium or phosphate significantly raised the autoflocculation efficiency at pH values of 10 and 11. Also, the simultaneous addition of these two ions allowed an autoflocculation efficiency of 80% to be reached at pH values of 8 and higher (Lavoie, 1987). Again, the onset of stationary phase decreased the autoflocculation efficiency across the pH ranges studied. This was observed even with the addition of extra phosphate and calcium ions into the culture medium (Lavoie, 1987).

In the same study, bioflocculation with native bacteria to the wastewater was also investigated. With the same species of algae, the quantities of extracellular polymers and

proteins varied during the course of incubation and did not appear to be correlated with the age of the cultures (Lavoie, 1987). As seen in Figure 2.7 below, extracellular polymers, proteins, extracellular sugars and capsular sugars were measured from the culture medium. Both extracellular sugar and capsular sugars seemed to increase continually until the end of the culture growth period (Lavoie, 1987). The EPS concentrations never exceeded 120 mg/L, the range of extracellular protein was between 0 and 7 mg/L, and the maximum concentrations for extracellular sugar and capsular sugar were 5.2 and 25.4 mg/L respectively (Lavoie, 1987).

The appearance of capsular sugars in the Lavoie study coincided with a loss of flocculation efficiency. The capsular biopolymers were postulated to protect the algal surface charges and hinder the ability of calcium and phosphate ions to co-precipitate with the algal cells (Lavoie, 1987).

Microalgal sedimentation improved with ageing, increasing drastically around the declining growth phase for both cultures as seen in Figure 2.8 (Lavoie, 1987). The percentage of algal recovery (settling efficiency) was lowest during the exponential growth phase, increasing rapidly during the onset of the declining growth phase, and staying constant around the stationary phase of growth (Lavoie, 1987).

Figure 2.7-Measurements of concentrations of various extracellular products of *Scenedesmus obliquus* over the growth curve, both A and B represent two different batch cultures of *Scenedesmus obliquus* (Source: Lavoie, 1987)

Figure 2.8-Effect of culture age on algal sedimentation in A) Darkness and B) Under illumination. Sedimentation duration was 1 hour (closed circle), 2 hours (open circle) and 4 hours (closed box). The arrows denote the onset of declining growth phase (early stationary) (Source: Lavoie, 1987)

The Lavoie study concluded that the amounts of EPS produced during the ageing of cultures or the low calcium and phosphate concentrations in the secondary effluent medium were insufficient to allow adequate bioflocculation or autoflocculation (Lavoie, 1987). The EPS produced at 120 mg/L was much less than the 500 mg/L observed by Pavoni (1974). Also, the relatively low native bacterial population (as measured in the wastewater) in the *Scenedesmus* batch cultures reduced the possibility of algal-bacterial bioflocculation (Lavoie, 1987). However, using controlled laboratory conditions of pH and known addition of certain concentrations of calcium and phosphate ions to the wastewater medium, the *Scenedesmus* cultures were shown to autoflocculate (Lavoie, 1987).

Autoflocculation also studied by Sukenik and Shelef (1984), using both laboratory and outdoor experiments with cultures of *Scenedesmus dimorphus* and *Chlorella vulgaris*. In the outdoor experiment, a pure culture of *Scenedesmus dimorphus* was grown in a HRAP with a specified amount of calcium chloride and magnesium sulfate, to affect the autoflocculation process of the algae (not a wastewater medium as in the Lavoie study). After eight days of batch growth, the carbon dioxide was turned off, and the *Scenedesmus* were observed to aggregate and form fragile flocs that settled to the bottom with a corresponding rise in pH to around 9. After initiation of autoflocculation in the outdoor ponds (a 24 hour time period), the *Scenedesmus* removal was up to 96% with a corresponding decrease in alkalinity, calcium, and orthophosphate concentrations (Sukenik, 1984). To further test the effects of pH on algal flocculation and settleability, the outdoor pond pH was varied in the range of 2.5 to 10.5. A small algal cell removal percentage of 40% was observed for the outdoor culture at pH 3 (Figure 2.9). No flocculation was observed for pH 5 to 7.5, while above pH 8.5, 98% removal of algae was achieved (Figure 2.9). These results the outdoor cultures of algae suggest that pH is an important factor in the induction of autoflocculation of algal cultures (Sukenik, 1984).

In the Sukenik study, laboratory experiments found three ions were specifically responsible for the autoflocculation phenomenon: magnesium, calcium, and phosphate (Sukenik, 1984). Calcium ions caused flocculation at pH 8.5 and above, while magnesium only caused flocculation at pH 10.5 and above (Sukenik, 1984). In addition, the absence of orthophosphate with calcium addition only did not allow for flocculation over the entire alkaline range (Sukenik, 1984). The absence of calcium with only orthophosphate in the medium also did not cause any flocculation within the same pH range (Sukenik, 1984). Magnesium, on the other hand, did not require the presence of either calcium or phosphate at the pH range of 10.5 and above for effective flocculation to occur (Sukenik, 1984). Therefore, calcium and phosphate ions must be present together for autoflocculation within the pH range of 8.5 to 10.5.

The algal surface charge during a flocculation and sedimentation test was measured in the Sukenik study over the pH range shown in Figure 2.10. The algal surface electric charge was neutralized at a pH around 9.0 and above this value a positive surface charge was measured according to Figure 2.10 below (Sukenik, 1984). The precipitate formed with the algal cells had a net positive surface charge and was observed to be composed of primarily calcium and orthophosphate ions (Sukenik, 1984). As the pH increased past 9, the dissolved concentrations of both orthophosphate were shown to decrease rapidly. This result showed that these two ions are only available to form a precipitate past the critical pH of 9 (Figure 2.10) (Sukenik, 1984).

Figure 2.9-pH variation with algal removal percent in the outdoor *Scenedesmus obliquus* cultures (Source: Sukenik, 1984)

Figure 2.10-Simulated algal flocculation with A) algae concentration and cells zeta potential and B) dissolved calcium and orthophosphate after flocculation and sedimentation (Source: Sukenik, 1984)

Similar to the outdoor results found in the Sukenik study, Pavoni (1974) found that pH ranges affected algal removal efficiencies. Maximum algal autoflocculation occurred at a pH of 3 as seen in Figure 2.11 (Pavoni, 1974). This study indicates that natural flocculation

is maximized when the surface charge of the algal biocolloid is minimized or at/around the isoelectric point (Pavoni, 1974). At this point, electrostatic repulsive forces will be reduced to a minimum, thereby allowing the bio-colloids to assume interparticle distances sufficient for Van der Waals forces to promote optimum flocculation (Pavoni, 1974). Similar results were obtained for addition of cationic polymers, where optimum algal removal was achieved when the pH was reduced to around 3 (Pavoni, 1974). Adequate removal was also found to take place at pH values above 9 and up to 11 (Pavoni, 1974).

Figure 2.11-Variation of algal removal percent and pH values for algal culture (Source: Pavoni, 1974)

Pavoni extended his main theory of bioflocculation to algae in addition to bacterial cells (Pavoni, 1974). Pavoni (1974) reported that algal flocculation was governed by the physiological state of a microorganism, where the algae did not produce EPS until they entered a restricted phase of growth (stationary phase) (Pavoni, 1974). Second, a direct correlation was found between algal extracellular polymer production and bioflocculation,

since the EPS to algal biomass ratios dramatically increased during algal flocculation (Pavoni, 1974). The surface coverage phenomenon, where 50% of the algal cell surface must be covered, was postulated as the mechanism for bioflocculation (Pavoni, 1974). Third, cell surface charge investigations indicate that surface potential reduction is not a necessary precursor to algal bioflocculation (Pavoni, 1974). Fourth, the major compositional makeup of algal EPS is polysaccharides, proteins, RNA, and DNA, which impart negative surface charge characteristics (Pavoni, 1974). Finally, the mechanism of algal bioflocculation can be seen as the interaction of EPS, accumulating at the surface during stationary phase of growth, and electrostatically or physically bridging the cells into a three-dimensional matrix dense enough to settle under quiescent conditions (Pavoni, 1974).

For the culture of algae used (remained unidentified in the study), EPS production was highest at early stationary phase of growth, and leveled off into later stationary phase of growth (Pavoni, 1974). Moreover, the ratio of algal EPS to algal cells decreased drastically until later stages of growth (Pavoni, 1974). This result suggests that some cultures of algae exude more EPS during earlier stages of growth as compared to the algal cells present. Both culture turbidity and cell dry weight were shown to peak late after around 40 days of growth, showing that the culture was very dense (almost zero percent transmission).

Tenney (1973) observed that the mechanisms behind chemical and autoflocculation of algae were essentially the same. Tenney reported that long chain, high molecular weight polymers exuded by both algae and bacteria attach themselves between particles and divalent metal ions (bridging), causing flocculation (Tenney, 1973). Tenney found that as the algae reaches early stationary phase (declining growth), less cationic polymer was needed to improve flocculation up to fifty percent (Figure 2.12). This may be due to the

measured fact that algae studied produced sufficient amounts of extracellular polymer to induce bioflocculation during late log to early stationary phases.

Figure 2.13-Effect of algal concentration at a fixed pH of 3 for cationic polyelectrolyte doses to increase algal removal (Source: Tenney, 1973)

Tenney also studied the effect of surface coverage of algal cells on the bioflocculation processes. Through increasing the available surface area (or available sites for polymeric bridging-the area for bioflocculation to occur) by increasing cell concentration, it was found that more cationic polymer was required (Figure 2.13 above). According to the same Figure (2.13), Tenney studied algal density up to 350 mg/L with a pH of 3, and concluded that algal concentration differences promote differences in dosages and effectiveness of cationic polymer. Similar to Pavoni's results, Tenney observed that optimum cationic flocculant dosage was achieved at lower pH values (2 to 4), and not

higher to moderate values (Figure 2.14). Tenney concluded that this effect was due to: 1) due to the isoelectric point of algae around pH of 2 to 3, electrostatic repulsion is lowest, and 2) polymeric bridging will be greater due to a greater polymer expansion.

Fig. 7. Effect of growth phase on algal flocculation with cationic polyelectrolyte (Dow C-31): (a) algal growth curve; (b) concentration of polymer required for 50% flocculation.

Figure 2.12-Effect of growth phase on cationic polyelectrolyte dosage to attain 50% flocculation/removal of algae (Source: Tenney, 1973)

Fig. 5. Effect of pH on algal flocculation with constant dosage of cationic polyelectrolyte (10 mg/liter, Dow C-31).

Figure 2.14-Effect of pH on algal removal with constant dosage of cationic polyelectrolyte (Source: Tenney, 1973)

Section 2.4.3.3 Effect of Light Intensity and Dark Conditions on Algal EPS and Settleability

Light intensity during algal growth and darkness during settling also have been investigated to improve algal settleability. In natural situations, only a fraction of light (often as low as 1%) penetrates the water column and is available for algae for photosynthesis (Shipin, 1999). Several adaptive and metabolic strategies are exhibited by microalgae to overcome light limitations (Shipin, 1999). To obtain a secure light source in these situations, algae are known to excrete flocculating substances of polymeric nature, which are usually acidic (Shipin, 1999). These algal EPS exudates allow suspended solids such as clay particles to flocculate and sink, thereby reducing the turbidity and allowing more light to be obtained. Thus, manipulating light intensity by varying exposure periods during growth could have an effect on improved bioflocculation. Benemann (1980) also observed the potential of a “dark sedimentation” process in which 90% removal of

algal TSS was achieved (compared to a control settling pond exposed to the light) by the operation of facultative, high rate and covered settling ponds in series, but the mechanism of this process was uncertain.

The application of light and dark conditions to enhance algal settleability was investigated in integrating ponds and activated sludge systems using the PETRO concept (Shipin, 1999). The PETRO system consists of oxidation ponding to treat the bulk organic load of wastewater (up to 70%) followed by a trickling filter or activated sludge process as a polishing step, hence the name, "Pond Enhanced Treatment and Operation" (Shipin, 1999). Effective microalgal removal using the trickling filter process was a main emphasis of the PETRO design (Shipin, 1999). In fact, microalgae acted as heterotrophs in the dark, contributing to mucilage production in the trickling filter itself (Shipin, 1999). Biofilm slime, predominantly EPS in nature, was produced by the microalgae experiencing stress transfer from two different types of growth (mixotrophy and heterotrophy) (Shipin, 1999). Under light conditions, algae followed mixotrophy (growth on organics in the light) and turned heterotrophic in the dark, in which microalgae used low molecular weight organics such as amino acids, monosaccharaides and volatile fatty acids (Shipin, 1999). A full-scale plant study and corresponding laboratory scale study were completed to better understand the process behind the improved treatment efficiency achieved from the trickling filter variant in the PETRO concept. The field study showed that seasonal algal blooms in the oxidation ponds preceding the activated sludge process characteristically resulted in an enhanced EPS content of the sludge flocs (Shipin, 1999). Moreover, the EPS content directly fluctuated with the algal concentration, showing the algae in the ponds contribute to the EPS produced in the activated sludge process variant (Shipin, 1999).

In this same study, experiments were carried out in the lab with *Chlamydomonas* species (Shipin, 1999). *Chlamydomonas* was observed to grow heterotrophically in the dark on a variety of organic compounds used in the lab such as glucose, amino acids, glycerol, ethanol, and acetate and to produce EPS when these conditions were altered. EPS production was more significant when the transition was from light to dark, confirming findings that some algae produce EPS after the stationary phase transfer from the mixotrophic to heterotrophic mode of growth (Shipin, 1999). In this way, stress-induced flocculation was one reason for algal removal in the PETRO activated sludge process and trickling filter (Shipin, 1999). In addition, EPS production was observed to start gradually in the mid logarithmic phase of growth and accelerate rapidly reaching a peak during stationary phase of growth (Figure 2.15) (Shipin, 1996). Algal EPS concentration was found to increase in magnitude after 10 days of growth if growth was switched from mixotrophy to heterotrophy. Overall, the microalgae from the oxidation ponds (grown mixotrophically) enter the trickling filter or activated sludge process (heterotrophy encouraged), which induces stress conditions to provide a stimulus for EPS overproduction (Shippin, 1996).

Figure 2.15-EPS production response of a microalgal species to changes from A) heterotrophy to mixotrophy and B) mixotrophy to heterotrophy (black box with dashed line is EPS production; filled circle with black solid line is algal biomass production) (Source: Shipin, 1999)

Another study involving *Scenedesmus obliquus* produced similar settling results to the PETRO system's light and dark conditions (Lavoie, 1987). When cultures were settled under dark conditions, the *Scenedesmus* was observed to settle more rapidly (Lavoie, 1987). Settling tests under illumination, on the other hand, caused the pH to rise to 10, under which conditions floc formation or enhanced settleability were not observed (Lavoie, 1987).

Two explanations were postulated for better settling in the dark. First, illumination, as a physical factor, is known to cause thermal convection currents within the settling apparatus (Lavoie, 1987). Second, algae continue photosynthesizing, even during settling conditions under illumination, and produce oxygen bubbles that could cause the algal cells to re-suspend or float to the surface (Lavoie, 1987).

Sukenik (1984) also studied light dependency during autoflocculation for outdoor cultures of *Scenedesmus dimorphus*. To determine the effects of light on autoflocculation, the cultures were first grown autotrophically for six days (Sukenik, 1984). Following this period, one pond was covered, while the other was left open to the sunlight, and the autoflocculation experiment was begun (Sukenik, 1984). In the light exposed cultures, the removal of algae was much quicker and autoflocculation was achieved with a rise in pH to 9 (Sukenik, 1984). However, in the covered pond cultures, pH did not rise, autoflocculation was not observed, and the culture settled rather poorly (Sukenik, 1984). Thus, it was determined that light is necessary for autoflocculation. This result was the opposite of that reported in the Lavoie study of *Scenedesmus obliquus*. However, better settling of dark cultures from the Lavoie study could have resulted from a difference in the medium used in the Sukenik study (wastewater versus a defined medium), where autoflocculation was not observed in either the dark or illuminated cultures. Therefore, the effect of light on settling may depend on both the culture medium and presence of elevated levels of calcium and phosphate. These factors may allow the illuminated samples to settle better than dark samples due to the autoflocculation phenomenon. Above all, both studies indicate that the algae possess inherent individual qualities of settling under light and dark conditions, and cannot be grouped or classified based on one attribute.

Section 2.4.3.4: Effects on Settling of Algal Surface Charge, Morphology, and Size

Surface charge, morphology and size of algae cells have a tremendous impact on their ability to flocculate and to settle (Pieterse, 1978). Algae cells, like colloidal particles, are negatively charged and relatively small under all pH conditions. Due to these like charges and the development of an electric double layer around the cells, algal cells often repel one another and stay in suspension (Pieterse, 1978). However, unlike their abiotic colloidal counterparts, algae cells possess characteristics that make them flocculate and settle differently (Pieterse, 1997). Algae cells may be elongated in shape or may be arranged in filaments or colonies, or the cells can have long spines that could affect the efficiency of flocculation (Pieterse, 1978). Some algal cells swim with flagella, further affecting the way algal cells flocculate and settle (Pieterse, 1997). In addition, algal cells are alive, as compared to colloidal particles, and metabolic processes move the cells (Pieterse, 1978).

One important difference between abiotic colloidal particles and algal cells is the physiological ability of the cells to respond to differences in conditions concerning their immediate environments (Pieterse, 1978). These metabolic processes include assimilation of carbon dioxide and other nutrients, production of oxygen, as well as exudation of cell byproducts such as EPS (Pieterse, 1978). In general, these metabolic processes have been shown to affect the flocculation ability and settling characteristics of most microalgae species (Pieterse, 1978).

Surface charges of algal cells are characterized by charged organic surfaces from carboxyl or amino groups that react with water and accept or donate protons (Pieterse, 1978). Thus, the pH of the solution affects the surface charge through protonation or deprotonation. For example, the measured charge density of a species of *Chlorella* over a

wide pH range (2.5 to 11.5) showed an isoelectric point around a pH of 7-8 (Figure 2.16; Ives, 1959). At a constant pH, the zeta potential and charge density are reported to vary from genus to genus of algae (Ives, 1959). Thus, the intensity of the surface charge is a function of pH, algal species, ionic strength of the medium, and other environmental conditions (Shelef, 1984). Algal surface charge can be further influenced by ionization of inorganic functional groups at the algal cell wall and the selective adsorption of ions from the culture media (Shelef, 1984).

Fig. 1. *Chlorella*: Charge density—pH graph

Figure 2.16-Variation of charge density with pH of a species of *Chlorella* (Source: Ives, 1959)

Algal size and morphology can also affect settling. One study examined 30 species of problem algae in coagulation and sedimentation processes in the water treatment plants throughout Korea (Choi et al., 2006). Most of the algae selected were found to be difficult to settle due to needle or long cylinder morphology, which created a greater buoyancy effect in the water since the longer algae made less dense flocs (Choi et al., 2006). The problem algae

studied were diatoms including *Syndra acus*, *Nitzschia acicularis*, *N. fruticosa*, and other types included green algae such as *Scenedesmus* and *Ankistrodesmus* (Choi et al., 2006). Removal rates of these problem algae were measured using a settling column while taking samples at a predetermined height before and after adding the algae cells (Choi et al., 2006). Removal rates ranged from 31 to 98%, and settling velocities were different for each species and gradually increased with age, algal size and volume, and water temperature (Figure 2.17; Choi et al., 2006). Moreover, the settling velocities of algae were closely correlated with changes in morphology rather than algal density of the cell itself, with the most extreme morphology being a needle-like morphology (Choi et al., 2006). Lastly, the effect of morphology was related with growth phase, where longer algae improved settling and smaller algae decreased settling later in growth during stationary phase (Choi et al., 2006).

Figure 2.17-Measured settling velocities of different problem species of algae (the circled points represent *Scenedesmus*). The left figure includes the diatom species studied (Source: Choi, 2006)

Section 2.4.3.5: Effects of Algal EPS on Settling and Flocculation

The effect of algal exudates alone (primarily biopolymer) on settling was first reported in a patent by Schenck (1976). In this work, algae were cultivated until high culture densities were achieved. After this phase of growth, the patent proposed placing the algae in an environment limited in nitrogen, causing the cells to shift from a growth phase in which protein production dominated to a growth phase where extracellular polymer production dominated (Schenck, 1976). The deficiency in nitrogen was found to produce high molecular weight polymers exhibiting strong flocculating activity (Schenck, 1976). The polymers exuded by the species of *Chlamydomonas* and *Chlorella* were found to be ethanol precipitable, anthrone sugar reacting materials, having a molecular weight of approximately 100,000 (Schenck, 1976). Several experimental approaches validated the patented findings of Schenck (1976).

The first experiment reported in Schenck's approach involved the green algal species of *Chlamydomonas mexicana*. Over a growth period of 10 days, the carbohydrate production, flocculation value (how well the algae cells flocculated) and viscosity of the algal culture all increased after growth ceased during the end of the exponential growth phase (Figure 2.18). At the end of the exponential growth phase, the cellular nitrogen dropped to 5% of the dry weight, which corresponded with an algal flocculant production phase. For this particular species of *Chlamydomonas*, no flocculant was produced until the cellular nitrogen content fell below 5 %. In addition, in this study the rate of flocculant production during exponential growth was one third less than that during stationary phase of growth (Schenck, 1976).

Figure 2.18-Relation of growth, flocculation and carbohydrate production of a *Chlamydomonas* culture (Source: Schenck, 1976)

The second approach used by Schenck involved inoculating two different cultures of the same species of *Chlamydomonas*, but doubling the nutrient medium composition of nitrogen of one culture. The culture inoculated with lower nitrogen content in the media was shown to reach a cellular nitrogen content of below 5% and to produce flocculant. The culture with twice the nitrogen content did not reach below 10 % cellular nitrogen content, and no flocculant was produced. This result demonstrated that manipulating the nitrogen content of the medium allowed more desirable flocculant properties of the green algae species (Schenck, 1976).

Phosphorus effects were further also in the Schenck patent. A culture of *Chlorella pyrenoidosa* was grown under two conditions: one with sufficient nitrogen and

phosphorus, and the other with sufficient nitrogen but limited phosphorus. Even though identical growth kinetics were observed, the low phosphorus level in the second culture resulted in an absence of flocculating activity. These results suggest that low cellular phosphorous levels cause depolymerization of polysaccharides or inhibit their formation (Schenck, 1976). However, under certain circumstances, the cells with low phosphorus were shown to reverse their behavior when exposed to light. The study suggested that this species of *Chlorella* was composed of “D” or “dark” cells that were small but with high photosynthetic and low respiratory activity. When these “D” cells were transferred to a medium deficient in nitrogen, they were transformed into “L” cells or “light” cells with comparatively higher respiratory activity and lower photosynthetic activity. “L” cells were capable of producing polysaccharides at much lower cellular phosphorous concentrations. Therefore, the L cells were observed to produce flocculants when the phosphorous levels were deficient (Schenck, 1976). Overall, this study determined that cellular phosphorus levels should be maintained above 0.5 % in all species of green algae if flocculent production is desirable (Schenck, 1976).

The final approach of the Schenk patent focused on the abilities of certain species of *Chlamydomonas* to enhance coagulation and flocculation with solids in wastewater. Results indicate stronger, larger and denser flocs were formed with all species observed due to the flocculant produced by the algae upon addition with lime to each of the specified wastewater samples (Schenck, 1976). The jar test procedure used illustrated that a rapid mixture period, followed by a gentle flocculation allowed the algae and lime combination to form more readily settleable flocs as compared to the mixture of wastewater with lime addition alone.

