Characterization of surface colonization by microalgae using Botryococcus braunii and Dunaliella tertiolecta
by Narendren Jayawickramarajah

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
Attachment and detachment of colonies of two micro-algae, Dunaliella tertiolecta and Botryococcus braunii, to glass, aluminum, steel and Teflon were examined. Images were taken from static and flow systems using white light microscopy and florescence microscopy using staining and auto-florescence techniques. Dunaliella tertiolecta attached more readily to all the tested surfaces than Botryococcus Braunii. Both Dunaliella tertiolecta and Botryococcus Braunii detached more rapidly from Teflon than aluminum or steel. Dunaliella tertiolecta appeared to form a biofilm structure, but while large self-adhering colonies of Botryococcus Braunii attached to the surfaces, biofilm structures similar to bacterial biofilms were not observed. Evidence was found from morphology of overlaid florescence images suggesting a possible synergistic relationship between smaller organisms (presumably bacteria) and micro-algae on surfaces.

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APPROVAL

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Narendren Jayawickramarajah

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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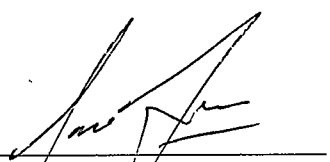
Signature 

Date 4/21/03

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ABSTRACT

Attachment and detachment of colonies of two micro-algae, *Dunaliella tertiolecta* and *Botryococcus braunii,* to glass, aluminum, steel and Teflon were examined. Images were taken from static and flow systems using white light microscopy and florescence microscopy using staining and auto-florescence techniques. *Dunaliella tertiolecta* attached more readily to all the tested surfaces than *Botryococcus Braunii.* Both *Dunaliella tertiolecta* and *Botryococcus Braunii* detached more rapidly from Teflon than aluminum or steel. *Dunaliella tertiolecta* appeared to form a biofilm structure, but while large self-adhering colonies of *Botryococcus Braunii* attached to the surfaces, biofilm structures similar to bacterial biofilms were not observed. Evidence was found from morphology of overlaid florescence images suggesting a possible synergistic relationship between smaller organisms (presumably bacteria) and micro-algae on surfaces.

INTRODUCTION

Studies of microbial attachment and detachment on surfaces are essential in understanding and controlling processes in aquatic systems used for industrial and environmental purposes. To date, a great amount of research has been done on the adsorption of bacterial cells to a substratum, but little has been done on algal biofilms. Further knowledge regarding surface adhesion of useful nonbacterial organisms such as micro-algae would be valuable in the future. Two micro-algal species, *Botryococcus braunii* and *Dunaliella tertiolecta*, were used in this study to examine algal biofilms. The organisms catalyze bio-reactions that could be useful in trimming down the emission of greenhouse gases into the atmosphere. This research might provide information that could be used to grow functional algal-bioflims.

Many systems have been employed to study the adhesion of cells to a given surface. The systems can be broken down into two different categories: static and flow. The static system is mainly described as a "no flow" condition. It is a system in which the fluid doesn't move in a coordinated manner relative to the substratum. The flow system on the other hand involves the proper control of hydrodynamic conditions (Scheuerman, 1996). Flow systems are preferred to static systems because a continuous inlet and outlet stream can be maintained. Nutrients can be fed to the system and harmful wastes could be washed out. Furthermore, the natural environments of these aquatic organisms involve flow conditions.

For this research, both static and flow systems were used to study different aspects of micro-algal cell adhesion and colonization. The flow cell was the device of

choice to study surface attachment in a flow system, because it allows observation of microbial colonization process *in situ* (Scheuerman, 1996). Additionally, different coupons (types of materials or surfaces) could be inserted into the system to compare the variation in attachment and colonization of microorganisms on different surfaces.

Goal of Thesis

To examine attachment, detachment and morphology of adhesion of two micro-algal species on solid surfaces.

Objectives of Research

1. Characterize relative attachment tendency and colonization of two different micro-algal species (*Botryococcus braunii* and *Dunaliella tertiolecta*) on glass under static conditions.
2. Characterize relative attachment tendency of these two organisms on various surfaces (stainless steel, aluminum, glass, Teflon) under flow conditions.
3. Compare detachment rates of these two micro-algae from three surfaces (stainless steel, aluminum, Teflon) under flow conditions.
4. Characterize biofilm formation and morphology of micro-algae and bacterial association on different surfaces (stainless steel, aluminum, Teflon) under flow conditions.