Ramus (1980) devised a similar method to enhance algae biopolymer production by nitrogen limitation. Ramus developed a framework for a new and improved process for the synthesis of algal biopolymers by shifting from a growth phase favoring growth to a stationary phase where biopolymers are produced under nitrogen limitation (Ramus, 1980). The Ramus patent was based on a two-stage production system. The first stage relied on cultivating the algae in a continuous growth system and keeping the growth phase at a fixed exponential rate (Ramus, 1980). The next stage of growth was under batch operation with a nitrogen-starved medium that allowed the algae to stop cell division and focus on biopolymer production (Ramus, 1980). As seen in the Table 2.5, biopolymer production increased dramatically during the 20 days of batch growth for the species *Porphyridium aerugineum* (Ramus, 1980). Yields of biopolymers reached 1.73 and 4.22 g/L respectively for two different cultures (Ramus, 1980).

Table 2.5-Measurments of biopolymers produced as a function of culture age for two different cultures of *Porphyridium aerugineum* (Source: Ramus, 1980)

Culture A		Culture B	
Batch Culture Age (days)	Biopolymer (g/L)	Batch Culture Age (days)	Biopolymer (g/L)
9	0.06	9	0.06
10	0.07	10	0.13
11	0.1	11	0.23
12	0.16	12	0.39
14	0.55	14	0.94
16	0.65	16	1.59
18	0.9	18	2.49
20	1.73	20	4.22

Tenney et al. (1969) reviewed the parameters affecting flocculation and settling with cationic polyelectrolytes. This early study focused on three parameters including algal growth phase, algal concentration, and pH on optimum cationic flocculant dosing (Tenney et al., 1969). Other factors such as degree and extent of agitation, algal size, temperature

and electrolyte concentration were included in research but are not discussed here. Effect of growth phase was studied using a batch culture of *Chlorophyta* over a growth period of 12 days. As the algae entered the declining growth phase, the concentration of added cationic polymer was much smaller due to the accumulation of more extracellular polymers by the algae themselves (Tenney et al., 1969). Tenney et al. observed that the accumulation of algal extracellular material occurred during the stationary phase to early declining growth phase (Figure 2.19). Moreover, these algal biopolymers were of sufficient length, like cationic polymers, to form bridges between the algal particles (Tenney et al., 1969).

Figure 2.19-Algal concentration plotted against extracellular metabolites (algal EPS) over the growth curve for *Chlorophyta* (Source: Tenney, 1969)

Section 2.5 Bioflocculation and EPS

Bioflocculation is termed as the spontaneous flocculation of both algal and bacterial cells to form readily settleable flocs to improve gravitational sedimentation and microalgal harvesting without the use of chemical or physical methods of separation (Benemann and Oswald, 1996). Previous sections covered studies concerning flocculation without the addition of other bioflocculants or microorganisms to improve microalgal harvesting.

Bioflocculation studies, as covered in the upcoming sections, included the use of other biological agents that were native or non-native to the wastewater environment incorporating bacterial EPS from bioflocculants or return activated sludge. These microorganism provided an additional source of EPS to improve microalgal harvesting.

EPS production has been shown to occur in both prokaryotic and eukaryotic (algae, fungi) microorganisms (Wingender, 1999). EPS is defined as, "...an extracellular polymeric substance of biological origin that participates in the formation of microbial aggregates" (Wingender, 1999). Moreover, the broader term polymeric substance now incorporates a varied composition of proteins, nucleic acids, phospholipids and humic substances other than polysaccharides (Wingender, 1999). EPS is known to exist in two main forms: capsular or tightly bound and dissolved or loosely bound (Wingender, 1999). Capsular EPS attaches to the cell surface by either non-covalent interactions or covalent interactions through binding with lipid-A molecules or phospholipids (Wingender, 1999). Functions of both capsular and dissolved EPS include mediation between the cell and the environment, serving as a protective barrier to prevent desiccation, as well as aggregating bacterial cells to form either flocs or biofilms to increase cell density and culture stability (Wingender, 1999).

A direct correlation has been observed between EPS accumulation and microbial flocculation as reviewed by Pavoni et al. (1972). Pavoni et al. (1972) first observed that bacterial flocculation takes place only after the microorganisms have entered stationary phases of growth. Pavoni et al. (1972) also determined that extraction of EPS from a well-flocculated bacteria suspension followed by a subsequent re-addition of extracted polymer resulted in re-flocculated suspensions. The addition of the same extracted EPS to stable

inorganic dispersions of silica, alumina and kaolinite produced well-flocculated suspensions.

Throughout the Pavoni et al. (1972) investigation, pH was found to be the most important factor in determining the appropriate dosage and efficacy of extracted polymer. It was observed that bacterial supernatant polymer had increased flocculation ability at pH ranges above 7 and below 3. Throughout pH ranges of 3 to 7, especially at pH 7, flocculation ability was not as prevalent (Pavoni et al., 1972). Pavoni's study inferred that exuded polymeric material contains functional groups that are primarily anionic and nonionic in most neutral pH ranges (Pavoni et al., 1972). Thus, polymer colloid bonding occurs when both constituents possess negative charges, implying that a physical interaction, as opposed to electrostatic, is the primary mechanism of attachment (Pavoni et al., 1972). Pavoni observed that the interaction of high molecular weight exocellular polymers could bond either electrostatically or physically to bridge the cells of a dispersion forming a three-dimensional floc matrix large enough to settle. However, a reduction in electrostatic repulsion is not necessary for bioflocculation to occur (Pavoni et al., 1972).

Section 2.5.1 Large-Scale Bioflocculation Studies to Improve Harvesting of Microalgae

Full-scale bioflocculation for the harvesting of algae has already been studied extensively Benemann (1980, 1981). Benemann's experiments in 1976 to 1981 focused on both "pond isolation" for algae removal and "high-rate pond bioflocculation" techniques (Benemann & Oswald, 1996). Pond or phase isolation proposed by Benemann et al. (1981), involves the retention of oxidation pond effluent as a batch culture until a large proportion of the algal cells settle out of suspension. Due to isolation, the algal cells settle as a result of improved conditions for bioflocculation with native bacteria present in the wastewater, including increased physical interactions from an increase in contact time. Isolation also

was postulated to introduce a nutrient limited environment for a specified period of time, which allows both the bacteria and algae to produce more EPS, thereby enhancing bioflocculation (Benemann et al., 1981). In a different approach for high-rate pond bioflocculation, Benemann et al. (1981) used a flocculent culture of algae and maintained conditions suitable for that flocculent culture to be dominant and to improve settling within the high rate pond itself.

Benemann et al. (1981) found that removals in isolation ponds ranged from 81% to 95% TSS and averaged 88%. Benemann et al. also found that factors such as the behavior of cultures with large grazing organisms, mixing approach, and physical parameters of the pond (depth, shape, etc.) had little or no effect on algal removals. The greatest effects on settling were algal sludge seeding and manipulation of growth conditions during pond operation (Benemann et al., 1981). For instance, when algal sludge was recovered at the end of the previous experiment and seeded into the growth pond culture, bioflocculation was more rapid. An algal removal of 80% was reached in 21 days in the seeded pond compared to 29 days in the control (Benemann et al., 1981).

Manipulation of growth conditions was found to be the most effective means for reducing the required length of the isolation periods (Benemann et al., 1981). Growth conditions such as nutrient stress shortens the time the algal culture takes to enter the stationary growth phase, where polymer accumulation is largest, thereby shortening the isolation periods (Benemann et al., 1981). Growth at relatively high light intensity tended to reduce required isolation periods due to increased algal photorespiration and loss of algal cell viability (Benemann et al., 1981). The combination of bacterial and algal extracellular polymers was found to be important to reduce pond isolation periods (Benemann, 1981).

Benemann et al. (1981) developed another technology termed high rate pond bioflocculation in which flocculent algal cultures could be produced within properly operated high rate ponds. Using flocculent cultures of *Microactinium*, Benemann et al. (1981) showed that cultures grown with a moderate dilution rate were more settleable than those with a high dilution rate (80% for moderate to 76% removal for high). Benemann et al. concluded that a combination of both of these techniques (pond isolation and high rate pond bioflocculation) is the most feasible option for the harvesting of microalgae (Benemann et al., 1981). Pond isolation is hindered by the long detention time necessary and high rate bioflocculation is variable in the degree of settleability it can maintain (Benemann et al., 1981). Thus, control of bioflocculation processes have shown modest success, but it still remains hard to scale up to achieve year round harvesting efficiencies of above 95 % with concentrate around 1.5 to 3 % solids (Benemann and Oswald, 1996; Pavoni et al., 1972).

Similar studies by Al-Shayji in Kuwait showed that algal production (kg/ha.d) depended on the efficiency of floc formation and settling (Al-Shayji, 1994). These studies used pilot scale high rate algal ponds fed by municipal wastewater followed by a sedimentation tank. *Scenedesmus* species dominated the culture growth (Al-Shayji, 1994). A minimum of a 2-hour residence time was needed in isolating settling tanks to achieve adequate separation of the algae (Al-Shayji, 1994). Most of the time during pilot scale operation, effluent suspended solids were less than 20 to 30 mg/L (Al-Shayji, 1994). This study showed that the floc formation depended on the weather and operational conditions including variations of the flow rate and pond depth (Al-Shayji, 1994). In many cases, the settled material varied by the hour of the day by as much as 20-30%, illustrating that these results were highly variable with the relatively short detention times used for separation (Al-Shayji, 1994). Like Benemann et al.'s (1981) results, Al-Shayji found that using pond

isolation required a multiple day retention time for increased algal removal efficiency. Since Al-Shayji's study only relied on a fixed isolation time of two hours, results using the isolation technique were more variable according to the operational conditions encountered (Al-Shayji, 1994).

The effect of carbon dioxide addition on algal bioflocculation in experimental high rate ponds was also studied (Park, 2010). The predominant algal cultures included *Scenedesmus*, *Microactinium*, and *Pediastrum* (Park, 2010). Using a high rate algal pond with a short four-day hydraulic retention time, nearly double the algal production was observed with carbon dioxide addition compared to the control pond with no carbon dioxide addition (Park, 2010). Overall, using a simple gravity settler and three-hour retention time, a consistent settling efficiency of greater than 60% was achieved for the carbon dioxide supplemented culture (Park, 2010). Park concluded that the algal biomass grown in wastewater treatment high rate algal ponds with carbon dioxide addition may be harvested efficiently with a retention time of three hours or less (Park, 2010).

Section 2.5.2 Settling Characteristics of Activated Sludge

Settling in activated sludge wastewater treatment systems is reviewed briefly in this section to provide context for algae settling. In activated sludge, microbial EPS is a major component of the activated sludge floc matrix (Li, 2007). Different stages of growth in the activated sludge process correspond to better treatment performance and settling (and bioflocculation) of the microorganisms present as a result mainly of the microbial EPS produced. The logarithmic and lag phases of growth are not significantly associated with any floc formation; rather, bioflocculation is observed during later stages of growth during declining growth (Gerardi, 2002). Bacterial floc formation is greatest when the maximum bacterial population is reached and the bacteria have aged significantly to produce fibrils,

polysaccharides, and polyhydroxybutyrate (PHB) granules (Gerardi, 2002). In addition, changes in the microbial community structure can influence settling. For example, during endogenous phase of growth, the formation of filaments extends the floc formation to form dense, strong flocs (Gerardi, 2002). The presence of rotifers also serves to provide significant cropping of the dense flocs, allowing the flocs to become heavier and more tightly bound (Gerardi, 2002).

One study examined the microbial EPS within the activated sludge matrix as a function of solids retention time (SRT) and carbon source addition (Li, 2007). Li found that the activated sludge EPS includes both loosely bound and tightly bound components (Li, 2007). The production of tightly bound EPS component was not affected by the SRT, but the loosely bound EPS content increased as the sludge SRT increased (Li, 2007). Li also found that sludge fed on glucose contained more EPS than sludge grown on acetate. Even though the glucose-fed sludge had more available EPS, the acetate-fed sludge performed better in terms of bioflocculation, sludge sedimentation and compression, as well as sludge dewaterability (Li, 2007). As the SRT was increased for both of the carbon sources, the sludge flocculation and separation improved (Li, 2007). In the case of acetate-fed sludge the loosely bound EPS was shown to have a negative effect on the bioflocculation and sludge water separation (Li, 2007). Li's study argued that type and composition of EPS is essential for strong, dense floc formation, as floc formation and cell attachment was weakened by excessive loosely bound EPS resulting in weaker bioflocculation, greater cell erosion and retarded sludge water-separation (Li, 2007).

Badireddy et al. (2010) investigated the effects of EPS composition on activated sludge settling and showed that bioflocculation improved with total carbohydrates and proteins in the activated sludge sampled. Flocculation of the bacteria was inhibited during

exponential growth phase when EPS content was low and acidic in nature (Badireddy et al., 2010). This poor bioflocculation performance was attributed to the combination of decreased polymeric interactions and electrostatic repulsion (Badireddy et al., 2010). Similarly, a higher fraction of uronic acids to total EPS was found to increase the negative charge of the microorganisms during exponential growth, thereby reducing their ability to flocculate (Badireddy et al., 2010). However, enhanced bioflocculation was achieved during stationary growth phase given greater amounts of EPS were present over the uronic acids present (uronic acids with respect to EPS was low) (Badireddy et al., 2010). Therefore, better bioflocculation was obtained with higher concentrations of proteins and carbohydrates (EPS), possibly because the polymeric interactions and bridging cancelled out the electrostatic repulsions (Badireddy et al., 2010). Badireddy et al. also found that EPS compounds such as proteins, carbohydrates and alcohols promoted microorganisms to flocculate, while functionalities such as carbonyl, carboxylate, acetal, and hemiacetal/hydrocarbons hampered bioflocculation (Badireddy et al., 2010).

In a study by Pan et al. (1999), algal biomass was shown to improve the dewatering characteristics and bioflocculation capability of activated sludge. In this study, sludge with *Pediastrum*, *Ankistrodesmus*, and some diatom algal species was aged in a container free of sunlight. After 5 days of sludge storage, significant flocculation was found between the sludge particles and the algae by microscopic analysis (Pan et al., 1999). On the tenth day, the flocculation began to breakdown and the algae and its exudates were degraded by the bacteria (Pan et al., 1999). It was found that as the algae aged, more EPS was released and the flocs became larger due to more advanced flocculation (Pan et al., 1999).

Over the course of the sludge storage, the pH and the total solids content remained unchanged (Pan et al., 1999). The TOC, zeta potential, and bound water content, however,

decreased throughout storage (Pan et al., 1999). The zeta potential was shown to decrease until the fifth day, where algae and the largest presence of EPS, bearing negative charges, were shown to be responsible (Pan et al., 1999). After this period, the charge reversed due to the fact that the bacteria gradually degraded both the algae and its exudates (Pan et al., 1999). Given that the TOC stayed constant during the first six days, algal biomass and EPS degradation was seen to stabilize before dropping for the remaining period as bacteria degraded the EPS components (Pan et al., 1999). Sludge conditioning through use of a cationic polymer also was observed to be best after 5 days of ageing when the most algal exudates were present (Pan et al., 1999). Thus, over the aging process algae and its exudates significantly changed the surface properties of the algae-containing sludge, affecting the conditioning by allowing more bioflocculation to occur with less cationic polymer dosage (Pan et al., 1999).

Section 2.5.3 Addition of Bioflocculants to Improve Microalgal Harvesting

Multiple researchers have developed bioflocculants that can significantly improve flocculating efficiency and settleability of algal cultures. Bioflocculants studied include organisms such as autoflocculating microalgae and floc-forming bacteria of activated sludge matrices (Salim et al., 2011). This section will discuss studies using first bacterial bioflocculants (Section 2.5.3.1), then algal bioflocculants (Section 2.5.3.2), as well as floc-forming bioflocculants (i.e. RAS addition, Section 2.5.3.3) isolated from wastewater treatment plants.

Section 2.5.3.1 Addition of Bacterial Flocculants to Improve Microalgal Harvesting

Harvesting of algae with the addition of bacterial bioflocculants has been studied recently by several research groups (Oh et al., 2001; Kim et al., 2011; Lee et al., 2009). For example, Oh et al. (2001) conducted a study on the harvesting of *Chlorella vulgaris* using the

bacterium *Paenibacillus* (AM49) as a bioflocculant. In this study, the strain AM49 increased flocculating efficiency by up to 93%, with flocculation efficiency increasing with increasing concentration of bioflocculant (Oh et al., 2001). The culture age of *Chlorella vulgaris* used was fixed at 6 days (early stationary phase) and the study did not investigate settling at different algal growth phases (Oh et al., 2001). The AM49 strain was tested with a variety of other microalgae, including *Scenedesmus quadricada* and *Anabaena flos-aquae*, and flocculation efficiency ranged from 39 to 95% as compared to 93 % for *Chlorella vulgaris* (Figure 2.20) (Oh et al., 2001). Poor settling with bioflocculant addition was observed for *Anabaena flos-aquae* and *Microcystis aeruginosa*, which shows that improved harvesting of microalgae, depends on the type of bioflocculant used (Figure 2.20).

Figure 2.20-Flocculation of AM49 with different species of Microalgae with a fixed dose of bioflocculant (Ana-Anabaena flos-aquae; Mic-Microcystis aeruginosa; Bot-Botryococcus braunii; Sce-Scenedesmus quadricada; Sel-Selanastrum capricornutum; Chl-Chlorella vulgaris) (Source: Oh et. al, 2001)

A parallel study was conducted on the harvesting efficiency of a bacterial bioflocculant added to a pure *Scenedesmus* culture (Kim et al., 2011). The same bacterial

strain used in the study above, *Paenibacillus polymyxa* AM49, was researched as the principal bioflocculant. The bacterial culture was added to a high-density, late stationary phase, culture (16 days of growth) of *Scenedesmus* species at a concentration of 1% (v/v). Bioflocculant production by this bacterium, as measured by EPS concentration of the bacterial strain used, increased to the late exponential growth phase and then remained constant during the stationary phase of bacterial growth, following a close relationship with its growth curve (Kim et al., 2011). In this study, flocculation was monitored with addition of chemical co-coagulants, such as ammonium chloride, potassium chloride, calcium chloride, ferric chloride and ammonium sulfate. These co-coagulants had a significant effect on the settling of *Scenedesmus* and the flocculation increased with increasing electrical charges of the cations (Kim et al., 2011). However, increasing the concentration of the inorganic coagulants used, especially at higher culture densities of *Scenedesmus*, resulted in higher flocculation efficiencies (Kim et al., 2011).

An earlier study focusing on bacterial flocculation for algae harvesting used a mixed microbial culture to improve the flocculation of a marine microalgae (Lee et al., 2009). This study involved growing a marine species of algae, *Pleurochrysis carterae*, in a photobioreactor, where cultures near the end of logarithmic phase of growth and beginning stationary phase were used for flocculating experiments (Lee et al., 2009). Cultures of *Pseudomonas stutzeri* and *Bacillus cereus* were added as potential bioflocculants (Lee, 2009). This study varied the substrate type (glucose, acetate, and glycerine) and concentration (0.1 and 0.5 g/L) for growth of the microbial bioflocculants as well as the mixing time for addition of bioflocculants to the microalgae (between 6 and 24 hours). Marine microalgal flocculation using all three types of substrate was substantially enhanced at longer mixing times with around 90 to 95% removal of algal cells (after 24 hours) (Lee et al., 2009). At shorter mixing times, flocculating efficiency was worse for all substrate types

and concentrations, with a 45 to 55% range in removal of algal cells. At higher doses of organic carbon, larger volumes of flocs were evidenced, but the recovery efficiency (percent algal cells removed) remained relatively constant (Lee et al., 2009).

In this study a lack of nutrients was identified as a major factor that stresses the microbes to form more EPS (Lee et al., 2009). However, the volume of EPS produced was not related to the flocculation observed, as a more tightly bound EPS was more desirable given that this type of EPS produced the best flocculation (Lee et al., 2009). This study also concluded that tightly bound EPS can be achieved by reducing the organic carbon, increasing the mixing time and using the right dosage of microbes (Lee et al., 2009). Lastly, proper mixing time was crucial to increase the frequency of contact between the microalgae and the EPS, where too little mixing resulted in poor floc formation and over mixing caused the flocs to break into smaller fragments (Lee et al., 2009). Without this mixing intensity, *P. carterae* was allowed to settle under gravity, and researchers found that the flocs formed were too loose and difficult to separate properly from the culture media (Lee et al., 2009).

Section 2.5.3.2 Addition of Algal Flocculants to Improve Microalgal Harvesting

Bioflocculation of a non-flocculating microalgal culture can also be induced by adding flocculating microalgae (Salim et al., 2011). A preliminary study used the naturally flocculating diatom *Skeletonema* to successfully flocculate a non-flocculent culture of *Nannochloropsis* (Salim et al., 2011). Similarly, *Chlorella vulgaris* and *Neochloris oleoabundans* (non-flocculent cultures) were grown and combined with the following flocculating microalgae: *Scenedesmus obliquus*, *Tetraselmis suecica*, and *Ankistrodesmus falcatus* in order to improve microalgal harvesting (Salim et al., 2011). All three flocculating microalgae improved recovery efficiency of the two non-flocculent cultures when they were applied at a higher concentration, although doubling the concentration of flocculating

microalgae did not double the recovery efficiency of the previously nonflocculent culture (Salim et al., 2011). Mixing of the flocculating microalgae with the nonflocculent cultures prior to sedimentation increased the initial settling rates considerably (Salim et al., 2011). Micrographs from this study (e.g., Figure 2.21) show the improved algal flocculation over an eight-hour period for different combinations of flocculating and non-flocculating microalgae. Pictures A-C taken represent the non-flocculating microalgae that are present as single cells before floc formation is observed. Similarly, pictures D-F show flocculating species of microalgae used. After sedimentation, non-flocculating microalgae can be observed embedded into the flocs formed by the flocculating microalgae, supporting the observation that the addition of flocculating microalgae improves the recovery of non-flocculating microalgal species (Salim et al., 2011).

Figure 2.21-Photomicrographs of results observed with different flocculating microalgae, (A) and (B)-*Chlorella vulgaris*; (C)-*Neochloris oleoabundans*; (D)-*Ankistrodesmus falcatus*; (E)-*Scenedesmus obliquus*; (F)-*Tetraselmis suecica*; (G)-*C. vulgaris* with *A. falcatus*; (H)-*C. Vulgaris* with *S. obliquus*; and (I)-*N. oleoabundans* with *T. suecica* (Source: Salim, 2011)

Another promising strategy is recycling of settled algal species in high rate algal ponds as proposed by Park and Craggs (2011). Recycling settled algal biomass resulted in an increased dominance of *Pediastrum* species (76 to 99% dominance) as compared to the control HRAP (0-98% dominance) (Park & Craggs, 2011). In this study, recycling harvested algal biomass was found to preferentially select algae that settle more rapidly. The increased dominance of the *Pediastrum* species allowed an improved harvest efficiency of greater than 85% as compared to the control pond, which demonstrated an efficiency of

only 60% (Park & Craggs, 2011). The settling results depended on the extent of algal species domination within the algal HRAP's. If *Pediastrum* was dominant at least 80% over all other species, it exhibited 99.2% harvesting efficiency over a 24-hour period and resulted in 2.5 to 3% solids after twenty-four hours of gravity sedimentation (Park & Craggs, 2011). If *Pediastrum* was less dominant and the culture was afflicted by poor settleable unicellular algae (*Thalassiosira* species), only about 1.5 to 2% solids were reached after 24 hours of gravity sedimentation (Park and Craggs, 2011). Recycling increased the average size and biovolume of the dominant *Pediastrum* by 13-30% and 50-80% respectively, which further suggests good settling (Park and Craggs, 2011). Thus, recycling of harvested algal biomass is a simple and effective operational strategy to maintain dominance of readily harvestable algal species (Park and Craggs, 2011).

Section 2.5.3.3 Addition of Return Activated Sludge (RAS) to Improve Microalgal Harvesting

An early study focusing on return activated sludge (RAS) addition to improve the settling of microalgae involved culturing a readily settleable algal-bacterial biomass that provided high-level treatment for use in HRAP's (Gutzelt et al., 2005). Using both a laboratory scale and pilot scale reactor system, Gutzelt et al. (2005) grew a suspension of *Chlorella vulgaris* and added activated sludge from a municipal WWTP. The laboratory system was run as a sequential batch reactor (SBR) and the pilot scale system as a continuously stirred tank reactor (CSTR). Within four weeks of operation, the ratio of Chl. A of the supernatant phase to the total Chl. A in the laboratory reactor decreased from 95% to 0.26% (Gutzelt et al., 2005). In addition, the effluent biomass TSS of the lab scale SBR system was 1 to 1.5 g/L (Gutzelt et al., 2005). Sludge volume indices of 80 to 120 ml/g and sedimentation after only ten minutes of the entire biomass proved that the pilot scale

system also functioned well (Gutzelt et al., 2005). Results indicated that in both the laboratory and pilot-scale systems, algal bacterial aggregates could be developed successfully with *Chlorella vulgaris* and wastewater species (Gutzelt et al., 2005).

Another study using the same sequencing batch reactor (SBR) approach was operated in New Zealand to reproduce the Germany study (Valigore et al., 2012). The SBRs were operated with a combination of native microalgae to the wastewater treatment oxidation ponds as well as bacteria fed from primary treated wastewater (Valigore et al., 2012). The solids retention times over the time course of the experiment were varied from 4 to 40 days (Valigore et al., 2012). Results signified that longer SRTs in the SBR system enhanced biomass settleability, while shorter HRTs enhanced productivity (Valigore et al., 2012). Together, the greatest settleability and productivity of algal biomass was attained when settleable flocs were recycled to achieve longer SRTs than HRTs (Valigore et al., 2012). Therefore, the longer SRTs were more effective due to biomass recycling which reduced the growth rate, thereby facilitating better floc formation and sedimentation (Valigore et al., 2012). Finally, at these long SRTs, biomass productivity was found to be up to 31 g/day/m² of solids, corresponding with a biomass settleability ranging from 70 to 95% (Valigore et al., 2012). This study proved that SBRs operating with longer SRTs and short HRTs allow the production of a large amount of biomass for conversion to biofuel, as well as a corresponding improved bioflocculation and sedimentation of the biomass grown (Valigore et al., 2012).

In a similar study on RAS addition, effects of bacterial community, algal settleability, and carbon/nutrient removals in a series of photobioreactors were investigated (Su et al., 2012). The inoculum ratios of each bioreactor used ranged from 1:1 algae to activated sludge, 1:5 and 5:1 respectively (Su et al., 2012). In this study, activated sludge alone settled

the best, while the algae alone settled the worst (Figure 2.22; Su et al., 2012). The 1:5 algae to sludge ratio performed the second best, followed by 1:1, 5:1 and 10:1 indicating that activated sludge improved algae settling (Figure 2.22; Su et al., 2012). Analysis of the bacterial community structure for different inoculum ratios of RAS showed that differing algae to bacteria inoculation ratios had an effect on the bacterial community composition, possibly contributing to improved removal efficiencies (Su et al., 2012).

Figure 2.22-Settleability of biomass (algal and bacterial) with different RAS inoculation ratios (Source: Su, 2012)

CHAPTER 3: METHODS AND MATERIALS

The methods and equipment for microalgal and bacterial growth, settling test procedures, evaluation of settleability performance, as well as EPS quantification are described in this chapter. This research was focused on culturing and analyzing the settling characteristics of two species of algae: *Scenedesmus sp.* and *Chlorella vulgaris* with use of the bacterium *Burkholderia cepacia* as the biofloculant.

3.1 Initial Growth of Algal and Bacterial Cultures

Algal cultures obtained from the UTEX collection were first grown in 250 mL flasks using WC media, as described in Table 3.1 below. The two species of algae were *Chlorella vulgaris* (UTEX # 265) and *Scenedesmus sp.* (UTEX # 1589). WC medium is a defined growth medium, designed for use with most fresh water, green microalgal species (Andersen, 2005). In these experiments, Tris base was used as the primary buffer, instead of Glycylglycine. Additional stock solutions of vitamins and trace metals were also required at concentrations listed in Tables 3.2 and 3.3. The only procedure that differed for the preparation of the media, was preparing the vitamin stock solution, which required filter sterilization by a 0.2 µm filter, and then needed to be stored at -20°C.

Table 3.1-Composition of WC medium and concentration of chemical components
(Source: Andersen, 2005)

Component	Concentration in Medium (M)
Tris (Buffer)	4.13×10^{-3}
NaNO_3	1.00×10^{-3}
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	2.50×10^{-4}
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.50×10^{-4}
NaHCO_3	1.50×10^{-4}
$\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	1.00×10^{-4}
K_2HPO_4	5.00×10^{-5}
Vitamin Stock	See Table 3.2
Trace Metal Stock	See Table 3.3

Table 3.2-Composition of Vitamin Stock solution for WC medium
(Source: Andersen, 2005)

Component	Concentration in Final Medium (M)
Thiamine $\cdot$ HCl (vitamin B1)	2.96×10^{-7}
Biotin (vitamin H)	2.05×10^{-9}
Cyanocobalamin (vitamin B12)	3.69×10^{-10}

Table 3.3-Composition of Trace Metals stock solution for WC medium
(Source: Andersen, 2005)

Component	Concentration in Final Medium (M)
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	1.17×10^{-5}
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	1.17×10^{-5}
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	4.01×10^{-8}
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	7.65×10^{-8}
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	4.20×10^{-8}
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	9.10×10^{-7}
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	2.48×10^{-8}
H_3BO_3	1.62×10^{-5}

To keep conditions sterile for the algae, the 250-mL flasks and the WC media were autoclaved for 50 minutes at 121°C. The algae from the agar slants received from UTEX were revived in the WC media by transferring with a sterilized inoculating loop into sterile medium. The algae were placed in 250-mL flasks with sterile silicone enclosures and mixed under an 8-hour illumination using a light rack apparatus with specifications according to the Figure 3.1. The temperature and pH were not controlled during these initial inoculum-growing procedures. Optical density was monitored daily for both the *Scenedesmus* and *Chlorella* cultures for 2 weeks of the first batch culture using a Spectronic 20 spectrophotometer (Milton Roy Company, remanufactured by Update Instrument, Inc. Madison, WI). Illumination was provided by eight fluorescent Vita Lite® 40-Watt light bulbs, equivalent to 403 W/m² per bulb (or 1610 W/m² for the entire light rack assuming the distance from the bulb to the flasks was 3.5 inches). Lithonia Lighting manufactured the four ballasts. This process of growth of inocula was continued for two more batch inoculations using the same technique described above before stepping up growth to larger 2-L Schott Duran bioreactors, and later 4-L jar bioreactors that are described in more detail in the following section (Figures 3.2).

Figure 3.1- Light rack apparatus used for algal illumination for growth

Figure 3.2-Light rack apparatus in use in the laboratory

To preserve the newly obtained cultures, the algal cultures from the first batch culture broth were inoculated onto sterile petri dishes (Fisher) using two different media recipes: WC medium as described above and Bristol Medium as specified by the UTEX website and described in Table 3.4. To each 1-L of media, 1.5 % agar or 15 g were added, mixed sufficiently, and autoclaved at the same conditions specified above. The plates were then inoculated with first batch culture of algae from the broth growing for 6 days, and algae were allowed to grow on the agar under illumination for two weeks. The plates were then refrigerated at 4°C until future inoculation was needed. This process of plating was completed for several batch inoculations.

**Table 3.4 - Composition of Bristol Medium used for plating of algal cultures
(Adapted from the UTEX website)**

Composition	Amount (mL/L)	Stock Solution Concentration (g/400 mL DI)	Final Concentration (mM)
NaNO ₃	10	10	2.94
CaCl ₂ * 2H ₂ O	10	1	0.17
MgSO ₄ *7 H ₂ O	10	3	0.3
NaCl	10	1	0.43
KH ₂ PO ₄	10	7	1.29
K ₂ HPO ₄	10	3	0.43

The bacterium *Burkholderia cepacia* was grown to provide EPS for bacterial bioflocculation experiments. The *B. cepacia* was obtained as a lyophilized culture from the ATCC culture collection (ATCC #10856). The ATCC culture tube filled with 20 mL of nutrient broth (BD 23400) as directed by the ATCC guidelines. The nutrient broth had a composition shown in Table 3.5. With addition into the culture test tube and sufficient agitation, 5 mL of the bacterial culture was transferred into four different, 250 mL flasks containing 75 mL of the nutrient broth. The four flasks were covered with sterile closures and incubated on a Junior Orbit shaker (Labline Instruments, Inc. Melrose Park, Illinois), shaking at constant

rotational speed of 200 rpm, and allowed to grow for 4 days. Following this growth period, the *B. cepacia* cells were transferred from the nutrient broth to 75 mL of MMS-3 media (Table 3.6).

Table 3.5-Composition of Nutrient Broth BD 23400, added to 1 Liter of DI, and pH adjusted to 6.8
(Source: ATCC website)

Composition	Concentration (g/L)
Peptone	5
Beef Extract	3

The ionic strength was adjusted to 0.05 M with sodium nitrate. Sodium chloride was substituted two times during growth with sodium nitrate and the calculated adjusted concentrations for both species can be viewed in the Table 3.7. Finally, the pH was adjusted to 6 before autoclaving.

Table 3.6- Modified MMS-3 Media (Source: Nelson et al., 1996)

Composition of Modified MMS-3	
Composition	Concentration (mg/L)
99% Anhydrous Glucose	79
CaCl ₂ * 2H ₂ O	30
MgSO ₄ * 7H ₂ O	35
KNO ₃	15
(NH ₄) ₂ SO ₄	120
KH ₂ PO ₄	7
NaHCO ₃	0.84

Table 3.7 - Concentrations of compounds used to adjust ionic strength to .05 M (in modified MMS-3 media)

Composition	Concentration (g/L)
NaCl	2.635
NaNO ₃	3.832

The second batch inoculation of bacteria was aseptically inoculated with 3.75 mL of bacterial broth from the four initial 250-mL flasks into four new 250-mL flasks each filled with 75 mL of defined MMS-3 medium. Growth was maintained for 3 days before the third batch culture was created, scaling up growth to 2-L bioreactors. During this

same time, the *B. cepacia* cultures were inoculated on sterile petri dishes (Fisher) for culture preservation with MMS-3 medium. First and second batch cultures were inoculated onto the plates aseptically, and grown for 4 days at room temperature. Each new batch culture was inoculated monthly onto defined media MMS-3 plates for culture preservation.

3.2 Scale-Up of Growth of Algal and Bacterial Cultures

From the second batch culture, initial scale up of both the *Chlorella* and *Scenedesmus* cultures to the third batch culture was done in 1-L flasks grown in sterile conditions with defined WC medium. These third batch cultures were sparged with a blend of air and 5% carbon dioxide (v/v), blended with a Matheson gas controller (Model 665). This ratio was maintained using a calibration curve of the airflow device shown in Figure 3.3. The red line indicates the relationship between the average velocity of the calibration device and the flowmeter readings at higher flowrates, while the blue line indicates the same trend at lower flowrates.

Figure 3.3-Calibration curve used to regulate airflow and carbon dioxide input for Mattheson Model 602/603

The air was obtained from an aquarium pump (Top Fin) and was consistently allowed to enter the flasks. However, the carbon dioxide was restricted during hours when photosynthesis was not taking place to maintain pH control. Thus, carbon dioxide inputs were stopped or partially closed with a metal clamp when pH dropped below 6.5 and was started again when the pH climbed to around 9 or 10. The pH was monitored daily with an autoclavable probe (Mettler Toledo, Cole Parmer: Vernon Hills, Illinois). Optical density was monitored during growth using a UV 1700 Pharma Spec model spectrophotometer (Shimadzu) at 625 nanometers.

Following two to three weeks of growth, scale up of the algal cultures involved transferring 100 mL of third batch culture inoculum into autoclaved 2-L bioreactors filled with 1800 mL of autoclaved WC media. The bioreactors were constructed using water-jacketed beakers (Schott Duran) to allow temperature control with a Haake model DC3 water bath. As above, carbon dioxide was blended at 5% with air and pH was constantly monitored with a Mettler Toledo probe. Cultures were stirred at 150 rpm using a stir bar and stir plate (Fisher). Temperatures were maintained at 25°C. Temperature was also monitored with a thermometer placed inside the bioreactor to confirm readings with the water jacket system. Again, the fourth batch culture growth was monitored with optical density readings taken daily using the Spectronic 20 spectrophotometer.

To obtain a larger volume of algae for settling tests, a final scale up of growth consisted of transferring 100 mL of fourth batch culture inoculum into autoclaved 4-L clear glass jars filled with 4-L of autoclaved WC media (Figure 3.4). The tops of these jars had ports for filter sterilized air inlets, sterilized filter air outlets, as well as a pH probe/sampling port that was attached to another autoclaved 250-mL filter flask (Fisher) with a bulb to transfer samples out of the reactor. Millipore air filters, with pore size of 0.2 microns, were used to filter the air in and air out. From this fifth batch culture on, carbon

dioxide input was not needed, because air addition by an aquarium pump was sufficient for algal growth. No temperature control or pH control was used in the 4-L bioreactors, and growth of the cultures was usually sustained at a pH between 8 and 9, and an average room temperature of 21°C. Growth of these cultures was monitored daily using optical density at 625 nanometers with a UV 1700 Pharma Spec model spectrophotometer (Shimadzu). Dry weight and optical density measurements were correlated well for both *Scenedesmus* and *Chlorella* (Figures 3.5 and 3.6) with r^2 values of 0.96 to 0.99. 15-20 mL samples were taken for EPS analysis once every other day.

Figure 3.4-Growth of algae in a 4-L bioreactor equipped with filter flask for sample removal

Figure 3.5-Relationship developed between dry weight and optical density of *Scenedesmus*

Figure 3.6-Relationships developed between dry weight and optical density of *Chlorella*

The scale up of *B. cepacia* from second to third batch cultures also involved growing the bacteria in a sterile, 2-L bioreactor with air addition and a constant monitoring of pH. Figure 3.7 shows the two-liter bioreactors used with *B. cepacia* cultures. The scale up consisted of transferring 75 mL of the bacterial broth in MMS-3 with 1800 mL of autoclaved

MMS-3 media. Water was circulated and heated or cooled by a VWR Scientific Products by Polyscience model 1162 circulation heater. Unlike the algal cultures, if pH dropped below 6.0, a solution of 10% sodium hydroxide was added drop wise into the bioreactor to maintain a pH of at least 7.0. *B. cepacia* growth was consistent in first dropping the pH during exponential growth, and raising the pH during the onset of declining growth and stationary growth phases. Thus, pH generally needed only to be increased, as during later stages of growth, the pH remained constant at around 8.5. Like the algal bioreactors, a sampling flask was attached to the bioreactor apparatus and controlled with a bulb. Optical density was measured daily using a UV 1700 Pharma Spec model spectrophotometer (Shimadzu). In addition, samples were taken every other day for EPS measurement.

Figure 3.7 - 2-liter bioreactor setup used to grow *B. cepacia* cultures

3.3 Settling Test Procedures

Settling tests were conducted to measure the settling efficiencies for *Scenedesmus* and *Chlorella* cultures. Settling and bioflocculation effects were measured at different stages of growth, with and without bioflocculant addition. Effects of pH, light intensity and light vs. dark settling conditions were also tested. Experiments concerning nutrient deprivation of bioflocculant added and the effect of augmenting the carbon source through the addition of CO₂ were also performed.

To make best use of the relatively small volume of algae grown, modified settling apparatus were constructed out of acrylic using silicone glue. The modified settling apparatus has the same vertical dimensions as a traditional “squared beaker” test apparatus (Philips & Bird), but is scaled down to a volume of 400-mL (circular model) and 300-mL (square model) (Figures 3.8 and 3.9 and Tables 3.8 and 3.9). The sampling ports were constructed to be 1.875 inches above the bottom of the settling column to prevent any effects due to re-suspension. The outlets of the settling columns were equipped with glass tubing and rubber stoppers, and a clip to control the flow out of the columns. The complete set-ups are shown in Figure 3.10. To prevent excess algae and bacteria accumulation, the columns were cleaned thoroughly with 70% ethanol before and after each settling test.

Figure 3.8-Visual representation of rectangular settling columns constructed

Table 3.8-Approximate dimensions of rectangular settling apparatus built

Feature	Dimensions
Height (H)	8 inches
Top Width (L)	1.5 inches
Area of Top	2.25 inches squared
Height to Bottom of Sampling Port (H_p)	1.875 inches
Diameter of Sampling Port (D_p)	0.5625 inches
Approximate Volume	300 mL
Shape	Rectangular

Figure 3.9-Visual representation of cylindrical settling column

Table 3.9 - Approximate dimensions of cylindrical settling apparatus built

Feature	Dimensions
Height (H)	8 inches
Diameter (D)	2 inches
Height to Bottom of Sampling Port (H_p)	1.875 inches
Diameter of Sampling Port (D_p)	0.5625 inches
Approximate Volume	400 mL
Shape	Cylindrical

Figure 3.10-Illustration of Settling Columns in use

Each settling test was conducted over a 24- hour time period. Samples were collected once at the beginning, and after 0.5, 1, 2 and 24 hours. Before each sampling, the outlet tube was drained for about 5-10 seconds to flush the outlet tube. After the 2-hr and 24-hr samples were collected, they were labeled and refrigerated immediately until cell counting was conducted.

Suspended algae in the settled samples were quantified by cell counts using a hemocytometer (Fisher) with an Olympus microscope at 400X magnification, using phase contrast. The Olympus microscope used was a CX4RF model equipped with an Infinity 2 digital camera and Infinity Analyze/Capture software. Prior to counting, the algal and bacterial samples were deflocculated using sonification with a Branson Sonifer Model 250 at half of the full output (setting 6 to 8) for 8-10 minutes for the *Chlorella* cells and 6 to 8 minutes for the *Scenedesmus* cells. The *Scenedesmus* cells required a shorter time for deflocculation because the flocs were less dense compared to the tiny, densely packed

clusters of *Chlorella*. In some cases, samples needed to be repeatedly sonified (usually not more than twice). Each sample was counted at least 5 times to provide adequate statistics. Extreme outliers, calculated by a relative standard deviation of greater than 90%, were not included.

The hemocytometer counting grid consists of a middle grey square area surrounded by four equally sized grey areas (Figure 3.11). Within this middle grey area, there are 25 individual boxes. The area of the entire counting grid is 1 mm by 1 mm and the depth is 0.1 mm. Therefore, the volume of the area counted is 0.1 mm³. Thus, counting a sample within a known area of the grid allows one to calculate the number of cells per milliliter by multiplying the cell counts in the grid by a factor of 10,000.

Figure 3.11-Representation of a typical hemocytometer counting grid seen at 400x magnification

Cell counts were normalized to provide a consistent comparison to unsettled counts. For good statistics, counts had to be at least 100 to 130 cells. For less dense samples

(usually during earlier stages in the growth curve), counting 100 cells usually involved counting the entire grid of the hemocytometer. For more dense samples (usually found during later stages of the growth curve), counting usually involved one or two rows of the hemocytometer for *Scenedesmus*, and one large box to two rows within the large box of a hemocytometer for *Chlorella*. Before counting *Chlorella* cells, the samples had to set for approximately three minutes for the amount of *Chlorella* cells to stabilize. For an unknown reason, *Chlorella* cells seemed to multiply considerably for the first three minutes when the samples were placed on the hemocytometer.

The settleability of algae was evaluated by graphing the normalized cell concentration vs. settling time. Normalized cell concentration was calculated by dividing the average counted cell concentration by the original concentration (C/C_0). Throughout this research, normalized cell removal efficiency will refer to the percentage of cells *removed* by settling in the specified time.

3.4 EPS Analysis (Dissolved EPS/Filtrate Only)

Both bacterial and algal EPS were quantified by a filtration and dialysis method. The 20-mL to 30-mL samples were filtered through 1.2- μ m glass fiber G4 filters (Fisher) to remove cells, and then placed in dialysis tubing with a 6,000 to 8,000 MWCO (Fisher Brand). Low molecular weight compounds pass through the selective membrane of the dialysis tubing, while high MW polymers such as EPS are retained. The samples were exhaustively dialyzed in the refrigerator at 4 degrees Celsius by changing the DI water daily for 7 days straight. This allows for the elimination of low molecular weight compounds, leaving the higher molecular weight polymers to analyze by total organic carbon (TOC) analysis.

TOC was analyzed using a Shimadzu TOC-VSH TOC analyzer coupled with ASI-V sampling port and TN measuring unit. A calibration curve was made using standards from 15 mg/L and 5 mg/L stock solutions of organic carbon. The results of the calibration

demonstrate that the correlation between these points is linear (Figure 3.12, $r^2 = 0.976$). All samples were acidified using 1 N HCl to allow the instrument to remove any inorganic carbon in the form of carbonate or bicarbonate. The samples were run with continual standard checks, as well as a blank DI sample for Quality Assurance/Quality Control.

Figure 3.12-TOC calibration curve used for EPS Analysis and corresponding r -squared value

CHAPTER 4: RESULTS AND DISCUSSION

To examine repeatability of data, the following experiments were repeated in three separate sets. The first set of experiments focused on observing settling of *Chlorella* and *Scenedesmus* over their growth curves with and without *B. cepacia* addition. Experiment 1 examined settling up to early stationary phase of growth. Experiment 2 followed up on this approach to assess the repeatability of the data, and presented new physical methods to improve settling focused on mixing time of the *B. cepacia* and algal cells. Experiment 2 also prolonged the settling tests to focus on later growth stages (up to mid stationary phase). Experiment 3 further repeated the two initial experiments, but expanded on the physical factors such as dosage of *B. cepacia* bioflocculant and effect of settling during later growth stages (up to late stationary). Both experiment 2 and 3 quantified EPS production for both algal and bacterial cells over the growth curve to evaluate the effects of EPS production on settling. These first three experiments are described in Sections 4.1 and 4.2.

After the first general experiments, results focused on the effects of different environmental conditions on algal settleability. Wastewater samples with and without RAS addition and aeration were analyzed to compare results achieved within the laboratory and based on real world applications (Section 4.3). Conditions investigated included light/dark conditions during settling and light intensity during growth (Section 4.4), pH (Section 4.5), starvation of *B. cepacia* cells (Section 4.6), and carbon dioxide supplementation for algal growth (Section 4.7). In addition, settling was observed for older cultures of both algal species without bioflocculant addition (up to death phase) (Section 4.8). Within these results, effects of certain physical factors such as mixing intensity, and dilution of algal cells were incorporated. All of these experiments also included quantification of EPS and the correlation between EPS and improved settling. Detailed graphs depicting all results with

labeled section headings are provided in the Appendix (Page 227). In addition, a compilation of micrographs for several experiments taken is presented in the Appendix section (Page 217).

4.1 Settling of *Scenedesmus* and *Chlorella* Without Added Bioflocculant

The first investigations focused on measuring the settling of pure *Chlorella* and *Scenedesmus* cultures without any bacterial cell addition or EPS. Both cultures were grown under batch conditions, so one of the objectives was to determine the effect of growth phase on settling. Results and discussion included cover Experiments 1, 2, and 3 beginning with the growth curves, followed by settling results with *Scenedesmus* and *Chlorella* without cell addition, and ending in EPS correlation with settling (Experiments 2 and 3). Settling of older cultures of *Scenedesmus* and *Chlorella* (close to the death phase) without added bioflocculants was also explored in Section 4.1.10. In addition, autoflocculation of algal cells was investigated for both cultures of microalgae at a higher pH (Section 4.1.11) by comparing results to studies found in the literature.

4.1.1 Growth Curves of *Scenedesmus* and *Chlorella*: Experiment 1

Growth curves exhibited in preparation of cultures of *Scenedesmus* and *Chlorella* are shown in Figures 4.1 and 4.2. The growth of *Scenedesmus* was slower than that of *Chlorella*, but both reached a maximum OD of 0.4 to 0.5. As OD data is missing for *Chlorella* it is difficult to tell what growth pattern was obtained after 7 days of growth. Similarly, growth for *Scenedesmus* after sixteen and up to eighteen days of growth from the graph is not discernible. Thus, analysis of results for this experiment reflects growth of *Chlorella* cultures observed in Experiments 2 and 3 as a reference.

Figure 4.1-Growth curve of *Scenedesmus* over an 18-day growth period using optical density at 625 nm

Figure 4.2-Growth curve of *Chlorella* over a 14-day growth period using optical density at 625 nm

4.1.2 Effect of Growth Phase on Settling of *Scenedesmus*: Experiment 1

Settling of *Scenedesmus* alone were measured as a baseline and to establish the growth period where harvesting is most feasible. Figures 4.3 A through 4.3 C show the settling of *Scenedesmus* at varying growth stages. Figure 4.3 A shows normalized cell concentration after settling up to 24 hours, while Figure 4.3 B shows detailed settling over two hours, and Figure 4.3 C summarizes the results for two hour settling. Days 4 and 5 of settling are not included on part B since 2-hour samples were not taken for this experiment. It is apparent that the oldest *Scenedesmus* culture, grown for 18 days, exhibited the best settling based on normalized cell concentration (C/C_0 ; lower value is a better settling rate). At the end of 2-hour settling about 50% removal was observed after 18 days of growth (Figure 4.5 B and C). This corresponds with the latest time on the growth curve, or the onset of mid stationary phase. This result indicates that settling of *Scenedesmus* cultures improves in later phases of growth.

Figure 4.3- Settling based on normalized cell concentration remaining over A) a twenty four hour period for the *Scenedesmus* culture B) a two hour period C) Over eighteen days of growth plotted against the growth curve. The red line indicates optical density results over certain days sampled at 625 nm by a spectrophotometer

4.1.3 Effect of Growth Phase on Settling of *Chlorella*: Experiment 1

Figure 4.4 (A, B,C) depicts a normalization of the settling of *Chlorella* at three different stages of growth. The youngest culture after 7 days of growth has the best removal overall with over 90% removal by settling in a two hour period. In contrast to *Scenedesmus*, *Chlorella* settled better earlier in its growth curve (mid exponential phase of growth). Results from both 24 hours of settling and 2 hours of settling (Figure 4.4 A and B) indicate that the younger cultures of *Chlorella* have the most potential for harvesting without the use of any type of bioflocculant. Settling was much better than for *Scenedesmus* cultures, with above 90 % removal after only two hours of settling for cultures with 7 days of growth. Figure 4.4 C shows the cell removal over a 2-hour period across the entire 14-day growth period. Again, a higher value indicates a lower removal rate.

Figure 4.5 after illustrates how much more efficient *Chlorella* settles than *Scenedesmus* at similar stages of growth curve without the addition of a bioflocculant. After two hours of settling, *Chlorella* exhibits almost ten times the removal as compared to *Scenedesmus* based on the remaining cells after settling for a 2-hour period. However, 24-hour results are almost identical based on the normalized cell concentration remaining.

Figure 4.4- Settling based on normalized cell concentration remaining over A) a twenty four hour period for the *Chlorella* culture B) a two hour period C) over fourteen days of growth plotted against the growth curve. The red line indicates optical density results over certain days sampled at 625 nm by a spectrophotometer

Figure 4.5-Normalized cell concentration plotted against time over a 24-hour period for both the *Chlorella* and *Scenedesmus* cultures. The results are compared when *Scenedesmus* completed 10 days of growth and *Chlorella* 8 days.

4.1.4 Growth Curves of *Scenedesmus* and *Chlorella*: Experiment 2

Settling tests with *Scenedesmus* and *Chlorella* were repeated under the same conditions as Experiment 1 to determine the reproducibility of the previous results on the effect of growth phase on settleability. Growth curves for *Scenedesmus* and *Chlorella* are shown in Figure 4.6 and 4.7 for Experiment 2. The algae cultures were harvested in similar stages of late exponential growth to mid stationary growth when the settling tests were run, similar to Experiment 1. Higher growth of both cultures differs from Experiment 1 due to a switch in instruments used to measure optical density (Spec 20 to UV Pharma Spec 1700). In addition, the growth of cultures is more clearly defined as compared to Experiment 1.

Figure 4.6-Growth of Scenedesmus over a fourteen-day period, with optical density taken at 625 nm

Figure 4.7-Growth of Chlorella over a fourteen-day period, with optical density taken at 625 nm

4.1.5 Effect of Growth Phase on Settling of *Scenedesmus*: Experiment 2

The settling results of *Scenedesmus* during three different phases of growth are shown in Figure 4.8. The more mature *Scenedesmus* cells showed improved settling (Figure 4.8 A and B). These results match the earlier Experiment 1, reaffirming the conclusion that *Scenedesmus* settles better towards the early to mid stationary phase of growth. Figure 4.11 C explicates, over the entire growth curve, the measured settling efficiency based on normalized cell concentration remaining increased dramatically (from C/C_0 of 0.96 to 0.40), just within a range of four days. Thus, results from settling Experiment 2 with no addition of *B. cepacia* cells or filtrate support the initial finding during Experiment 1 that *Scenedesmus* settles the best towards the middle of stationary growth phase (past 10 days of growth).

Figure 4.8- Settling based on normalized cell concentration remaining of *Scenedesmus* control columns (no cell or filtrate addition) over growth curve (10 day period) for A) a twenty four hour period B) a two hour period and C) compared with growth phase (plotted against optical density at 625 nm) (Experiment 2).

4.1.6 Effect of Growth Phase on Settling of *Chlorella*: Experiment 2

In Experiment 2 *Chlorella*'s settling without *B. cepacia* cell addition was again far better at early stages of growth (Figure 4.9 A, B and C). At mid exponential growth phase it is evidenced that *Chlorella* will settle faster than in late exponential/early stationary phases. Again this result concurs with Experiment 1 data, which suggested that *Chlorella*'s settling was hindered during later stages in the growth curve (i.e. early to mid stationary phases).

Figure 4.9- Settling based on normalized cell concentration remaining of *Chlorella* over growth curve for A) a 24 hour period B) a 2 hour period and C) compared with growth phase (plotted against optical density at 625 nm)

(Experiment 2)

4.1.7 Growth Curves of *Scenedesmus* and *Chlorella*: Experiment 3

A third set of settling experiments was run to further demonstrate the reproducibility of the results of *Scenedesmus* and *Chlorella* settling. A focus of the new experiments was to investigate algae settling during later stages of growth including mid to late stationary phases. Since these growth phases had not been covered completely, longer waiting time between settling tests was instigated to allow for more culture to be available during later stages of growth with minimal disruption of cell concentration. As seen in growth curves for *Scenedesmus* and *Chlorella* below (Figures 4.10 and 4.11), growth was steady without abrupt changes in cell density. From both growth curves it appears that stationary phase is reached by day 12 to 14.

Figure 4.10-Growth Curve of Scenedemus using Optical Density at 625 nm over a fifteen day period

Figure 4.11-Growth Curve of Chlorella using Optical Density at 625 nm over a fourteen day period

4.1.8 Effect of Growth Phase on Settling of *Scenedesmus*: Experiment 3

Settling of *Scenedesmus* was best after 15 days of growth for both 24 and 2 hours of settling (Figure 4.12 A,B,C). Maximum cell removal of up to 70 % was observed at 15 days of growth, which based on the growth curve, Figure 4.10, corresponds inbetween early to mid stationary phase. These results are similar to results seen in previous Experiments (1,2), except that this new experiment extended into longer growth periods.

Figure 4.12-Settling based on normalized cell concentration remaining for the same *Scenedesmus* culture over the growth curve with no bioflocculant addition for A) a twenty four hour settling period B) a two hour settling period and C) a comparison of growth phase (plotted against optical density at 625 nm over the growth curve) (Colors correspond to days of growth on all graphs) (Experiment 3)

4.1.9 Effect of Growth Phase on Settling of *Chlorella*: Experiment 3

For *Chlorella*, normalized cell removal efficiency was again observed in the mid exponential phase of growth (Figures 4.13 A,B, C). This result is in agreement with the previous Experiments (1 and 2) with *Chlorella*. However, cell removal efficiency increased from Day 13 to Day 15 of growth, a result that was not observed in the previous experiments (Figure 4.13 C). Thus, three separate experiments all exhibited significantly better settling of *Chlorella* in exponential growth (mid exponential growth).

Figure 4.13-Settling based on normalized cell concentration remaining for the same *Chlorella* culture over the growth curve with no bioflocculant addition for A) a twenty four hour settling period B) a two hour settling period and C) a comparison of growth phase (plotted against optical density at 625 nm over the growth curve) (Colors correspond to days of growth on all graphs-note color change from *Scenedesmus*) (Experiment 3)

4.1.10 Settling of Older Cultures of *Scenedesmus* and *Chlorella* Without Biofloculants

Settling of the oldest *Scenedesmus* and *Chlorella* samples used in this research were compared to the settling of cultures at mid stationary phase of growth. This analysis was used to investigate whether partially dead cells in the samples help the settling of either algal culture. Older cultures (around 30 days old) of *Scenedesmus* were observed to settle about the same as young cultures, although the older culture settled slightly better in the 2-hour period and slightly worse over the 24 hour period as compared to cultures at mid stationary phase of growth (Figure 4.14 A and B). The older culture of *Chlorella* was very dense (large dry weight) and more difficult to measure cell concentration. The older culture of *Chlorella* settled very slowly as compared to the younger culture, with the largest cell concentration observed after 2 hours of settling ($C/C_0=2.5$). Cells from the top of the column were so slow in reaching the sampling port that after 2 hours of settling the cell concentration at the port was very high (Figure 4.14 B).

Figure 4.14-Settling of Young (mid stationary phase-15 days) versus Old (death phase-30 days)cultures based on normalized cell concentrations remaining of A) *Scenedesmus* B) *Chlorella* and C) Comparing settling between cultures over a 2-hour time period (instead of 24 hours)

4.1.10.1 EPS Correlation With Settling Results of Older Cultures of *Scenedesmus* and *Chlorella* Without Addition of Biofloculants

EPS was measured for the older cultures to determine the relationship, if any, of EPS concentration with settleability for older cultures. Older cultures (past 30 days of growth) produced almost twice the EPS as mature cultures in mid stationary phase (Figure 4.15). For *Scenedesmus*, settling of cultures in the death phase was similar to that in mid stationary phase (Figure 4.14) even though the EPS concentration was much higher in the death phase (Figure 4.15). For *Chlorella*, settling was poor for cultures in the death phase (Figure 4.14) even though the EPS concentration was much higher (Figure 4.15). Clearly, some other factor is affecting settling of the algae cultures in the death phase other than the EPS.

Figure 4.15-EPS productions of old (over 30 days of growth) and young (mid stationary growth phase-15 days old) cultures of *Chlorella* and *Scenedesmus*

4.1.11 Analysis of the Autoflocculation Phenomenon

Autoflocculation was postulated to rely on the presence of three ions in certain concentrations: calcium, magnesium and orthophosphate (Sukenik, 1984). Sukenik (1984) used a culture medium with similar concentrations of calcium, magnesium and orthophosphate ions of 25, 75, and 17.5 mg/L respectively. To examine the autoflocculation phenomenon in this study the composition of the WC medium (Table 4.1) used in these experiments was compared to the solution chemistry of autoflocculation experiments reported in literature. The WC medium used in this study lacked an extra 2.25 mM (250 mg/L) of calcium chloride and 0.6 mM (72.22 mg/L) magnesium sulfate relative to the Sukenik (1984) medium concentrations. In another study, Lavoie used secondary effluent from a wastewater treatment plant as the medium, containing 9.5 mg/L ammonium and 2.2 mg/L of orthophosphate (Lavoie, 1987). Both studies covered autoflocculation concerning two *Scenedesmus* species.

Table 4.1-Composition of WC Media for Autoflocculation Analysis

Component	Concentration (mM)	Concentration (mg/L)
Tris (Buffer)	4.13	500.30
NaNO ₃	1.00	84.99
CaCl ₂ · 2H ₂ O	0.25	36.75
MgSO ₄ · 7H ₂ O	0.15	36.97
NaHCO ₃	0.15	12.60
K ₂ HPO ₄	0.05	6.8

Lavoie (1987) found that natural flocculation with the wastewater medium (much lower concentrations of orthophosphate compared to WC) was possible for this *Scenedesmus* culture up to a pH of 11 and before stationary phase. This was due to the fact that orthophosphate concentrations were used up at the beginning of stationary phase (Lavoie, 1987). This result parallels results obtained with this research, where *Scenedesmus* settling was improved after seven days of growth (in the same range as Lavoie) to achieve up to 50% sedimentation (Figure 4.87). However, as the culture age increased, the orthophosphate in the WC medium was likely used up. This is proved given after 14 days of growth, no settling of *Scenedesmus* was observed at a pH of 11 without *B. cepacia* cell addition (Figure 4.82). This result corresponds with Lavoie's study, where low orthophosphate concentrations in the medium during later *Scenedesmus* growth resulted in poor autoflocculation and settling.

Sukenik's (1984) study also showed that necessary conditions within the culture medium needed to be met prior for autoflocculation to occur. These conditions include a concentration of orthophosphate between 0.1 mM to 0.2 mM and 1.5 mM to 2.5 mM calcium in the medium and a pH at or above a range of 8.5 to 9 (Sukenik, 1984). It was determined that these conditions were met by the *Scenedesmus* culture used in this research early on in the growth phase (when the pH was raised to 11), before orthophosphate concentrations were limiting. Poor settling results for *Chlorella*, on the other hand, indicated that possibly orthophosphate ions were used up faster, providing less of a chance for autoflocculation to occur. However, without measuring the concentrations of these two ions, there was no way of directly proving this hypothesis. Based on the published literature, autoflocculation might be improved during later stages in growth by enrichment of the culture media with additional calcium, magnesium and phosphate. This research proved that during early

stages in growth, enrichment of the culture media was determined not necessary for improved autoflocculation of *Scenedesmus* cultures only.

4.2 Settling of *Scenedesmus* and *Chlorella* with *B. cepacia* Biofloculants

This section focused on measuring the settling based on normalized cell removal of pure *Scenedesmus* and *Chlorella* cultures with *B. cepacia* bacterial biofloculant addition and were covered in Experiments 1, 2 and 3. The effect of biofloculant addition over the growth curve of both algae cultures is discussed in Experiment 1 (Section 4.2.2). Physical factors such as the mixing time were also explored as an important treatment studied throughout Experiment 2 (Section 4.2.3) to find the optimum time for the bacterial and algal cells to adhere together, overcome surface repulsion, and rely on physical forces (Van der waals) of attraction. In addition, physical factors affecting settling such as the dosage of *B. cepacia* cells or filtrate required further investigation in Experiment 3 (Section 4.2.4).

4.2.1 Growth Curves of *B. cepacia* cultures used for Biofloculant Addition

The growth curves of *B. cepacia* cultures used in Experiment 1 are shown in Figures 4.16 and 4.17. It was important to establish the bacterial growth curve because more EPS is reported during stationary growth phase (Pavoni, 1972). Bacterial growth curves are similar for this experiment in that they peak after 100 hours of growth and level off accordingly (signifying the onset of stationary growth phase), although the maximum OD was different.

The *B. cepacia* bacterial growth curve for Experiment 2 is shown in Figure 4.18 and characteristically peaked around 72 hours of growth and into mid stationary phase. *B. cepacia* was harvested during early to mid stationary phases of growth, when EPS production was expected to be optimal. The OD values are smaller in comparison to other growth curves since this experiment used an older spectrophotometer (Spec 20) as

compared to the UV 1700 Pharma Spec spectrophotometer (Shimadzu) used in the other experiments. Growth was also uncharacteristically high at later stages (Figure 4.18).

In previous Experiments (1,2), *B. cepacia* cultures showed not as much growth as desired. Therefore, new techniques were employed to assure better growth, presumably for higher EPS production. New methods included inoculating the bioreactor with an older culture of *B. cepacia* to ensure the cells would be conditioned to whatever new environment they would be placed in. This improved technique resulted in a much higher cell density, as indicated in Figure 4.19. The growth characteristically hit a maximum after 48 to 72 hours and declined steadily until around ten days, where OD increased. This increase in OD could be attributed to a number of possible effects; for example, OD may have been affected by changing cell size. Thus, after 10 days of growth, death phase could have been reached given the OD increased as cells became smaller (due to a lack of nutrients). The resulting smaller cells could have contributed to a greater light scattering effect.

Figure 4.16-Growth curve of *B. cepacia* over a 250-hour growth period using optical density

Figure 4.17-Growth curve of a second batch culture of *B. cepacia* over a 250-hour growth period using optical density

Figure 4.18-Growth of *B. cepacia* over a 250-hour period, with optical density taken at 625 nanometers

Figure 4.19-Growth curve of *B.Cepacia* at 26 °Celsius using optical density at 625 nm over a 350-hr period

4.2.2 Effect of *B. cepacia* Bioflocculant Addition on Settling of *Scenedesmus* and

***Chlorella*: Experiment 1**

Settling of both *Chlorella* and *Scenedesmus* were measured after mixing with *B. cepacia* cells or *B. cepacia* filtrate. The filtrate contained high molecular weight presumed to be dissolved EPS from *B. cepacia*. In this first experiment a fixed mixing time of five minutes was used. Figure 4.20 (A, B) illustrates the same algal culture of *Scenedesmus* as used in previous experiments settled with and without *B. cepacia* bacterial cells and filtrate over 24-hour and 2-hour settling periods. The culture of *Scenedesmus* used was in the late stationary phase (18 days of growth) and the *B. cepacia* was harvested during early stationary phase of growth.

The observed-settling rate of *Scenedesmus* was greater than that observed previously for the controls without *B. cepacia* or filtrate. Addition of *B. cepacia* cells allowed *Scenedesmus* to settle at a higher rate during the course of the 24-hour period as well as the 2-hour period (Figure 4.20). Cell filtrate addition also improved *Scenedesmus* settling, although not as much as cell addition. Thus, both the capsular fraction and dissolved fraction of the *B. cepacia* EPS appears to enhance settling.

Results for *Chlorella* mixed with *B. cepacia* cells and filtrate can be observed in Figure 4.21 (A, B) depicting normalized cell concentration removal over a twenty-four and two hour period, respectively. An older culture of *Chlorella* was used (after 14 days of growth), which resulted in relatively decreased settling for the control without cell or filtrate addition. For *Chlorella* cultures, *B. cepacia* cell addition made settling worse than the control over a 2-hour period. Filtrate addition, on the other hand, significantly improved the settling of the *Chlorella* culture over both the 24- hour and 2-hour period. These results suggest that *B. cepacia* filtrate, with dissolved EPS, enhanced settling. The effect of *B. cepacia*

cells was quite different for *Chlorella* compared to *Scenedesmus*. The *B. cepacia* cells improved the settling of *Scenedesmus* and hindered the settling of *Chlorella*.

Figure 4.20-Comparison of *B. cepacia* cell, filtrate and no cell addition to an 18-day old *Scenedesmus* culture using normalized cell concentration remaining as a function of settling time over A) a 24- hour period and B) a 2-hour period

Figure 4.21-Comparison of *B. cepacia* cell, filtrate and no cell addition to a 14-day old *Chlorella* culture using normalized cell concentration remaining as a function of settling time over A) a 24-hour period and B) a 2-hour period

4.2.3 Effect of *B. cepacia* Bioflocculant Addition on Settling of *Scenedesmus* and

***Chlorella*: Experiment 2: Varying Contact Time**

In addition to repeating Experiment 1, Experiment 2 also was used to investigate the effect of varying contact times between *B. cepacia* cells, filtrate and algae before settling tests were conducted. For experiment 2, three settling tests were run using algae samples collected during days 6 and 8 of growth.

The first settling test involved *Scenedesmus* and *Chlorella* cells along with the addition of *B. cepacia* cells on Day 6 of growth. For *Scenedesmus*, varying contact times with *B. cepacia* cells of 5 and 15 minutes were used. Contact times for *Chlorella* with *B. cepacia* cells were 5, 15, and 30 minutes. All algae and bacterial cells were mixed uniformly during the various contact times using stir plates at 750 rpm and then added immediately to the settling columns.

This second settling test, like the first, involved addition of *B. cepacia* cells at different contact times on Day 8 of growth. In this test, *Scenedesmus* and *B. cepacia* were mixed at 5, 15, and 30-minutes. *Chlorella* and *B. cepacia* cells were mixed at 5 and 15-minutes

The third settling test, for *Scenedesmus*, focused on settling with *B. cepacia* filtrate at 15 and 30-minute contact times on Day 10 of growth. *Chlorella* was mixed with *B. cepacia* filtrate at 5, 15 and 30 minute contact times. It is important to note that the third settling test contacted the algae with *B. cepacia* filtrate, not cells.

The effects of *B. Cepacia* cells on the settling of *Scenedesmus* with different contact times are shown in Figures 4.22 (A, B), and Figure 4.23 (A, B). Figure 4.22 (A, B) is for *Scenedesmus* cells after 6 days of growth at two contact times of 5 and 15 minutes with *B. cepacia*, while Figure 4.23 (A, B) is for *Scenedesmus* cells after 8 days of growth with three

different contact times (5, 15 and 30 minutes). These results are summarized in Figure 4.24. For the 6-day-old *Scenedesmus* culture, the addition of *B. cepacia* cells increased two hour settling significantly (Figure 4.22 B) similar to that observed in Experiment 1. However, at least with a 5-minute mixing time, the 24-hour settling was actually hindered by *B. cepacia* cells (Figure 4.22A). *B. cepacia* cells also improved the settling for the 8-day-old *Scenedesmus* culture, but the effect was much smaller (Figure 4.24).

For the 6-day-old *Scenedesmus* culture, the effect of increasing the mixing time was small (Figure 4.24). Figure 4.22 (A, B) shows that the *B. cepacia* cells work the best to settle the *Scenedesmus* at a contact time of around 15 minutes for both settling time periods. For the 8-day-old culture, longer contact times increased settling, where up to 30 minutes was best (Figure 4.24). However, as compared to the 6-day-old culture the overall settling efficiencies were low (Figure 4.24). The effect of *B. cepacia cells* addition was more pronounced for *Scenedesmus* harvested at 6 days, when settling without *B. cepacia* were observed to be poor. These results suggest that a longer contact time of around 30 minutes, at a growth phase closer to early stationary, coupled with the addition of *B. cepacia* cells might have a positive effect on the settling of *Scenedesmus*.

The effect of adding *B. cepacia* filtrate on the settling rate of *Scenedesmus* after 10 days of growth is shown in Figure 4.25 (A and B). In this case, filtrate did not improve the settling rate of *Scenedesmus*. (Figure 4.25 A, B). In fact, both the 15 and 30-minute contact times made the settling between 0 and 2 hours worse compared to no filtrate added at all. After 24 hours of settling, the filtrate had little to no effect on the settling rate of *Scenedesmus* (Figure 4.25 A).

Figure 4.22-Effect of *B. cepacia* cell addition on settling of *Scenedesmus* using normalized cell concentrations remaining after 6 days of growth over A) a 24-hour settling period and B) a 2-hour settling period

Figure 4.23-Effect of *B. cepacia* cell addition on settling of *Scenedesmus* using normalized cell concentrations remaining after 8 days of growth over a A) 24-hour settling period and B) over a 2-hour settling period

Figure 4.24-Effect of *B. cepacia* cell addition and contact time on the measured settling of *Scenedesmus* based on normalized cell concentration remaining after 6 and 8 days of growth over a 2-hour settling period. The legend denotes contact time between cells (Experiment 2)

Figure 4.25- Settling of *Scenedesmus* using normalized cell concentrations remaining with and without *B. cepacia* filtrate addition after 10 days of growth over a A) 24-hour settling period and B) 2- hour settling period

The effect of *B. cepacia* cell addition on settling of *Chlorella* is shown in Figures 4.26 and 4.27 at various mixing times. For *Chlorella*, the effect of adding of *B. cepacia* cells was very different for *Chlorella* cultures harvested at 6 days compared to 8 days (Figure 4.28). For the 8-day-old *Chlorella* culture, *B. cepacia* cells improved settling, while 6-day-old *Chlorella* cultures actually settled worse with *B. cepacia* cells (Figure 4.28). Negative results were also observed for *Chlorella* in Experiment 1. In general, longer mixing times tended to accentuate the *B. cepacia* cell effect, whether positive or negative.

Figure 4.29 (A, B) shows the settling of *Chlorella* with and without *B. cepacia* filtrate added. Filtrate added at a contact time of five minutes (shorter contact time) had the most beneficial effect on settling over both a 2 and a 24- hour settling period. This result agrees with earlier results from Experiment 1 that filtrate addition has a beneficial impact on the settling of *Chlorella*. This result also concurs with the effect of mixing time observed with *B. cepacia* cell addition (shorter mixing time has a better effect). The extent of improved settling at this mixing time of five minutes, however, was more pronounced for *Chlorella* with the addition of *B. cepacia* filtrate. The greatest cell removal was observed with filtrate addition after ten days of growth with a contact time of five minutes, removing about 75% of cells within two hours, and up to 99% of algal cells within 24 hours of settling.

Figure 4.26-Effect of *B. cepacia* cell addition on settling of *Chlorella* using normalized cell concentrations remaining after 6 days of growth over a A) 24-hour period and B) 2-hour settling period

Figure 4.27-Effect of *B. cepacia* cell addition on settling of *Chlorella* using normalized cell concentrations remaining after 8 days of growth over a A) 24-hour settling period and B) 2-hour settling period

Figure 4.28-Effect of *B. cepacia* cell addition and contact time on settling of *Chlorella* based on normalized cell concentration remaining after 6 and 8 days of growth over a 2-hour settling period. The legend denotes contact time between cells (Experiment 2)

Figure 4.29-Settling of Chlorella based on normalized cell concentrations remaining with and without *B. cepacia* filtrate addition after 10 days of growth over a A) 24-hour settling period and B) 2-hour settling period.

4.2.4 Effect of *B. cepacia* Bioflocculant Addition on Settling of *Scenedesmus* and

***Chlorella*: Experiment 3: Varying Dosage of Bioflocculants**

In Experiment 3, bioflocculation experiments with *B. cepacia* cells and filtrate were reported, this time using different doses of bacterial cells and filtrate. In prior experiments, a volumetric ratio of 100 mL of bacterial cell suspension was added to 200 mL of each algal culture prior to settling (1:2). The volumetric ratio of bacterial filtrate was also generally 1:2, but sometimes 3:5. In Experiment 3, the ratio of *B. cepacia* cells to *Chlorella* was decreased, as was the ratio of *B. cepacia* filtrate to *Scenedesmus*. The new ratio was set by halving the existing *B. cepacia* input to 50 mL and increasing the *Scenedesmus* to 250 mL for a ratio of 1:5. A ratio of 100 mL of *B. cepacia* filtrate to 300 mL *Scenedesmus* (1:3) was also used.

Addition of *B. cepacia* cells increased the settling of *Scenedesmus* significantly for all stages in the growth curve (Figure 4.30). Figure 4.28 details the results summarized in Figure 4.30 for cell addition and is included in the Appendix section as a reference. The effect was particularly dramatic when the *Scenedesmus* culture reached 15 consecutive days of growth (Figure 4.30). In fact, the removal efficiency increased by a factor of 7 with *B. cepacia* cell addition at this later stage in algal growth (Figure 4.30).

With *B. cepacia* filtrate addition, the rate of *Scenedesmus* settling also increased with increasing algal growth as seen in Figure 4.30. Figure A.2 (Appendix) details the results summarized in Figure 4.30 for filtrate addition and is included in the Appendix section as a reference. However, the settling rate did not improve as

drastically with filtrate addition as it did with whole cell addition. Filtrate addition helped the 15 day old culture the least, suggesting that *B. cepacia* requires some form of capsular EPS in later stages of growth to improve settling (Figure 4.30). Again, detailed Figures (A.1, A.3, A.4 and A.5) are available in the Appendix section that illustrate both the 2 and 24 hour settling of cell and filtrate addition over the growth curve for *Scenedesmus*.

The effect of reducing the dose of *B. cepacia* filtrate by about one half on settling of *Scenedesmus* can also be seen in Figure 4.30, which show the results of a settling test performed on *Scenedesmus* during the middle stage of stationary algal growth. Reducing the dose of *B. cepacia* filtrate actually improved settling for *Scenedesmus* over a 2-hour period (not 24) as compared to full strength filtrate addition.

Figure 4.30- Comparison of settling based on normalized cell concentration remaining over a two hour settling period for a *Scenedesmus* culture with no *B. cepacia* addition, cell addition, and filtrate addition, and ½ filtrate addition over 15 days of growth (Colors refer to columns in the legend) (Experiment 3)

For *Chlorella*, *B. cepacia* cell addition in Experiment 3 during earlier stages of algal growth resulted in better normalized cell removal, but only when a half dose of cells was used (Figure 4.31). After 15 days of *Chlorella* growth, *B. cepacia* had no effect on settling (Figure 4.31). Similar results were observed in Experiment 2 in which *B. cepacia* cells enhanced settling of a 6-day-old *Chlorella* culture with a short mixing time. Again, detailed Figures (A.6, A.7 and A.8) are available in the Appendix section that illustrate both the 2 and 24 hour settling of cell addition over the growth curve for *Chlorella*. Also, in Experiment 2, less effect of *B. cepacia* cells was observed for the 8-day-old *Chlorella* compared to the 6-day-old *Chlorella*, but *Chlorella* cells were not used after 8 days of growth (only filtrate during 10 days). The additional growth time employed in Experiment 3 showed the continuing trend of less effect of *B. cepacia* cells later in the growth curve of *Chlorella*.

B. cepacia filtrate addition greatly improved settling of *Chlorella* early in growth (late exponential stages) (Figure 4.31). Detailed Figures (A.10, A.11 and A.12) are available in the Appendix section that illustrate both the 2 and 24 hour settling of filtrate addition over the growth curve for *Chlorella*. This result proves that *B. cepacia* filtrate has the most prominent effect on improving the settling of *Chlorella* as compared to cell addition or no cell addition at all (agreeing with previous results from Experiment 1 and 2). After 15 days of growth, however, settling of pure algal cells was not affected by filtrate (Figure 4.31). Thus, filtrate addition has little effect on *Chlorella* in later growth phases (mid stationary).

B. cepacia cell and filtrate additions had reverse trends for *Scenedesmus* and *Chlorella*. For *Chlorella*, *B. cepacia* cells and filtrate had a greater effect for early growth phases (mid exponential), while for *Scenedesmus* this effect was greater for later growth phases (up to mid stationary).

Figure 4.31-Settling of *Chlorella* based on normalized cell concentrations remaining after a 2-hour settling period compared over 6, 13, and 15 days of growth for no *B. cepacia* cell addition, with *B. cepacia* cell addition, with *B. cepacia* filtrate addition, and with *B. cepacia* ½ cell addition (Experiment 3)

4.2.5 Results of EPS Analysis and Correlation with Algal Settleability: Experiment 2

Both the bacterial EPS and algal exudates (polymeric in nature) were quantified to determine if EPS played a role in improving bioflocculation. Quantifying EPS was achieved through a standard filter and dialysis method as described in the Methods and Materials chapter. Samples were taken every other day during growth of all bacteria and algae cultures to analyze polymeric exudates. Filtrate measurements of dissolved EPS were also taken when settling tests were run with filtrate addition. When settling tests were run, samples of all cells (bacterial and algal) were ran in order to allow correlation of settling with EPS measurements

EPS production by *Scenedesmus* was sporadic and reached an apparent peak EPS concentration above 20 mg/L at 8 days during mid exponential phase of growth (Figure 4.32). *Chlorella*, contrarily, produced more EPS later on in its growth curve, around early stationary phase (Figure 4.33). At 10 days of growth, *Chlorella* produces more EPS than *Scenedesmus*, around 25 mg/L. *B. cepacia* produces most of its EPS around 10 days of growth or middle to late stationary growth phase at a concentration similar to *Chlorella* around 25 mg/L (Figure 4.34).

At 10 days of growth, EPS production of *Scenedesmus* was moderate to high (Figure 4.32) and this corresponds to when better settling was observed for *Scenedesmus* (Figure 4.30). Thus, *Scenedesmus* exhibits improved settling during later stages of growth when its own EPS production is high. Addition of *B. cepacia* filtrate at this same time of growth (10 days) hindered settling of *Scenedesmus* cells. At this day in growth (10), dissolved EPS of the filtrate was around 25 mg/L (Figure 4.34). Thus, *Scenedesmus* does not benefit from the increased dissolved EPS from *B. cepacia* at later stages in growth. During later stages of growth, when EPS production of *Scenedesmus* is larger, capsular EPS, rather than dissolved EPS from *B. cepacia* filtrate, seems to aid in the settling of *Scenedesmus* the most.

For *Chlorella*, EPS concentration increased to early stationary phase (Figure 4.33). The best settling without *B. cepacia* addition was observed at eight days of growth, when EPS production was not at a maximum. Settleability also decreased into mid stationary phase where EPS production was maximum. Therefore, the settling of *Chlorella* is less affected by the production of its own EPS. However, after 10 days of growth (where settling is judged poor) *Chlorella* settled better with addition of *B. cepacia* filtrate. Therefore, dissolved EPS (from bacteria in later stages of growth) aids in the settling of *Chlorella*

vulgaris, with a maximum effect during early stationary phase. These tests are repeated in Experiment 3 to assess the repeatability of these initial EPS correlations with settleability.

Figure 4.32-Dissolved EPS concentration of *Scenedesmus* plotted against its growth curve measured until 12 days of growth. Green arrows represent days in which settling tests were run (Experiment 2)

Figure 4.33-Dissolved EPS concentration of *Chlorella* over a 12-day growth period plotted against the growth curve measured at 625 nanometers by optical density. Green arrows represent days when settling tests were run (Experiment 2)

Figure 4.34-Dissolved EPS concentration of *B. Cepacia* over a 12-day growth period plotted against the growth curve measured at 625 nanometers by optical density. Green arrows represent days when settling tests were run (Experiment 2)

4.2.6 Results of EPS Analysis and Correlation with Algal Settleability : Experiment 3

EPS concentrations produced over the growth curves of *Scenedesmus*, *Chlorella* and *B. cepacia* are presented in Figures 4.35 through 4.37. The dry weight is included for each of the algal cultures for comparison. The EPS concentrations fluctuated but an increasing trend was observed in EPS production of *Scenedesmus*, *Chlorella* and *B. cepacia* (Figures 4.35-4.37). Settling of *Scenedesmus* after 15 days (stationary phase) of growth was confirmed to be much better than earlier growth phases. This result suggests that during mid stationary phase of growth, settling of *Scenedesmus* without *B. cepacia* addition is influenced greatly by its own dissolved EPS (Figure 4.35). This result concurs with the previous Experiment 2 results.

B. cepacia cell addition in later growth stages for *Scenedesmus* improved the settling rate of *Scenedesmus* to almost twice that with no cells. Dissolved EPS in the *B. cepacia* filtrate, added after 15 days of growth did not significantly improve the settling rate of *Scenedesmus* as compared to cell addition alone. For this reason, it is assumed that

Scenedesmus benefits more at later stages in growth from *B. cepacia* capsular EPS, and not dissolved EPS. From results in the previous section, *B. cepacia* filtrate addition (dissolved EPS) has more of an effect on settling during earlier stages of growth (Figure 4.30), where *Scenedesmus* dissolved EPS production is at a minimum (Figure 4.35 below). Current results also illustrate that when *Scenedesmus* produced smaller amounts of EPS, filtrate helped in improving settling, especially earlier on in growth, just not to the same extent.

EPS correlations with *Chlorella* settling were found much different than that for *Scenedesmus*. Even though dissolved EPS production by *Chlorella* was at a maximum after 13 days of growth, settling was poor. Settling of *Chlorella* were highest early on in the growth curve, when EPS concentrations were low. Thus, the settling rate of *Chlorella* does not appear to be affected by its own dissolved EPS production. This result agrees with previous Experiment 2 results.

Chlorella demonstrated improved settleability with filtrate addition during the mid exponential growth phase (Figure 4.31). The effect of *B. cepacia* filtrate on settling is not as pronounced during early to mid stationary phase of growth when *Chlorella* is observed to be affected by a higher amount of its own EPS (Figure 4.31). These results again concur with Experiment 2 results that *Chlorella* was more affected by filtrate in mid exponential phase, when production of its own dissolved EPS was at a minimum. In addition, the settling of *Chlorella* was improved greatly with one half the cell addition during early stationary phase of growth (Figure 4.31). This corresponded to about 6 mg/L of dissolved EPS from *B. cepacia* and 12 mg/L EPS of dissolved produced by *Chlorella* itself (Figure 4.36). This result also shows that at later stages in growth, *Chlorella* improves settling with less dissolved EPS and may be more affected by *B. cepacia* capsular EPS (from cell addition).

Figure 4.35-EPS measurements compared to dry weight observed over a 15 day growth period for *Scenedesmus* (Green arrows indicate when settling tests were run) (Experiment 3)

Figure 4.36-EPS measurements compared to dry weight observed over a 15 day growth period for *Chlorella* (Green arrows indicate when settling tests were run) (Experiment 3)

Figure 4.37-EPS measurements compared to optical density observed over a 15 day growth period for *B. cepacia* (Green arrows indicate when settling tests were run)

4.3 Settling of Mixtures of Algal Pond Water and Primary Treated Wastewater With RAS Treatment

4.3.1 Effect of RAS Addition on Settling of Mixtures of Algal Pondwater and Primary Treated Wastewater: Experiment 1

The first experiment conducted on wastewater samples from the San Luis Obispo wastewater treatment facility focused on a fixed RAS ratio addition, where samples were either aerated or not. The wastewater samples included a defined volume of algal pond water blended with primary wastewater. RAS addition was also varied to determine the optimal dose to increase algal settleability. Mixture compositions were calculated as algal pond water: food (primary wastewater) and food: RAS ratios. Table 4.2 shows the different combinations used in this experiment. A defined ratio of about 4:1 for *algal pond water* to *primary wastewater (food)* was used for each of the four samples. The difference between

both samples 1a and 2a compared to 1 and 2 was that 1a and 2a were aerated for a specified period of time with RAS addition.

Table 4.2-Defined Volumes of Wastewater samples used compared to RAS addition for comparison of settling in this study

Sample	Volume of Pond water (mL)	Volume of Primary Wastewater (mL)	Volume of RAS (mL)	Total Volume (mL)	Aeration
1	800	200	0	1000	None
2	728	182	91	1000	None
1a	800	200	0	1000	Aerated
2a	728	182	91	1000	Aerated

Samples 1 and 1a did not have any RAS addition. RAS addition was set at 10% of the total volume for *both* samples 2 and 2a. For samples 2 and 2a with RAS addition, the defined ratio of primary wastewater (food) to RAS (bacteria) was 2:1.

Without aeration, little or no settling was observed with or without RAS addition (Figure 4.38 A). RAS addition slightly improved the settling of these non-aerated samples (Figure 4.38 A). Aeration improved settling significantly (Figure 4.38 B). With aeration, settling was initially improved slightly with RAS addition over the first half hour, but after two hours of settling, the solids settled better without RAS addition (Figure 4.38 B).

Figure 4.38- Effect of adding a certain fixed dose of RAS to A) an non-aerated mixture of raw primary municipal wastewater samples and algae pond water from the San Luis Obispo Wastewater Treatment Facility over a 2-hour settling period and B) an aerated sample with and without RAS treatment over a settling period of 2 hours

4.3.2 Effect of RAS Addition on Settling of Mixtures of Algal Pond Water and Primary Treated Wastewater: Experiment 2

For RAS experiment 2, samples were collected in February and all samples aerated prior to performing settling rate experiments. The ratio of RAS to algal pond water to primary wastewater for each sample is shown in Table 4.3. The amount of RAS in samples with RAS addition was fixed as in Experiment 1, at around 10% of the total volume. Dilution with primary wastewater resulted in algal pond water (greater) to RAS volume ratios of 1:1, 1:2 and 2:1. A pure algal pond water sample was also tested as collected from the SLO wastewater treatment plant with no primary wastewater addition.

Table 4.3: Classification of samples used for analysis with and without RAS addition

Primary Wastewater: Algal Pond Water	Volume of Algal Pond water (mL)	Volume of Primary Wastewater (mL)	Volume of RAS (mL)	Total Volume (mL)
2:1	300	600	0	900
1:1	500	500	0	1000
1:2	600	300	0	900
2:1 W/RAS	300	600	100	1000
1:1 W/RAS	450	450	100	1000
1:2 W/RAS	600	300	100	1000

The cell concentrations measured included all countable bacterial and algal cells. The algae treatment pond during this period was characterized by mostly algal species of *Chlorella* and *Ankistrostrum*, with very little *Scenedesmus* present.

Normalized settling over 24 hours, with and without RAS addition, is presented in Figure 4.39 and the 2 hour settling efficiencies are summarized in Figure 4.40. For algae pond water alone, settling was mediocre, with about 30% of cells removed after 2 hours (Figure 4.40). When algae pond water was mixed with primary wastewater (without RAS addition), the 2 hour settling efficiencies either got better or worse, depending on the ratios used (Figure 4.40). Without RAS addition, little or no settling was observed for the 2:1 and 1:2 primary wastewater to algae pond water samples (33% algae and 66% algae, respectively) at 2 hours (Figure 4.40), but nearly 60% solids removal was observed for the 1:1 sample with 50% algae. However, after 24 hours, all three mixtures settled with over 80% removal of cells (Figure 4.39).

RAS addition dramatically improved the settling of the 1:2 and 2:1 wastewater to algae pond samples at 2 hours, but actually made settling worse for the 1:1 samples (Figure 4.40). Similarly, after 24 hours of settling, RAS addition improved the settling of the 2:1 ratio, but made settling worse for the 1:1 ratio (Figure 4.39). At 24 hours, RAS had no observable effect on settling of the 1:2 ratio (Figure 4.39).

Figure 4.39-Normalized cell concentration as a function of settling time over a 24-hour period for different wastewater pond cultures with or without RAS addition at A) 2:1 ratio B) 1:1 ratio and C) 1:2 ratio of primary wastewater to algae pond water (Red line signifies no RAS addition, blue line signifies RAS addition)

Figure 4.40-Comparison of settling based normalized cell concentration remaining after a 2-hour settling period for samples with RAS/Aeration and without addition, and with varying volumetric ratios of primary wastewater to algae pond water

4.3.3 Correlation of EPS with RAS addition to Wastewater Samples: Experiment 2

Measured EPS concentrations were similar for all samples, including pure algae pond water (Figure 4.41). The 1:1 mixture without RAS addition had slightly more dissolved EPS than the sample with RAS addition itself and this could possibly explain why settling was better without RAS addition for this ratio. For other the 1:2 and 2:1 ratios of primary wastewater and algae pond water, samples with RAS had slightly higher dissolved EPS concentrations and for these ratios RAS addition helped settling. Therefore, it appears that increased EPS aids in settling, but it is difficult to make this conclusion from these data because large variations in EPS concentration were not observed.

Figure 4.41- Dissolved EPS measurements for samples with and without RAS addition or aeration, also including a sample of pure algal pond water

4.4 Effect of Light and Dark Cycles During Growth and Light and Dark Settling Conditions on the Settling of *Scenedesmus* and *Chlorella*

This section focused on improving the settling of *Scenedesmus* and *Chlorella* by first changing the light and dark exposure periods during growth (light/dark cycles). Light intensity (brightness) was not varied, but kept constant throughout growth. Normal light/dark cycles during growth consisted of an 8-hour illumination period followed by a 16-hour dark period. The following experiments, for both *Scenedesmus* and *Chlorella*, focused on changing the light (illumination) period to 48 hours of constant intensity prior to settling tests, with the elimination of a dark period. After this exposure, the algae were either settled in the light or in the dark without *B. cepacia* biofloculant addition (Section 4.43, 4.44), or with *B. cepacia*

biofloculant addition (Section 4.45, 4.46) to improve settling. During settling, dark conditions simply implied that the settling columns were covered to prevent light exposure, and light conditions implied the columns were not covered. Also, a control experiment with algae in mid stationary growth phase was conducted to compare settling trends in the light and dark without a change in light exposure during growth (Section 4.41). In addition, EPS measurements were made during growth to observe the effects of changing the light and dark cycle on EPS production and settling of algal cultures (Section 4.47).

4.4.1 Effect of Light and Dark Settling Conditions on Algal Settleability: Preliminary Analysis

A preliminary analysis of settling in light and dark environments was conducted. Two refrigerated samples of both algae species in the mid stationary phases of growth were taken, mixed for five minutes at 750 rpm and settled in either the light or dark. *Scenedesmus* cultures settled much better in the dark than in the light over 2 hours of settling (Figure 4.42 B). After 24 hours, settling was efficient regardless of light exposure during settling (Figure 4.42 A). For *Chlorella*, settling was similar in the dark and in the light (Figure 4.43 A,B), but settling was slightly better in the light for both settling times (2 and 24 hours).

The effect of light on settling of *Scenedesmus* and *Chlorella* is summarized in Figure 4.44. Here it is clear that light had the opposite effect on the two algae species with *Scenedesmus* settling best in the dark and *Chlorella* cells settling slightly better in the light.

Figure 4.42-Normalized cell concentration remaining as a function of settling time for *Scenedesmus* settling in the light or dark over a A) 24-hour settling period and B) a 2-hour period

Figure 4.43-Normalized cell concentration remaining as a function of settling time for *Chlorella* settling in the light or dark over a A) 24-hour settling period and B) a 2- hour period

Figure 4.44-Comparison of settling over a 2-hour period for both *Scenedesmus* and *Chlorella* cultures settled in the light/dark

4.4.2 Effect of Light and Dark Cycles During Growth and Light and Dark Settling Conditions on the Settling of *Scenedesmus* and *Chlorella*: Growth Curves

Scenedesmus settling tests during these experiments were conducted with two cultures of similar densities. For both cultures throughout growth, illumination periods before settling tests were changed to 48 hours with no dark periods on 6, 11, and 18 days of growth. Settling was then run in either the light or in the dark and with or without biofloculant addition. The second culture was a replicate of the first to compare settling results. *Chlorella* followed a different procedure where only two experiments were run both after 8 and 15 days of growth, but with the same light/dark cycles used above. A replicate culture was not used due to time constraints, but should be considered for future experimentation.

Growth curves for the two cultures of *Scenedesmus*, *Chlorella* and bacteria used in the light/dark experiments are shown in Figures 4.45, 4.46, 4.47, 4.48, and 4. 49. Growth for both cultures of *Scenedesmus* reached a maximum after eighteen days, but *Scenedesmus* culture 2 was a little denser (Figures 4.45, 4.46). *Chlorella*'s growth was consistent even

though light and dark cycles were differed (Figure 4.47). *B. cepacia* cell growth was similar for both cultures, reaching optical density levels above 1 within 5 days (Figures 4.48 and 4.49).

Figure 4.45-Growth Curve of Sc. 1 culture used in light/dark testing

Figure 4.46-Growth Curve of Sc. 2 culture used in light/dark testing

Figure 4.47-Growth Curve of Chlorella culture used in light/dark settling (Chl 1.1)

Figure 4.48-Growth Curve of B. cepacia 1 culture used in light/dark settling

Figure 4.49-Growth Curve of *B. cepacia* culture 2 used in light/dark settling tests

4.4.3 Effect of Light Intensity and Light/Dark Cycles During Growth on Settling of *Scenedesmus* Without *B. cepacia* Biofloculant Addition

For *Scenedesmus*, cultures harvested at 6 days, there was no significant effect of increased light exposure during growth or light exposure during settling (uncovered) on the average 2-hour settling efficiency between the two cultures (4.50). For cultures with the same increased exposure to light but settling in dark conditions (covered), better settling was observed earlier on in growth (Figure 4.50). In later stages (11 and 15 days), *Scenedesmus* cells settled better when they were exposed to additional light during growth and when they were settled uncovered.

These results were different than those observed in Section 4.4.1, which showed that *Scenedesmus* cells without additional light exposure settled better in mid stationary phase of growth (15 days) in dark (covered) conditions. Therefore, increasing the exposure to a constant light intensity with different light/dark cycles during growth affects the way

algae settle when exposed to either light or dark conditions of settling. It appears increasing the exposure to a constant light intensity helps *Scenedesmus* the most during early stages of growth, where previous settling was observed to be poor. In addition, settling in the dark after this additional light intensity may further improve the settling of *Scenedesmus* in earlier stages of growth.

Figure 4.50-Comparison of settling based on normalized cell concentration remaining for an average of *Scenedesmus* cultures (1 and 2) over 18 days of growth under light and dark conditions of settling over the growth curve with no bioflocculant addition. Before each test, each culture was exposed to 48 hours of constant light intensity and then settled in the light (not covered) or dark (covered). For Day 6, replicates were run and the error bars indicate the differences in results

4.4.4 Effect of Light Intensity and Light/Dark Settling Conditions on Settling of *Chlorella* Without *B. cepacia* Bioflocculant Addition

Overall, with additional light exposure, *Chlorella* settled better in early growth for both cultures settling in the light and in the dark (Figure 4.51). As compared to settling in the dark, extra light had little or no effect to improve settling in the light (uncovered) during early growth (8 days) (Figure 4.51). After 15 days of growth, settling was much lower for *Chlorella* settling in both the light and the dark exposed to additional light and settling in the

light and dark (Figure 4.51). Therefore, dark-settling conditions (covered) combined with an increased light exposure (up to 48 hours) improved the settling of *Chlorella* during later stages of growth (mid stationary phase) when settling was previously judged poor. Again, like *Scenedesmus*, results were opposite compared to the *Chlorella* culture in mid-stationary phase that was not exposed to an increased amount of light prior to settling (Section 4.4.1). This culture was observed to settle better in the light as compared to the dark during later stages of growth. Thus, increasing exposure to a constant light intensity appears to have an effect on the settling of both *Scenedesmus* and *Chlorella* during stages of growth where settling was previously inadequate for harvesting of algae cells.

Figure 4.51-Comparison of settling based on normalized cell concentration remaining for *Chlorella* over the growth curve of 18 days. Before both tests, light exposure was increased to 48 hours and settling took place in either the light or the dark (covered or uncovered)

4.4.5 Effect of Light and Dark Cycles and Light and Dark Settling Conditions on Settling of *Scenedesmus* With *B. cepacia* Biofloculant Addition

Early in the growth phase of *Scenedesmus* (6 days) without *B. cepacia* biofloculant addition, there was an increased settling effect of extra light during growth either for cultures settling in the light or dark (Figure 4.52). As compared to normal light/dark cycles after 8 days of growth for *Scenedesmus* (Figure 4.24), settling was most improved without biofloculant addition with an increase in exposure to light and settling in the dark. However, when *B. cepacia* cells are added as a biofloculant, *Scenedesmus* cells appear to settle much better in the dark and with extra light as compared to without biofloculant addition (Figure 4.52).

For mid stages of growth (early stationary phase of 11 days), *Scenedesmus* with *B. cepacia* biofloculant settled better in the dark with added light during growth, but the effect was less than that observed after 6 days of growth (Figure 4.52). In addition, the higher mixing intensity (DMI), as indicated by Figure 4.52, improved settling for algal cells in the dark with extra light addition during early stationary phase of growth. After 18 days of growth (mid stationary phase), cell removal of *Scenedesmus* is still improved by cell addition with extra light under dark settling conditions, but the effect is still diminished compared to earlier stages of growth (Figure 4.52). The different (higher) mixing intensity also made settling worse later on in growth (Figure 4.52).

These results demonstrate that the settling of *Scenedesmus* are improved by adding *B. cepacia* cells as a biofloculant, particularly under dark conditions of settling, when algal cells have been treated with extra light during growth (up to 48 hours). Moreover, this effect is diminished for longer periods of growth.

Figure 4.52-Comparison of settling for *Scenedesmus* with and without *B. cepacia* cell addition and constant light exposure of 48 hours before settling in the light or dark (covered or uncovered) over 18 days of growth. Error bars indicate samples that were replicated, with the average normalized cell concentration remaining after 2 hours shown

***DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 5 minutes followed by a 100-rpm flocculating period for the remaining 25 minutes before settling in the dark**

4.4.6 Effect of Light Intensity and Light/Dark Conditions on Settling of *Chlorella* With *B. cepacia* Biofloculant Addition

Addition of *B. cepacia* cells as a biofloculant improved the settling of *Chlorella* under light and dark settling conditions during early growth (Figure 4.53). This effect was slightly better under dark conditions. Filtrate additions under light and dark conditions did not improve settling of *Chlorella* under any conditions. Thus, for both *Scenedesmus* and *Chlorella* settling under dark conditions with additional light was improved early on in growth with *B. cepacia* cell addition, not filtrate. For 15 day-old *Chlorella* cultures (mid stationary phase of growth), the best settling in the dark and the light was observed without cell or filtrate addition. These results, even with an increase in light exposure, were consistent with previous results in Figure 4.31, where at 15 days in growth (mid

stationary), bioflocculants from *B. cepacia* did not help improve settling to a significant extent.

Figure 4.53-Comparison of settling based on normalized cell concentration remaining over a 2-hour settling period for *Chlorella* with and without *B. cepacia* biofloculant additions, with one sample mixed under a different intensity, using both light and dark conditions and over a 15-day growth period. The biofloculant addition is based on the order of the legend. All cultures were exposed to a 48-hour period of constant light intensity prior to settling

** DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 2 minutes followed by a 100-rpm flocculating period for the remaining 3 minutes before settling in the dark*

4.4.7 EPS Correlation Between Varying Light Intensity and Light/Dark Settling Conditions on Improved Settleability of *Scenedesmus* and *Chlorella*

EPS concentrations were measured in the light/dark experiments to see if EPS production was affected by prolonged exposure to a constant light intensity and to see if bacterial cell and filtrate addition increased the EPS to improve settling. EPS concentrations over the growth curve for both *Scenedesmus* cultures were similar, peaking later in mid stationary growth around 12 mg/L (Figure 4.54). This concentration of dissolved EPS was similar to that observed previously for growth at shorter exposure periods to light (different light/dark cycles). This suggests that varying exposure periods to a constant light

intensity does not have an effect on EPS production. Similarly, *Chlorella* showed no signs of increased EPS production due to changes in exposure to a constant light intensity (Figure 4.57).

Increased growth of *B. cepacia* cultures further increased dissolved EPS concentrations to values much higher than the typical values seen in previous experiments in this study (Figure 4.55 A and B). Figure 4.56 shows that *B. cepacia* 1 cells had the highest EPS production and increased over the growth curve while *B. cepacia* 2 cells had a more variable EPS production. The differences in EPS by the bacterial cultures may have resulted in differences in settling between both of the *Scenedesmus* cultures observed with larger error bars in Figure 4.52. For instance, during settling tests in later stages of growth of *Scenedesmus*, *B. cepacia* 1 had twice the dissolved EPS than the other culture. Also, dissolved EPS between the two cultures of *Scenedesmus* was similar. Thus, more of an error was observed in settling efficiencies with *B. cepacia* added to *Scenedesmus* cells in the light (Figure 4.52) than any other time throughout growth. This suggests that differences in EPS between the bacterial cultures resulted in improved settling of one culture of *Scenedesmus* over the other.

Figure 4.54-EPS concentrations plotted against dry weight expressed as TSS with A) Sc. 1 culture and B) Sc. 2 culture

Figure 4.56-Summary of EPS concentrations (Figure 4.75-4.76) plotted over 18 days of growth for both Scenedesmus and B. cepacia cultures used during settling tests. Error bars indicate replicates used with different cultures of bacteria and algae. Samples were taken from each culture separately before settling tests were run

Figure 4.57-Summary of EPS concentrations plotted over the growth curve for Chlorella and B. cepacia cells and filtrate used during settling tests. Samples were taken from each culture separately before settling tests were run

4.5. Varying pH to Improve Settleability of *Scenedesmus* and *Chlorella*

The pH during settling was varied from 3, to 7, to 11 without bioflocculant addition (Sections 4.5.2, 4.5.3) and with bioflocculant addition (Sections 4.5.4, 4.5.5) to test the effect of pH on settling of *Scenedesmus* and *Chlorella*. In these experiments, *Scenedesmus* cultures were treated with *B. cepacia* cells as compared to filtrate given that settling was improved to a greater extent in earlier experiments (1,2,3) with *B. cepacia* cell addition. Likewise, *Chlorella* cells were treated with *B. cepacia* filtrate as opposed to cells due to similar results in previous experiments (1,2,3). Dissolved EPS from both algae cultures and the bacterial bioflocculants used was measured and correlated with settling to further determine if there was an optimal pH for bioflocculant addition.

4.5.1 Growth Curves of *B. cepacia*, *Scenedesmus* and *Chlorella* used in pH experiments

Two cultures of *Scenedesmus* were grown for these two experiments with similar growth curves (Figure 4.58). Only one *Chlorella* culture was used, with the growth curve shown in Figure 4.59. Three cultures of bacteria were used over the course of this testing and their respective growth curves can be seen in Figure 4.60. Growth was not as great as observed in earlier experiments (light and dark experiments) for the *B. cepacia* cultures. The figures also show when the cultures were used for different pH settling tests (included in the captions).

Figure 4.58-Growth Curve of A) *Scenedesmus 1.1* used in pH testing and B) *Scenedesmus 1.2* used in pH testing

Figure 4.59-Growth Curve for Chlorella culture used in pH testing (Chl 2.1)

Figure 4.60-Growth curve of A) *B. cepacia* used with *Scenedesmus* settling test # 1 for pH variations, B) *B. cepacia* used in *Chlorella* settling test #1 and *Scenedesmus* settling test # 2 for pH variations, and C) *B. cepacia* used in *Chlorella* settling test # 2 for pH variations

4.5.2 Effect of Growth Phase on the Settleability of *Scenedesmus* with Varying pH

Lowering the pH to 3 without *B. cepacia* cell addition greatly improved the settleability of *Scenedesmus* early in the growth curve (5 days) (Figure 4.61). Increasing the pH to 11 only slightly enhanced settling of *Scenedesmus* (Figure 4.61). All cultures did not settle well at 14 days of growth regardless of pH (Figure 4.61). This poor settling late in growth was different than that observed in earlier experiments. At earlier stages in growth, the pH effect may be due to *Scenedesmus* reaching an isoelectric point where all charge is neutralized. As the culture increased in density, this effect was observed to diminish.

Figure 4.61-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period with variation in pH for *Scenedesmus* without cell addition over a growth period of 14 days

4.5.3 Effect of Growth Phase on the Settleability of *Chlorella* with Varying pH

Without *B. cepacia* filtrate addition, pH had a consistently strong influence on settling of *Chlorella*, with the best settling observed at a pH of 3 (Figure 4.62). This was observed for both 24 and 2-hour settling periods (A.25, A.26, 4.62). At a pH of 11, *Chlorella* settling was poor at both stages of growth (Figure 4.62). Again, results for *Chlorella* were

different than those for *Scenedesmus*. Lowering the pH to 3 improved settling during later growth stages for *Chlorella* as compared to early growth stages for *Scenedesmus*.

Figure 4.62-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period with variations in pH for *Chlorella* without cell addition over a growth period of 14 days

4.5.4 Effect of *B. cepacia* Cell Addition on the Settleability of *Scenedesmus* with Varying pH

During earlier stages in *Scenedesmus* growth, *B. cepacia* cell addition slightly improved settling at all three pH values tested, where a pH of three improved settling the most (Figure 4.63 A). Cell addition at pH 11 using a higher mixing intensity did not improve the settling of *Scenedesmus* early in growth (Figure 4.63 A).

For a later growth stage (14 days), *B. cepacia* cell addition improved settling only at a pH of 3 and 11, and had a negative effect at pH 7 (Figure 4.63 B). At a pH of 11, use of a higher mixing intensity markedly improved the settling of *Scenedesmus* at a later growth stage (Figure 4.63 B). At a pH of 3, a greater effect of cell addition on settling was observed as compared to early growth (Figure 4.63 B).

Figure 4.63-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period for *Scenedesmus* with *B. cepacia* cell addition over a wide range in pH during A) Early growth (5 days) and B) Late growth (14 days)

*** DMI (different mixing intensity)-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 5 minutes followed by a 100-rpm flocculating period for the 25 minutes before settling**

4.5.5 Effect of *B. cepacia* Filtrate Addition on the Settleability of *Chlorella* with Varying pH

During earlier stages of growth (6 days), filtrate addition only minimally improved settling of *Chlorella* at all pH ranges (Figure 4.64 A). Later in the *Chlorella* growth curve (14 days), a different trend in effect of filtrate on settling of *Chlorella* was observed. Filtrate addition at a pH of 11 improved the settleability of *Chlorella* from 20% cell removal without filtrate to 40% removal with filtrate addition (Figure 4.64 B). Filtrate addition at a pH of 7 also improved settling of *Chlorella* during later stages of growth, but not to the same degree (30% removal). Filtrate addition at pH 3 actually made settling worse during this later stage of growth. Increasing the mixing intensity with filtrate addition for a pH of 11 had no impact on settling earlier on in growth, but in later stages of growth the higher mixing intensity made settling worse (Figure 4.64 B).

Figure 4.64-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period for *Chlorella* over a varied pH range with and without *B. cepacia* filtrate addition during A) Early growth (6 days) and B) Late growth (14 days)

*** DMI (different mixing intensity)-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 2 minutes followed by a 100-rpm flocculating period for the remaining 3 minutes before settling**

4.5.6 EPS Correlation with Settleability of *Scenedesmus* and *Chlorella* at Varied pH

The dissolved EPS produced by the *Scenedesmus* and *B. cepacia* cells used for settling tests appears to increase for *B. cepacia* and to drop slightly for *Scenedesmus* from 5 to 14 days (Figure 4.65). Settling of *Scenedesmus* without biofloculant addition was improved at early stages of growth, when dissolved EPS production was slightly higher. *B. cepacia* cell addition had a greater effect on improving settling later on in growth, and at a higher pH and mixing intensity (Figure 4.63). This shows that larger concentrations of dissolved EPS from *B. cepacia* improved *Scenedesmus* settling during later stages of growth. As compared to a neutral pH, a pH of 3 and 11 combined with the larger concentration of dissolved EPS from *B. cepacia* had some effect in improving the settling of *Scenedesmus* during later growth stages (Figure 4.63, 4.65).

For the *Chlorella* experiments with *B. cepacia* filtrate, both the dissolved EPS of *Chlorella* and the dissolved EPS of the *B. cepacia* filtrate were higher during later phases of growth (Figure 4.66). *Chlorella* settling without filtrate was better at 6 days of growth than at 14 days of growth (Figure 4.64). Since EPS from *Chlorella* was greater at 14 days than at 6 days (Figure 4.66), this suggests that the increased EPS produced by *Chlorella* did not improve its own flocculation or settling. However, *B. cepacia* filtrate addition was much more effective to improve settling for the 14-day culture (at least at pH 7 and 11) (Figure 4.64) when EPS concentration in the filtrate was higher (Figure 4.66). Therefore these preliminary results suggest that dissolved EPS from *B. cepacia* is a more effective biofloculant than that of *Chlorella*, especially at higher pH ranges and later stages of growth.

Figure 4.65-Comparison of EPS production for settling tests involving pH changes of both Scenedesmus and B. cepacia cells over 14 days of growth

Figure 4.66-Comparison of EPS production for settling tests involving pH changes of both Chlorella and B. cepacia cells and filtrate over 14 days of growth

4.6 Addition of Nutrient Starved *B. cepacia* Cultures to Improve the Settleability of *Scenedesmus* and *Chlorella*

Nutrient levels during growth of the *B. cepacia* cultures were reduced to determine if starved *B. cepacia* cells further improve bioflocculation. In these experiments, the nitrogen and phosphorus in the growth medium were reduced by a factor of ten. The nutrient levels of all algal cultures were not changed as to only observe the effects of nutrient limitation on the bacterial (bioflocculant) cultures. In the literature, nutrient limitation has been shown to increase bacterial biopolymer production and to enhance bioflocculation with algae (Benemann and Oswald, 1996). Thus, algal settling was measured with addition of starved and not starved bacterial cultures and correlated with EPS production.

4.6.1 Growth Curves of *B. cepacia*, *Scenedesmus*, and *Chlorella*

Two cultures of *Scenedesmus* were used for these settling experiments. Growth of these two cultures differed in that growth of Culture 2 was faster than growth of Culture 1 (Figure 4.67). The growth of the *Chlorella* culture used showed similar behavior to previous growth (Figure 4.68). Growth increased slowly into late stationary phase around 20 days of growth, which is characteristic of previously grown *Chlorella* cultures.

Growth of the *B. cepacia* bacterial cultures used for addition to *Scenedesmus* cells is shown in Figure 4.69. Starved cultures grew much better than the non-starved cultures as the starved cultures reach a peak at an optical density of 0.3 as compared to about 0.18. Different *B. cepacia* cultures were grown for the *Chlorella* experiments. As compared to the bacterial cultures used for *Scenedesmus*, growth of the non-starved culture seemed much better than the starved culture used (Figure 4.70). However, data for the nutrient starved culture of *B. cepacia* used in *Chlorella* settling tests was missing between 4 to 8 days of growth, which may suggest that growth was possibly the same or higher than the non-

starved culture. Also, data for the non-starved culture is missing past Day 7, making it difficult to compare the growth of nutrient starved and non-starved cultures growth in later stages.

Figure 4.67-Growth curve results for Scenedesmus Cultures 1 and 2 used in bacterial nutrient starved settling tests

Figure 4.68 -Growth curve results for the Chlorella culture used in bacterial nutrient starved settling tests

Figure 4.69-Growth curve results for nutrient starved and non-starved *B. cepacia* cultures used for *Scenedesmus* settling tests

Figure 4.70-Growth curve results for nutrient starved and non-starved *B. cepacia* cultures used for *Chlorella* settling tests

4.6.2 Effect of Starved *B. cepacia* Cell addition on the Settleability of *Scenedesmus*

Two *Scenedesmus* cultures were studied for this investigation to provide replicates of the data obtained and error bars in Figure 4.71 indicate which tests were replicated. At 6 days of growth, the non-starved and starved *B. cepacia* cells both improved settling of *Scenedesmus* to the same extent (Figure 4.71). At 15 days of growth, the non-starved cells again improved settling of *Scenedesmus*, but the starved cells did not (Figure 4.71). These results suggest that reducing the Nitrogen and Phosphorus nutrient levels did not provide improved settling. However, these preliminary experiments were conducted only with an arbitrary reduction in Nitrogen and Phosphorus, and it is not known if the cultures were truly nutrient limited (i.e. nutrient levels in the medium were not tested). In addition, the different (higher) mixing intensity used did not affect settling of *Scenedesmus* for the 6-day cultures or the 15-day cultures (Figure 4.71).

Figure 4.71-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period between the addition of starved cells, non starved cells, no cell addition, and starved filtrate addition for *Scenedesmus* 2 over 15 days of growth. Error bars indicate which tests were replicated

*** DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for five minutes followed by a 100-rpm flocculating period for the remaining twenty five minutes before settling**

4.6.3. Effect of Nutrient Starved *B. cepacia* Filtrate Addition on the Settleability of *Chlorella*

For *Chlorella*, the effect of filtrate from starved and non-starved *B. cepacia* cultures on settling was tested. Replicates were made for starved and non-starved bacterial filtrate addition, as well as settling of *Chlorella* without biofloculant addition. Early on in growth, the addition of starved *B. cepacia* filtrate improved settling of *Chlorella* as compared to non-starved filtrate, but this settling was no better than *Chlorella* without any filtrate (Figure 4.72 A). Using ½ the dosage of filtrate improved *Chlorella* settling the most early on in growth, with starved cultures helping the most (Figure 4.72 A). However, since a different mixing intensity was used for the ½ filtrate with starved cells, this cannot be directly compared. Non-starved cell addition did not have an effect on *Chlorella* settling during early growth, and starved cell addition actually made settling worse than no cell addition (Figure 4.72 A). Using a different mixing intensity early in growth increased settling of *Chlorella* with starved cells and decreased settling with non-starved cells, but neither of these settling results was better than *Chlorella* settling alone (Figure 4.72 A).

Later in growth, filtrate additions had little effect on *Chlorella* settling using either starved or non-starved *B. cepacia* cultures (Figure 4.72 B). *Chlorella* settled slightly better with non-starved *B. cepacia* filtrate than with starved *B. cepacia* filtrate (Figure 4.72 B). Again, later in growth, halving the filtrate dosage improved settling of *Chlorella* compared to a full dose, but the settling was still no better than the control with no filtrate addition (Figure 4.72 B). Using a different mixing intensity with non-starved filtrate greatly improved the settling of *Chlorella* during later stages of growth as compared to starved filtrate addition at this same mixing intensity. Finally, similar to early growth, starved and non-starved cell addition had little or no effect on *Chlorella* settling later in growth (Figure 4.72 B).

Figure 4.72-Comparison of settling of *Chlorella* with and without bacterial bioflocculant cell/filtrate addition (starved for nutrients and not-starved for nutrients) based on normalized cell concentration remaining after a 2-hour settling period for *Chlorella* during A) Early growth (10-12 days) and B) Later growth (21-23). Error bars indicate which settling tests were replicated

*** DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 2 minutes followed by a 100-rpm flocculating period for the remaining three minutes before settling**

4.6.4 EPS Production by Nutrient-Starved *B. cepacia* cells, *Chlorella*, and *Scenedesmus* and Correlation with Improved Settling

Starved bacterial cells resulted in a higher production of EPS as compared to non-starved bacterial cells, especially at the beginning of the growth curve (Figures 4.73). This result may be due to the lack of nutrients encountered by the starved cells after around 12 to 15 days of growth. The highest EPS production was observed around 12 days of growth (Figures 4.73). The second highest peak was observed after 48 to 72 hours or once the bacteria encountered early stationary phase of growth (Figure 4.73). Overall, the nutrient starved cells had a higher average EPS production of 9.5 mg/L ($\pm$ 4.4 mg/L) as compared to 5.5 mg/L ($\pm$ 3.1 mg/L) for the non-starved cells.

The amount of EPS produced is about the same for each *Scenedesmus* culture on each day of growth (Figures 4.74 and 4.75). Even though EPS production values were similar, measured dry weight values of the second *Scenedesmus* culture show that it was denser, reaching about 700 mg/L during mid to late stationary phase of growth (as compared to 400 for *Scenedesmus* 1). Thus, it appears EPS production was not affected by culture density for *Scenedesmus*.

Scenedesmus settling increased across the growth curve without cell addition (Figure 4.71), which agrees with earlier observations. EPS production for *Scenedesmus* was higher later in growth, suggesting that settling was improved by its own dissolved EPS. Results also show that EPS concentrations of *Scenedesmus* were not significant early on in growth, which further suggest that *Scenedesmus* may favor the production of its own dissolved EPS to improve settling (Figure 4.75). This result confirms the observations from Experiments 2 and 3.

According to Figure 4.71, nutrient starved cells either had no effect or made *Scenedesmus* settling worse compared to non-starved cell addition. Even though, on average, nutrient starved *B. cepacia* cells produced more EPS, early in growth there was no improvement in *Scenedesmus* settling with starved *B. cepacia* versus non-starved *B. cepacia* cells. Moreover, during the later growth phase (15 days), settling of *Scenedesmus* was better with non-starved cell addition. Further experiments would be required with more nutrient conditions to completely understand the role of nutrient starvation on EPS production and its effect on algal settling.

EPS production of *Chlorella* increased throughout growth, peaking in late stationary phase for both trials (Figure 4.76). EPS concentrations in filtrate from starved and non-starved *B. cepacia* cells were similar-both in early and late growth (Figure 4.76). Relative to non-starved filtrate, starved filtrate additions improved *Chlorella* settling in early growth (10-12 days), but not during late growth (21-23 days) (Figure 4.72). Since EPS production was not very different for the starved and non-starved cells, differences in EPS production are not likely to be the cause of the different effects of bacterial cell starvation.

Figure 4.73-EPS production of A) nutrient starved *B. cepacia* and B) non-starved *B. cepacia* with optical density measurements over the growth curve (green arrows indicate when settling tests were run)

Figure 4.74-EPS production of A) *Scenedesmus* Culture 1 and B) *Scenedesmus* Culture 2 with dry weight measurements over the growth curve (green arrows indicate when settling tests were run)

Figure 4.75-Summary of EPS concentrations (Figure 4.117-4.120) plotted over 15 days of growth for both *Scenedesmus* and *B. cepacia* cultures used during settling tests. Samples were taken from each culture separately before settling tests were run. Error bars indicate differences in EPS production of both *Scenedesmus* cultures used

Figure 4.76-Summary of EPS concentrations of *Chlorella* and *B. cepacia* cultures used in settling tests for early stationary growth of *Chlorella* (10-12 days) and later stationary growth of *Chlorella* (21-23 days). Samples were taken from each culture separately before settling tests were run. Error bars indicate replicates of EPS measurements for both settling experiments

4.7 Effect of Carbon Dioxide Addition on the Settleability of *Scenedesmus* and *Chlorella*

Studies covered in the background section suggest that carbon dioxide supplementation in high-rate algal wastewater ponds helped increase the settling efficiency of the dominant algal species present in the wastewater (Craggs, 2011). Past studies at Cal Poly, San Luis Obispo have also indicated that wastewater algal species, when supplemented with concentrations of carbon dioxide below 5% (v/v) exhibit increased growth and culture density (Lundquist, 2010). To explore the effect of carbon dioxide on algal settling, this part of the research measured settling efficiencies of both *Scenedesmus* and *Chlorella* cultures with and without carbon dioxide supplementation (without biofloculant addition) (Section 4.72, 4.73). In addition, the effect of *B. cepacia* biofloculant addition (filtrate and cells) on increasing the settleability was studied using cultures of algae grown with and without the addition of carbon dioxide (Section 4.74, 4.75). Finally, the correlation between EPS production, improved algal settling and carbon dioxide augmentation was studied (Section 4.76).

4.7.1 Growth Curves of *B. cepacia*, *Scenedesmus* and *Chlorella*

Settling of *Scenedesmus* and *Chlorella* for a control culture grown on air (without carbon dioxide addition), and cultures grown with 5% carbon dioxide mixed with air during an 8-hour illuminated period constantly. During this period, the pH was maintained above 7. Just before settling tests, the amount of carbon dioxide input was increased as follows. The first settling test for both *Scenedesmus* and *Chlorella* (during early stationary growth) involved a four-hour constant addition of carbon dioxide prior to settling. The second settling tests used a larger supplementation (24 to 48 hours) to possibly induce further flocculation and enhance settleability.

Scenedesmus cultures with and without carbon dioxide supplementation reached about the same density during all growth phases (Figure 4.77). *Chlorella* growth was also similar with and without carbon dioxide (Figure 4.78).

Five *B. cepacia* cultures were grown to provide cells and filtrate for biofloculation experiments as seen in the figures below (Figure 4.79-83). Both 4.79 and 4.80 were used for settling tests with control cultures of *Chlorella* (no carbon dioxide addition), where Figure 4.79 culture is not starved. All of the bacterial cultures used were free from contamination and exhibited similar trends in growth to previous results. In addition, all 5 cultures reached an optical density of about 0.2, indicating minimal growth. For bacterial cultures in 4.80 to 4.83, missing data between days 4 and 8 accounts for the abnormal representation of growth.

Figure 4.77-Effect of carbon dioxide on growth for *Scenedesmus* cultures

Figure 4.78-Effect of carbon dioxide on growth for *Chlorella vulgaris* cultures

Figure 4.79-Growth of non-starved *B. cepacia* culture for use in *Chlorella* settling tests without carbon dioxide addition

Figure 4.80-Growth of starved *B. cepacia* culture for use in *Chlorella* settling tests without carbon dioxide addition

Figure 4.81-Growth of *B. cepacia* culture for use in *Scenedesmus* settling tests without carbon dioxide addition

Figure 4.82-Growth of *B. cepacia* culture for use in *Scenedesmus* and *Chlorella* settling tests with carbon dioxide addition

Figure 4.83-Growth of *B. cepacia* culture for use in *Scenedesmus* and *Chlorella* settling tests with carbon dioxide addition

4.7.2. Effect of Carbon Dioxide Addition on the Settleability of *Scenedesmus*

Scenedesmus settled slightly better without carbon dioxide addition in both early and late growth phases (Figure 4.84). Thus, throughout growth, 2 hour settling based on normalized cell concentrations show that carbon dioxide input has no effect on improving the settleability of *Scenedesmus* (Figure 4.84).

Figure 4.84-Comparison of settling based on normalized cell concentration after a 2-hour settling period for *Scenedesmus* with and without carbon dioxide addition from early (10-11 days) to late stationary phases (24-27 days) of growth

4.7.3 Effect of Carbon Dioxide Addition on the Settleability of *Chlorella*

Chlorella settled much better without carbon dioxide addition in early and late phases of growth (Figure 4.85). *Chlorella* also settled better in early stages of growth, which corresponds with previous results in Experiments 1,2 and 3 (Figure 4.85). 24- hour period settling results are similar to the 2-hour settling results, where cultures without carbon dioxide addition settled better (Figures A.40, A.41).

Figure 4.85-Comparison of settling based on normalized cell concentration after a 2-hour settling period for *Chlorella* with and without carbon dioxide addition from early stationary (10-11 days) to late stationary (23-32 days) phases of growth

4.7.4 Effect of Carbon Dioxide Addition and *B. cepacia* Cell Addition on the Settleability of *Scenedesmus*

For cultures treated with carbon dioxide, *B. cepacia* cell addition improved settling slightly during early stationary and late stationary phases of growth (Figure 4.86 A, B). Adding ½ the dose of *B. cepacia* cells provided no improvement in settling, and in fact resulted in very poor settling in some cases (Figure 4.86). For the control culture of *Scenedesmus* without supplemented carbon dioxide, settling was improved by *B. cepacia* cell addition during late growth phases, but cells had no effect early in the growth curve (Figure 4.86 B). During later growth, cell addition had the same effect on improved settling between cultures with and without carbon dioxide (Figure 4.86 B). Diluting the *Scenedesmus* cultures only had a significant effect for cultures with carbon dioxide during late stationary growth (Figure 4.86 B).

Cell addition with a different (higher) mixing intensity also improved settling dramatically during early stages of growth for cultures without carbon dioxide addition; however, changing the mixing intensity did not improve settling when carbon dioxide was supplemented (Figure 4.86). Changing the mixing intensity for both doses (1/2 and full) had more of an effect on cultures without carbon dioxide early on in growth as compared to cultures with carbon dioxide (Figure 4.86 A). This effect decreased into late stationary phase for both cultures (Figure 4.86 B).

Figure 4.86-Comparison of settling based on normalized cell concentration remaining after a 2-hour settling period for *Scenedesmus* with and without carbon dioxide addition, with and without *B. cepacia* cell addition at various doses and mixing intensities from A) Early growth (11-12 days) to B) Late growth (24 to 27 days)

*** DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 5 minutes followed by a 100-rpm flocculating period for the remaining 25 minutes before settling**

4.7.5 Effect of Carbon Dioxide Addition and *B. cepacia* Filtrate Addition on the Settleability of *Chlorella*

Without carbon dioxide addition, *B. cepacia* filtrate addition had little or no effect on the settling of *Chlorella* (Figure 4.87 A, B). However, with carbon dioxide supplementation, filtrate addition improved settling in late stationary phase of growth (Figure 4.87 B). One half the dose of filtrate for both cultures was better during early growth, especially for cultures without carbon dioxide addition (Figure 4.87 A, B). Diluting algal cultures seemed to have a greater effect on cultures with carbon dioxide addition as compared to without, especially during early growth (Figure 4.87 A, B). In addition, similar to *Scenedesmus*, using a different (higher) mixing intensity had a greater effect on improved settling for cultures of *Chlorella* without carbon dioxide addition as compared to with carbon dioxide addition (Figure 4.87 A, B).

Figure 4.87-Comparing settling based on normalized cell concentration remaining after a 2-hour settling period for *Chlorella* with and without carbon dioxide addition, with different doses of filtrate, mixing intensities, and diluted samples of *Chlorella* (1/2) during A) Early growth (10-11 days) and B) Late growth (23-32 days)

*** DMI-Indicates the cell addition had a 1,000 rpm initial mixing intensity for 2 minutes followed by a 100-rpm flocculating period for the remaining 3 minutes before settling**

4.7.6 Correlation of EPS with Carbon Dioxide Addition on the Settleability of *Scenedesmus* and *Chlorella*

Scenedesmus cultures produced slightly more dissolved EPS when not supplemented with carbon dioxide for early and late growth phases (Figure 4.88). Increased carbon dioxide supplementation prior to settling tests both for 4 hours and 48 hours did not increase EPS production by *Scenedesmus*. Carbon dioxide supplemented cultures of *Scenedesmus* also did not settle as well without biofloculant addition (Figure 4.84), indicating that less EPS produced by the carbon dioxide supplemented cultures decreased settling. Therefore, excess carbon dioxide did not improve the settling of *Scenedesmus* since less dissolved EPS was produced.

For *Chlorella*, dissolved EPS production was much lower with carbon dioxide supplementation (Figure 4.89). In addition, *Chlorella* cultures with carbon dioxide supplementation in general settled poorly as compared to cultures without carbon dioxide during both growth phases (Figure 4.87). Thus, comparing both cultures (with and without carbon dioxide supplementation) *Chlorella*'s settling was shown to improve with more of its own dissolved EPS at each growth stage. However, like previous results, settling was best during early growth phases when its dissolved EPS production was smallest. Adding *B. cepacia* filtrate to both cultures did not significantly improve settling during both growth phases (Figure 4.87). This was similar to previous results with starved/non-starved filtrate, where *Chlorella* settling was not improved by a larger concentration of dissolved EPS present in the *B. cepacia* filtrate.

Figure 4.88-EPS concentration of *Scenedesmus* and *B. cepacia* cells used in settling tests over A) early stationary phase of growth (10-11 days) and B) late stationary phase of growth (24-27 days). (W or W/Out CO₂ for *B. cepacia* corresponds to EPS production of bacterial cells used with either algal culture during settling tests)

Figure 4.89-EPS concentration of *Chlorella* and *B. cepacia* cells used in settling tests over A) early stationary phase of growth (10-11 days) and B) late stationary phase of growth (23-32 days). (W or W/Out CO₂ for *B. cepacia* corresponds to EPS production of bacterial cells or filtrate used with either algal culture during settling tests)

Chapter 5: Conclusions

One of the most important observations in this research was that different species of algae, i.e., *Scenedesmus* and *Chlorella vulgaris*, exhibit different settling characteristics and that their settling performances are influenced very differently by biofloculants such as bacterial cells and dissolved EPS. *Scenedesmus* settled faster in later growth stages, with the best settling observed in stationary phase (14 to 18 days). In contrast, *Chlorella* settling was less affected by growth phase, with slightly better settling observed in early stages of growth.

Algal EPS production increased with increasing growth for both algal species. Since *Scenedesmus* settling was greater for mature cultures with more growth and more EPS produced, this suggests that *Scenedesmus* settling is enhanced by its own EPS. In contrast, observed settling of *Chlorella* did not increase in later stages of growth when *Chlorella* EPS production was greater.

Two types of external biofloculants were tested: Bacterial cell suspensions of *B. cepacia*, and dissolved EPS produced by *B. cepacia*, which was introduced as a filtrate of the bacterial culture. The cell suspension included both the capsular and dissolved components of *B. cepacia* EPS. *Scenedesmus* settling was enhanced significantly by the cell suspension, and to a lesser degree by dissolved EPS in the filtrate. The higher the measured EPS concentration of the added cell suspension, the greater the enhancement of settling of *Scenedesmus*, especially in early growth phases when EPS production by *Scenedesmus* itself was low. In comparison, *Chlorella* settling was improved only by dissolved bacterial EPS in the filtrate, but this effect was variable, depending on the growth phase of the *Chlorella*.

Filtrate addition had the most impact on settling of *Chlorella* during early stationary phase, when settling of cells alone was poor.

In some cases lower concentrations of added *B. cepacia* cells or filtrate improved settling relative to the higher concentrations originally used. *Scenedesmus* showed better settling with a reduction of *B. cepacia* filtrate concentration (not cell dose) dosage, especially during mid stationary phase of growth. Comparably, *Chlorella* demonstrated better settling with a lower *B. cepacia* cell dosage (not filtrate) that was most effective early on in growth (mid exponential to late exponential phase).

Longer mixing times of algae and biofloculants improved settling of *Scenedesmus*. Once again, *Chlorella* settling behavior was the opposite of *Scenedesmus*, as better settling was observed at shorter mixing times. *Scenedesmus* cells settled best with a rapid mix followed by a gentle flocculation period, especially in early growth stages. *Chlorella* also settled better with this mixing scenario, but this mixing was more effective during later stages of growth (mid stationary).

Light/dark cycles also affected algal settling. *Scenedesmus* settled best in a dark environment, while *Chlorella* settled better with more light. Providing the algae cultures with 30-48 hours of continuous light exposure prior to settling tests affected each culture differently. *Scenedesmus* settled better after this added light exposure under dark conditions during early stages of growth (6 days) and under the light during later stages of growth (mid stationary). The extended period of light exposure during the growth of *Chlorella* improved settling for *Chlorella* early in early growth stages with and without cell addition using dark settling conditions. The extended period of light exposure did not change the EPS production of either algal culture.

Lowering the pH to three improved settling for both algae cultures without *B. cepacia* cell or filtrate addition in early growth stages. This result was similar to conclusions reported by Pavoni (1974) and Tenney (1969), showing that algae reach an isoelectric point (around pH of 3) where surface charge is reduced and where flocculation was improved significantly with synthetic cationic polymers. The effect of *B. cepacia* cell and filtrate addition, similar to the cationic polymer addition, was considerably greater when the pH was lowered to 3 for both algae cultures for *Scenedesmus* in early growth stages and for *Chlorella* throughout its growth curve.

Increasing the pH to 11 also improved settling efficiencies (up to 50%) of *Scenedesmus* during early stages in growth but had little or no effect on the settling rate of *Chlorella*. The mechanism for autoflocculation has been postulated as the formation of a net positively charged chemical precipitant that bridges with the negatively charged algal surface due to the presence of specific concentrations of ions (namely calcium, magnesium and phosphate) in the medium at a higher pH (Sukenik, 1984). When pH was higher due to increased photosynthesis of algal cultures, orthophosphate becomes deprotonated and precipitates with calcium ions at concentrations of 0.1 to 0.2 mM orthophosphate and 1.5 mM-2.5 mM calcium (Sukenik, 1984). This same effect was observed using *Scenedesmus* cultures grown on wastewater which early on in growth showed signs of autoflocculation (greater than 50%) at low orthophosphate levels of around 30-40 μ M at a pH of 11 to 12 (Lavoie, 1987). *Scenedesmus* exhibited this phenomenon early in growth since it had the required concentrations of ions in the medium (specifically orthophosphate) to induce autoflocculation according to the WC media recipe and Lavoie's result. Autoflocculation was not induced in *Chlorella*, since it was assumed to use up more orthophosphate during earlier stages of growth than what was required to create optimum conditions for this condition to occur.

For *Scenedesmus* cultures with *B. cepacia* cell addition at a higher pH, settling was improved during later stages of growth, especially with a different mixing intensity. Notably, *Chlorella* did not respond the same way, favoring a pH of 3 with *B. cepacia* filtrate addition over the growth curve.

Starving the *B. cepacia* cultures of nutrients resulted in a higher yield of EPS, and this improved bioflocculation of the *Scenedesmus* cultures. *Chlorella* settled better in earlier phases with starved *B. cepacia* filtrate and later with non-starved filtrate, showing settling was more affected by the amount of dissolved EPS added by the bioflocculant.

Carbon dioxide addition during algal growth increased the cell density of *Scenedesmus* significantly, but did not affect the growth of *Chlorella*. Carbon dioxide supplementation (up to 24 hours continuously before settling) resulted in poor settling for *Chlorella* cultures. Carbon dioxide addition also hindered settling of *Scenedesmus* for 2 hours of settling, but 24-hour settling results were dramatically improved (up to 99%). Carbon dioxide supplementation did not increase EPS production by either species of algae.

For a comparison of these pure-culture lab results to real-world applications, settling tests were conducted for samples from an algae-based treatment pond mixed with primary wastewater, with and without RAS addition. The algae pond water had a large amount of dissolved EPS, and during this sampling *Chlorella* was the dominant species. Without RAS additions, the cultures settled very poorly, but RAS: algae ratios of 1:3 or 1:6 improved settling. A ratio of algae pond water to primary wastewater of 1:1 with no RAS addition as well as the settling of the pure algal pond water demonstrated improved settling, suggesting that dissolved EPS in the pond wastewater improved algal settling.

RAS is known to contain highly bound capsular EPS and loosely bound dissolved components (Li, 2007). The loosely bound component was shown to negatively affect floc

formation and settleability as compared to the capsular portion of EPS in RAS (Li, 2007). EPS analysis of the algae pond water used in this study showed that the loosely bound component was not as prevalent as the capsular component of EPS.

From lab scale study, *Chlorella* are more affected by dissolved EPS (similar to loosely bound components in RAS) in a more concentrated dosage (1:2 ratio of biofloculant to algae). *Chlorella* have also been observed to improve settling with small dosages of *B. cepacia* cell suspensions, suggesting they are more sensitive to capsular bound EPS components of RAS. Thus, increasing the ratio of RAS to algae to 1:6 improves settling since *Chlorella* are less exposed to this tightly bound component, and are more likely to be influenced by the loosely bound, dissolved portion of RAS.

This was a different conclusion than results from the study by Su (2011) that showed an increase in sludge ratio to algae exhibited better settling (5:1 instead of 1:5 was better). This study also concluded that a 1:1 ratio settled well, which was not observed by the above results. Differences may be due to the algae species present and the RAS composition used (microbial community), which were not presented. Therefore, a feasible explanation and insight exists correlating both the lab experiments and wastewater field experiments on evaluating how one individual species may interact with another species in a complex community of microorganisms.

The first experiment with wastewater field samples also demonstrated the concept of HRT, when *Scenedesmus* species were dominant. In this experiment, RAS addition helped settling without aeration. *Scenedesmus* species characteristically do not settle well in early stages of growth. Therefore, improved settling could be achieved by possibly lengthening the HRT with RAS addition, and keeping the dose at or around 1:2 or 1:3 for RAS to algae (this dose was proven optimal for *B. cepacia* cell addition with *Scenedesmus*). Similar

improved settling results could be achieved with *Chlorella* by shortening HRT, thereby keeping *Chlorella* in a growth condition favorable for settling to occur.

The observation in this study that a bioflocculant can improve the settling of *Chlorella* and *Scenedesmus* species agrees with previous results indicating that bioflocculant addition was effective (Oh, 2001, Kim, 2011). For optimization of bioflocculation, these studies included small concentrations of co-flocculant such as calcium chloride or ferric chloride were required in addition to the bioflocculant. However, these studies were focused on commercial harvesting methods for biofuels and not specifically tailored for use in wastewater treatment. Effects of EPS production by bacteria on settling in wastewater applications has not yet been reported in the literature. The present study also examined the effects of physical factors on settling, such as mixing intensity, dosage, light/dark cycles, carbon dioxide addition, and nutrient starvation for improving the bioflocculation, which have not been widely reported. The results presented here should be useful for future wastewater applications in terms of the harvesting process.

For this research, reproducible results were achieved with the first three experiments. Based on these experiments, general trends in settling for both *Chlorella* and *Scenedesmus* were observed to be consistent. At times, for *Scenedesmus*, the normalized values exceeded one, which was thought to be due to resuspension. This increase in normalized concentration was due, however, to slower settling of algal cells, where more cells from the top of the column had reached the sampling port. A representation of the entire year's research is presented in the appendix compilation section. Repeatability of the second part of the results (focused on environmental conditions) may need to be revisited in future studies. Since this was a "screening" study, emphasis was not placed on achieving

reproducibility, and instead on studying the largest amount of environmental and physical effects on settling within the given time frame.

The most promising environmental conditions with bioflocculant addition to improve the harvesting of *Scenedesmus* and *Chlorella* achieved in this study were reviewed. It appears for *Scenedesmus*, much improved settling was observed with *B. cepacia* bioflocculant addition at a higher pH during later growth, especially with an increased mixing intensity. In addition, dark settling conditions were shown to improve the settling of *Scenedesmus* cultures during later stages of growth without bioflocculant addition. Increasing the exposure to light intensity followed by dark settling conditions further increased settling of *Scenedesmus* with bioflocculant addition. This effect was enhanced using a higher mixing intensity with bioflocculant addition. Lastly, adding bioflocculant (*B. cepacia* cells) followed by a higher mixing intensity at a neutral pH was shown to produce consistent improved settling of *Scenedesmus* cultures during early growth stages.

For *Chlorella*, improved settling at a higher pH with bioflocculant addition during later growth stages was observed, but not at a higher mixing intensity. Similarly, during later growth, *B. cepacia* filtrate addition improved settling of carbon dioxide supplemented *Chlorella* cultures to a greater extent than observed previously. Starved cultures of *B. cepacia* (filtrate) also had a greater impact on improving *Chlorella* settling during early growth and with a different mixing intensity. Finally, *B. cepacia* cell addition to cultures of *Chlorella* experiencing prolonged light exposure improved settling to a greater extent in the dark (during early growth).

Future studies would benefit from more accurate ways to quantify capsular EPS of pure algal cultures as well as RAS, such as EPS extraction methods described by Liu and Fang (2010). This reference concluded that a technique for extraction involving a

combination of formaldehyde and sodium hydroxide was the most effective and led to the least contamination of intracellular products. This EPS extraction procedure was not possible to use in the current study. More experimentation on optimizing the RAS ratio should also be conducted to correlate with the pure-culture lab studies. Another important addition to this work would be the quantification of surface charge and pH of zero charge for the algae cultures used, which would help in the interpretation of observed settling phenomena. Investigation into the settling effects of different algal species would extremely help comparison and classification for use in commercial scale applications. This study was a broad synopsis important in determining the fact that algae species can vary much differently in their settling and bioflocculation characteristics. Future studies could pinpoint certain improved, non-variable bioflocculation conditions with certain algal species present.

Research in conjunction with algal species dominance and control of HRT, as studied by Park and Craggs using activated algae, may help further pinpoint these differences and improve bioflocculation on a large scale. Thus, future research into the harvesting process of algae used in wastewater treatment may contribute to finding a suitable, cost-effective, and reliable method to meet the challenging scale up requirements that the production of algal biofuels entail.

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Appendix

Appendix: Section 1: Compilation of Results

Four Tables are presented that summarize the research conducted. The first two tables summarize settling effects of environmental conditions without biofloculant addition for *Chlorella* and *Scenedesmus*. The next two tables present a summary of the effect of biofloculant addition with the different environmental conditions studied. All effects are based on two-hour settling performances (not 24) over the growth curve. Up arrows signify an improved settling performance and down arrows signify a decreased settling performance. In general, for both species of algae: mid exponential phase (6-9 days of growth), early stationary (10-14 days of growth), mid stationary (15-22 days of growth), late stationary (23 to 32 days of growth), and death phase was (33 days and above). A key below each table explains variations in results.

For Tables 3 and 4, settling performance was compared to settling results of no cell or biofloculant addition for each trial. Settling results were based on normalized cell concentration remaining after two hours of settling. If the biofloculant addition improved settling performance compared to no cell addition it received an up arrow, while a down arrow signified the environmental condition/type of biofloculant/physical condition was not favorable for settling. If multiple stages of growth were favorable to increase settling, a pound (#) sign meant that better settling was observed in that particular growth phase compared to the others studied.

**Table 1: Effect on Settling of Different Environmental Conditions for *Scenedesmus* sp.
Without Biofloculant Addition**

Effect (Environmental Condition)	Growth Phase				
	Mid Exponential	Early Stationary	Mid Stationary	Late Stationary	Death Phase
Settling in the Dark	?	?	↑	?	?
Settling in the Light	?	?	↓	?	?
Settling in the Dark*	?	↑	↓	?	?
Settling in the Light*	?	↓	↑	?	?
pH 7 (neutral)	?	↑	↓	?	?
pH 3	?	↑	↓	?	?
pH 11	?	↑	↓	?	?
Carbon Dioxide Addition**	?	↓	?	↓	?
No Cell Addition	↓	↑	# ↑	↓	↑

*-Constant light exposure of 30 and 48 hours before settling in the light or dark for mid to late exponential cultures (30 hrs) and early/mid stationary cultures (48 hrs) respectively

?-Not studied, needs future attention

No Cell Addition-General settling trends without biofloculant addition (based on normalized cell concentration remaining after two hours of settling)

#-Best growth phase for settling without biofloculant addition (based on normalized cell concentration remaining after two hours of settling)

**.-Constant carbon dioxide addition of 4 to 24 hours before settling of early (4 hrs) and late stationary phase (24 hrs) cultures respectively

Table 2: Effect on Settling of Different Environmental Conditions for *Chlorella vulgaris* Without Biofloculant Addition

Effect (Environmental Condition)	Growth Phase				
	Mid Exponential	Early Stationary	Mid Stationary	Late Stationary	Death Phase
Settling in the Dark	?	?	↓	?	?
Settling in the Light	?	?	↑	?	?
Settling in the Dark*	?	↓	↑	?	?
Settling in the Light*	?	↑	↓	?	?
pH 7 (neutral)	?	↑	↓	?	?
pH 3	?	↑	↑	?	?
pH 11	?	↑	↓	?	?
Carbon Dioxide Addition**	?	↓	?	↓	?
No Cell Addition	# ↑	↓	↑	↓	↓

*-Constant light exposure of 30 and 48 hours before settling in the light or dark for mid to late exponential cultures (30 hrs) and early/mid stationary cultures (48 hrs) respectively

?-Not studied, needs future attention

No Cell Addition-General settling trends without biofloculant addition (based on normalized cell concentration remaining after two hours of settling)

#-Best growth phase for settling without biofloculant addition (based on normalized cell concentration remaining after two hours of settling)

** -Constant carbon dioxide addition of 4 to 24 hours before settling of early and late stationary phase cultures respectively

**Table 3: Effect on Settling of Different Environmental Conditions for *Scenedesmus* sp.
With Bioflocculant Addition**

Effects of Different Experimental Variables			Growth Phase			
Type of Bioflocculant	Environmental Condition	Physical Condition	Mid to Late Exponential	Early Stationary	Mid Stationary	Late Stationary
<i>B. cepacia</i> Cells	Settling in the Dark*	None	↑	# ↑	↑	?
<i>B. cepacia</i> Cells	Settling in the Light*	None	↓	↓	↑	?
<i>B. cepacia</i> Cells	Settling in the Dark*	Different Mixing Intensity (DMI)	?	# ↑	↑	?
<i>B. cepacia</i> Cells	pH 7 (neutral)	None	↑	?	↓	?
<i>B. cepacia</i> Cells	pH 3	None	# ↑	?	↑	?
<i>B. cepacia</i> Cells	pH 11	None	# ↑	?	↑	?
<i>B. cepacia</i> Cells	pH 11	Different Mixing Intensity (DMI)	↓	?	↑	?
<i>B. cepacia</i> Cells	Starved Nutrients (bacteria)	None	# ↑	?	↑	?
<i>B. cepacia</i> Cells	Starved Nutrients (bacteria)	Different Mixing Intensity (DMI)	↑	↑	# ↑	?
<i>B. cepacia</i> Cells	None	None	↑	↑	# ↑	?
<i>B. cepacia</i> Cells	None	30 Minute Contact Time	↑	# ↑	?	?
<i>B. cepacia</i> Cells	None	1/2 Dose	?	↓	?	↓
<i>B. cepacia</i> Cells	None	Different Mixing Intensity (DMI)	?	# ↑	?	↑
<i>B. cepacia</i> Cells	None	1/2 Dose + DMI	?	# ↑	?	↑
<i>B. cepacia</i> Cells	None	1/2 Algae + 1/2 Dose	?	?	?	↑
<i>B. cepacia</i> Cells	None	1/2 Algae + Full Dose	?	?	?	↑
<i>B. cepacia</i> Filtrate	None	None	↑	↑	# ↑	?
<i>B. cepacia</i> Filtrate	None	1/2 Dose	?	↑	?	?
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	None	?	# ↑	?	↑
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	Different Mixing Intensity (DMI)	?	# ↑	?	↑
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	1/2 Dose	?	↑	?	↓
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	1/2 Dose + DMI	?	↓	?	↑
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	1/2 Algae + Full Dose	?	↓	?	↑
<i>B. cepacia</i> Cells	With Carbon Dioxide (algae)**	1/2 Algae + 1/2 Dose	?	↓	?	↓

*-Constant light exposure of 30 and 48 hours before settling in the light or dark for mid to late exponential cultures (30 hrs) and early/mid stationary cultures (48 hrs) respectively

?-Not studied, needs future attention

#-Best growth phase for settling with bioflocculant addition and environmental/physical condition presented (based on normalized cell concentration remaining after two hours of settling)

**.-Constant carbon dioxide addition of 4 to 24 hours before settling of early and late stationary phase cultures respectively

Table 4: Effect on Settling of Different Environmental Conditions for *Chlorella vulgaris* With Bioflocculant Addition

Effects of Different Experimental Variables			Growth Phase			
Type of Bioflocculant	Environmental Condition	Physical Condition	Mid to Late Exponential	Early Stationary	Mid Stationary	Late Stationary
<i>B. cepacia</i> Cells	Settling in the Dark*	None	↑	?	↓	?
<i>B. cepacia</i> Cells	Settling in the Light*	None	↑	?	↓	?
<i>B. cepacia</i> Filtrate	Settling in the Dark*	None	↑	?	↓	?
<i>B. cepacia</i> Filtrate	Settling in the Light*	None	↓	?	↓	?
<i>B. cepacia</i> Filtrate	Settling in the Dark*	Different Mixing Intensity (DMI)	↓	?	↓	?
<i>B. cepacia</i> Filtrate	pH 11	None	↑	?	# ↑	?
<i>B. cepacia</i> Filtrate	pH 11	Different Mixing Intensity (DMI)	↑	?	# ↑	?
<i>B. cepacia</i> Filtrate	pH 7	None	↑	?	# ↑	?
<i>B. cepacia</i> Filtrate	pH 3	None	↑	?	↓	?
<i>B. cepacia</i> Cells	None	None	↓	↑	↓	?
<i>B. cepacia</i> Cells	None	Different Mixing Intensity (DMI)	?	↓	?	?
<i>B. cepacia</i> Cells	None	1/2 Dose	↑	?	↓	?
<i>B. cepacia</i> Filtrate	None	None	# ↑	↑	↓	↓
<i>B. cepacia</i> Filtrate	None	Different Mixing Intensity (DMI)	?	# ↑	↑	↓
<i>B. cepacia</i> Filtrate	Starved Nutrients (Bacteria)	None	?	↑	↓	↓
<i>B. cepacia</i> Filtrate	Starved Nutrients (Bacteria)	Different Mixing Intensity (DMI)	?	↓	↓	↓
<i>B. cepacia</i> Cells	Starved Nutrients (Bacteria)	None	?	↓	↓	?
<i>B. cepacia</i> Cells	Starved Nutrients (Bacteria)	Different Mixing Intensity (DMI)	?	↓	?	?
<i>B. cepacia</i> Filtrate	None	1/2 Dose	?	↓	?	↓
<i>B. cepacia</i> Filtrate	Starved Nutrients (Bacteria)	1/2 Dose	?	↑	?	↓
<i>B. cepacia</i> Filtrate	With Carbon Dioxide (algae)	1/2 Dose	?	↑	?	↑
<i>B. cepacia</i> Filtrate	With Carbon Dioxide (algae)	None	?	↓	?	↑
<i>B. cepacia</i> Filtrate	With Carbon Dioxide (algae)	Different Mixing Intensity (DMI)	?	↓	?	?
<i>B. cepacia</i> Filtrate	With Carbon Dioxide (algae)	1/2 Algae + 1/2 Dose	?	↑	?	?
<i>B. cepacia</i> Filtrate	With Carbon Dioxide (algae)	1/2 Algae + Full Dose	?	↓	?	↑
<i>B. cepacia</i> Filtrate	None	1/2 Algae + Full Dose	?	?	?	↓

*-Constant light exposure of 30 and 48 hours before settling in the light or dark for mid to late exponential cultures (30 hrs) and early/mid stationary cultures (48 hrs) respectively

?-Not studied, needs future attention

#-Best growth phase for settling with bioflocculant addition and environmental/physical condition presented (based on normalized cell concentration remaining after two hours of settling)

**.-Constant carbon dioxide addition of 4 to 24 hours before settling of early and late stationary phase cultures respectively

Appendix: Section 2: Compilation of Micrographs

Micrographs were taken of both the bacterial and algal cultures used in this research. The label beneath each micrograph describes the content of each.

Micrograph 1-Alcian Blue Staining and Visualization of TEP from a Chlorella sample from Dr. Brian Hampson, Cal Poly, San Luis Obispo (1000X) (Phase 3)

Micrograph 2-Alcian Blue Staining and Visualization of TEP in an algal floc from a Chlorella sample from Dr. Brian Hampson, Cal Poly, San Luis Obispo (1000X) (Phase 3)

Micrograph 3-Cluster of B. cepacia bacterial cells used as a bioflocculant in research (1000X-No Alcian Blue)

Micrograph 4-Larger floc of B. cepacia bacterial cells used as a bioflocculant in research (1000X-No Alcian Blue)

Micrograph 5-A *floc of Chlorella vulgaris cells used in research with added bioflocculant (B. cepacia filtrate) (1000X) (Phase 2-No Alcian Blue)*

Micrograph 6-A *floc of Chlorella vulgaris cells used in research with added bioflocculant (B. cepacia filtrate) (1000X) (Phase 2-No Alcian Blue)*

Micrograph 7-A floc of Scenedesmus cells used in research with added bioflocculant (B. cepacia cells) (1000X) (Phase 2-No Alcian Blue)

Micrograph 8-A floc of Scenedesmus cells used in research with added bioflocculant (B. cepacia cells) (1000X) (Phase 2-No Alcian Blue)

Micrograph 9-A floc of Scenedesmus cells used in research with added bioflocculant (B. cepacia cells) (1000X) (Phase 3-No Alcian Blue)

Micrograph 10-A floc of Scenedesmus cells used in research with added bioflocculant (B. cepacia cells) (1000X) (Phase 3-No Alcian Blue)

Appendix: Section 3: Compilation of Supplemental Figures: Section 4.2.4

Figure A.1- Settling of Scenedesmus based on normalized cell concentration remaining with *B. cepacia* cell addition for a A) 24-hour settling period, B) 2-hour settling period and C) a comparison of growth phase and cell addition (plotted against optical density at 625 nm) (Colors refer to days of growth and are the same throughout)

Supplemental figures for Section 4.2.4

Figure A.2- Settling based of *Scenedesmus* based on normalized cell concentration remaining for with *B. cepacia* filtrate addition for a A) 24-hour settling period, B) 2-hour settling period and C) a comparison of growth phase and cell addition (plotted against optical density at 625 nm) (Colors refer to days of growth and are the same throughout)

Supplemental Figures for Section 4.2.4

Figure A.3 -Normalized cell concentration remaining as a function of settling time for the same *Scenedesmus* culture with *B. cepacia* cell, filtrate, and no cell addition after 6 days of growth over a A) 24-hour settling period and B) 2-hour settling period

Supplemental Figures for Section 4.2.4

Figure A.4-Normalized Cell Concentration as a function of settling time for the same *Scenedesmus* culture with *B. cepacia* cell, filtrate, ½ filtrate, and no cell addition after 13 days of growth over a A) 24-hour and B) 2-hour settling period

Supplemental Figures for Section 4.2.4

Figure A.5-Normalized cell concentration as a function of settling time over a 2-hour period for the same *Scenedesmus* culture with *B. cepacia* cell, filtrate, and no cell addition after 15 days of growth

Supplemental Figures for Section 4.2.4

Figure A.6-Settling based on normalized cell concentration remaining for the same *Chlorella* culture over the growth curve with *B. cepacia* cell addition for a A) 24-hour settling period, B) 2-hour settling period and C) a comparison of growth phase and cell addition (plotted against optical density at 625 nm) (Colors refer to days of growth and are the same throughout)

Supplemental Figures for Section 4.2.4

Figure A.7-Settling based on normalized cell concentration remaining for the same *Chlorella* culture over the growth curve with *B. cepacia* filtrate addition for a A) 24-hour settling period, B) 2-hour settling period and C) a comparison of growth phase and filtrate addition (plotted against optical density at 625 nm) (Colors refer to days of growth and are the same throughout)

Supplemental Figures for Section 4.2.4

Figure A.8-Settling based on normalized cell concentration remaining for the same *Chlorella* culture over the growth curve with $\frac{1}{2}$ *B. cepacia* cell addition for a A) 24-hour settling period, B) 2-hour settling period and C) a comparison of growth phase and $\frac{1}{2}$ cell addition (plotted against optical density at 625 nm) (Colors refer to days of growth and are the same throughout)

Supplemental Figures for Section 4.2.4

Figure A.9-Normalized cell concentration remaining as a function of settling time for the same *Chlorella* culture with *B. cepacia* cell, filtrate, 1/2 cells and no cell addition after six days of growth over a A) 24- hour period and B) 2-hour period

Supplemental Figures for Section 4.2.4

Figure A.10-Normalized cell concentration remaining as a function of settling time for the same *Chlorella* culture with *B. cepacia* cell, filtrate, and no cell addition after thirteen days of growth over a A) 24-hour period and B) 2-hour period

Supplemental Figures for Section 4.2.4

Figure A.11-Normalized cell concentration remaining as a function of settling time over a 2-hour period for the same *Chlorella* culture with *B. cepacia* cell, filtrate, 1/2 cells, and no cell addition after fifteen days of growth (twenty four hour data was omitted)

Supplemental Figures for Section 4.4.3

Figure A.12-Effect of growth phase with no cell addition and dark settling conditions on A) Sc. 1 over 18 days of growth and B) Sc. 2 over 18 days of growth throughout a 24-hour settling period

Supplemental Figures for Section 4.4.3

Figure A.13-Effect of light and dark conditions of settling on A) Sc. 1 and B) Sc. 2 after 6 days of growth with no biofloculant addition

Supplemental Figures for Section 4.4.3

Figure A.14-Effect of Light and Dark conditions of settling for Sc. 2 after 11 days of growth with no bioflocculant addition

Supplemental Figures for Section 4.4.4

Figure A.15-Effect of A) Light conditions of settling and B) Dark conditions of settling on *Chlorella* over the growth curve of 15 days after exposure to light intensity of 30 hours before 8 days of growth and 48 hours before 15 days of growth

Supplemental Figures for Section 4.4.4

Figure A.16-Effect of Light and Dark settling conditions on Chlorella after A) 8 days of growth and B) 15 days of growth after exposure to light intensity of 30 hours before 8 days of growth and 48 hours before 15 days of growth

Supplemental Figures for Section 4.4.5

Figure A.17-Settling of *Scenedesmus* 1 with and without *B. cepacia* cell addition, different mixing intensity (DMI) and light/dark conditions after A) 6 days of growth B) 11 days of growth and C) 18 days of growth over a 24-hour settling period

Supplemental Figures for Section 4.4.5

Figure A.18-Settling of *Scenedesmus* 1 with *B. cepacia* cell addition and A) Dark settling conditions and B) Light settling conditions over 18 days of growth using a 24-hour settling period

Supplemental Figures for Section 4.4.5

Figure A.19-Settling of *Scenedesmus 2* with and without *B. cepacia* cell addition in light and dark conditions, including a different mixing intensity (DMI) over A) 6 days of growth B) 11 days of growth and C) 18 days of growth using 24-hour settling data

Supplemental Figures for Section 4.4.5

Figure A.20-Settling of *Scenedesmus* 2 with *B. cepacia* cell addition under A) Dark settling conditions and B) Light settling conditions over 18 days of growth using 24-hour settling data

Supplemental Figures for Section 4.4.6

Supplemental Figures for Section 4.4.6

Figure A.22-Settling of *Chlorella* with *B. cepacia* cell and filtrate addition with light and dark settling conditions and different mixing intensities over 15 days of growth during A) a 24-hour and B) a 2-hour settling period. All *Chlorella* cultures were treated with 48 hours of constant light exposure prior to settling.

Supplemental Figures for Section 4.5.2

Figure A.23- Effect of pH on *Scenedesmus* after 7 days of growth over a) 24-hour settling period and B) over a 2-hour settling period

Supplemental Figures for Section 4.5.2

Figure A.24-Effect of pH on Scenedesmus after 16 days of growth over a A) 24-hour settling period and B) over a 2-hour settling period

Supplemental Figures for Section 4.5.3

Figure A.25-Effect of pH on *Chlorella* settling after 6 days of growth over A) a 24 hour settling period and a B) 2-hour settling period

Supplemental Figures for Section 4.5.3

Figure A.26- Effect of pH on *Chlorella* settling after 14 days of growth over a A) 24-hour settling period and a B) 2-hour settling period

Supplemental Figures for Section 4.5.4

Figure A.27-Effect of settling on *Scenedesmus* with *B. cepacia* cell addition over a A) 24-hour settling period and B) over a 2-hour settling period at varying pH levels after 7 days of growth

Supplemental Figures for Section 4.5.4

Figure A.28-Effect of settling with *B. cepacia* cell addition over a A) 24-hour settling period and B) over a 2-hour period at varying pH levels after 16 days of growth

Supplemental Figures for Section 4.5.5

Figure A.29- Effect of varying pH range on *Chlorella* with *B. cepacia* filtrate addition after 6 days of growth over a A) 24-hour settling period and B) over a 2-hour period

Supplemental Figures for Section 4.5.5

Figure 4.30- Effect of varying pH range on *Chlorella* with *B. cepacia* filtrate addition after 14 days of growth over a A) 24-hour settling period and B) over a 2-hour period

Supplemental Figures for Section 4.6.2

Figure 4.31-Comparison of starved, non-starved and no *B. cepacia* cell addition, and different mixing intensities over A) 6 days of growth B) 11 days of growth and C) 15 days of growth on the settleability of *Scenedesmus 1*

Supplemental Figures for Section 4.6.2

Figure 4.32-Effect of the addition of A) starved cells and B) starved cells at a different mixing intensity on improving the settleability of *Scenedesmus 1* over 15 days of growth

Supplemental Figures for Section 4.6.2

Figure A.33-Comparison of starved, non-starved and no *B. cepacia* cell addition over A) 6 days of growth, B) 11 days of growth and C) 15 days of growth on the settleability of *Scenedesmus* 2

Supplemental Figures for Section 4.6.2

Figure A.34-Effect of the addition of A) starved cells and B) non-starved cells on improving the settleability of Scenedesmus 2 over 15 days of growth

Supplemental Figures for Section 4.6.3

Supplemental Figures for Section 4.6.3

Supplemental Figures for Section 4.6.3

Figure A.37-Comparison of settling of *Chlorella* based on normalized cell concentration remaining after a 2-hour settling period for A) starved versus non-starved filtrate B) $\frac{1}{2}$ the dose of starved/non-starved filtrate

Supplemental Figures for Section 4.7.2

Figure A.38-Normalized cell concentration remaining for pure cultures of *Scenedesmus* A) without carbon dioxide and B) with carbon dioxide addition over a 24- day period of settling

Supplemental Figures for Section 4.7.2

Figure A.39-Normalized cell concentration remaining for pure cultures of *Scenedesmus* with and without carbon dioxide input at A) early stationary phase and B) later stationary phase of growth over a 24- hour settling period

Supplemental Figures for Section 4.7.3

Figure A.40-Normalized cell removal of pure *Chlorella A*) without carbon dioxide and B) with carbon dioxide addition over the growth curve over a 24-hour settling period

Supplemental Figures for Section 4.7.3

Figure A.41-Normalized cell removal of pure *Chlorella* with and without carbon dioxide addition during A) early stationary phase of growth and B) later stationary phase of growth over a 24-hour settling period

Supplemental Figures for Section 4.7.4

Supplemental Figures for Section 4.7.4

Supplemental Figures for Section 4.7.4

Figure A.44-Normalized cell concentration remaining over a A) 24-hour and B) 2-hour period for *Scenedesmus* without carbon dioxide addition and *B. cepacia* addition at different doses and mixing intensities after 10 days of growth

Supplemental Figures for Section 4.7.4

Figure A.45-Normalized cell concentration remaining over a A) 24-hour period and B) a 2-hour period for *Scenedesmus* without carbon dioxide addition and *B. cepacia* cell addition at different doses and mixing intensities after 24 days of growth

Supplemental Figures for Section 4.7.5

Figure A.46-Normalized cell concentration remaining over a A) 24-hour settling period and B) 2-hour period for *Chlorella* with carbon dioxide addition (diluted and not diluted) and *B. cepacia* filtrate additions at various doses and mixing intensities after 11 days of growth

Supplemental Figures for Section 4.7.5

Figure A.47-Normalized cell concentration remaining over a A) 24-hour settling period and B) 2-hour period for *Chlorella* with carbon dioxide addition (diluted and not diluted) and *B. cepacia* filtrate additions at various doses after 32 days of growth

Supplemental Figures for Section 4.7.5

Figure A.48-Normalized cell concentration remaining over a A) 24-hour settling period and B) a 2-hour period for *Chlorella* without carbon dioxide addition and *B. cepacia* filtrate additions at varying doses, intensities, and addition of either starved or non-starved cultures over 10 days of growth

Supplemental Figures for Section 4.7.5

Figure A.49-Normalized cell concentration remaining over a A) 24-hour settling period and B) 2-hour period for *Chlorella* without carbon dioxide addition and *B. cepacia* filtrate additions at varying doses, intensities, and addition of either starved or non-starved cultures over 23 days of growth