BACKGROUND

Biofilms- An Overview

From the earth's very origins, organisms have evolved mechanisms and characteristics that enable them to survive in a changing environment. In submerged aquatic environments the ability of planktonic organisms to attach to surfaces may serve as a mechanism that insures survival and proliferation. Biofilms are biologically active matrices of cells and non-cellular material accumulated on a solid surface (Characklis et al., 1990). Studies done on micro-colonies suggest that cells only make up a small fraction of the volume within bacterial biofilm (Costerton and Stewart, 2001). The rest of the film volume is made up of water and substances secreted by the cells. These substances (extra cellular matrix) form a network which binds the micro colony together. Biofilm formation is caused by the presence of certain physical, chemical and biological processes occurring in the system. In the case of bacteria, cells found within a biofilm exhibit a different phenotype than a suspended cell. Bacteria are known to express specialized genes once they are attached. Studies suggest that biofilm formation is the preferred mode of existence; in nature (Costerton and Geesey, 1987). Bacterial biomass associated with sessile population exceeds that of the planktonic by 2-4 log units (Costerton and Geesey, 1987). Biofilms are confronted in many environments ranging from bacterial films in dental plaque to algal/fungal films which form slimy layers covering wetland rocks. The planktonic phase enables the microorganisms to spread and proliferate while the attached biofilm phase facilitates a microenvironment in which

organisms are protected from threats posed by the external environment. These threats can include chemicals that can be harmful to cells (i.e. antibiotics, chorine, and etc), phages, predators (i.e. protozoans), desiccation, UV light, and, to a limited level, minor temperature and PH changes (Chirac et al., 1985).

The initial steps regarding biofilm formation involves the transport and attachment of microbial cells from the bulk fluid to a support surface. In most cases attachment is made possible through the formation of a conditioning layer on the solid-liquid interface. Organic and inorganic molecules present in the aqueous environment adsorb to the surface, forming a conditioning film. The conditioning layer alters the physico-chemical properties of the support material. Changes in surface free energy, surface hydrophobicity and in the local electrostatic interactions enable microbial attachment (Kumar and Anand, 1998). Once attached, the organisms grow and multiply both laterally and upwards adjacent to the previously attached cells.

In the next phase the adjacent colonies grow together and upwards (towards the bulk fluid) forming a heterogeneous structure made up of microorganism, glycocalyx and fluid channels reaching from the substratum to the bulk fluid interphase (Bishop, 1997). The extra cellular polymeric secretion (EPS) forms a polymer gel that is interconnected by chemical and physical cross links. Currently used models assume that substrates, nutrients, inhibitors and electron acceptors are transported by diffusing from the bulk fluid through a liquid boundary layer at the surface of the film and are utilized by cells(in the biofilm) for growth (Characklis et al., 1990). Wastes and other products produced by reaction within the film diffuse out. The parameters that influence the diffusion are thought to be film density, age, thickness, porosity, speciation, and electrostatic

interactions (Characklis et al., 1990). Other minor forms of transport in and out of the biofilm are convection through the pores, sedimentation and cell motility.

'Detachment' or loss of biomass is also an important aspect of biofilm physiology. Biomass is lost due to erosion by fluid flow, abrasion caused by the collision between suspended particles and film constituents, and general sloughing. Resent research suggests that lytic extra cellular enzymes within the EPS matrix may trigger the release of cells from the attached biofilm state (Characklis et al., 1990). The progression of biofilm formation follows a familiar pattern. In a plot of biomass vs. time, the biomass accumulation seems to follow a sigmoidal relationship. The biofilm process can be arbitrarily split into three different phases: (1) the induction phase- which includes initial attachment and conditioning procedures, (2) the log accumulation phase- characterized by exponential growth and biomass production and (3) the plateau stage- in which the biomass, biofilm cell numbers and thickness show steady state values. The steady state is produced by an equilibrium between accumulation and detachment of biomass and may oscillate some.

Basic Transport and Kinetic equations relating to Biofilms

The most basic one-dimensional mass balance for a microbial species in a biofilm is thought to be:

(Eq1) $$\frac{\partial C_X}{\partial t} = -\frac{\partial j_X}{\partial z} + R_X$$

C_x = concentration of the organism (X), usually defined as mass of dry solid per unit biofilm volume [ML^{-3}]
J_x = mass flux perpendicular to the substratum per unit biofilm area [$ML^{-2}T^{-1}$]
R_X= net rate of microbial mass production per biofilm volume [$ML^{-3}T^{-1}$]
t= time [T]
z= distance perpendicular to the substratum

NOTE: The mass flux in this equation originates from biomass production within the biofilm. For example if the biofilm increase in volume because of cell division then the mass flux would be positive.

$$(\text{Eq2}) \quad j_X = U_F C_X$$

U_F= the velocity in which the biomass is displaced relative the substratum

The velocity U_F can be calculated as follows:

$$(\text{Eq3}) \quad U_F = \frac{1}{A_b}\frac{dv}{dt} = \frac{1}{C_{XF}}\int_0^z \sum_{i=1}^{Nx} R_{Xi}\,dz$$

A_b= biofilm area [L^2]
υ= biofilm volume between substratum and the z direction [L^3]
N_x= number of different species present in the biofilm
C_{xf}= total dry biomass of microbes per unit volume [ML^{-3}]

The equation defining biofilm thickness is described as:

$$(\text{Eq4}) \quad \frac{dL_z}{dt} = U_F(L_z) + U_a - U_d$$

L_z= biofilm thickness in the z direction [L]
U_a= increase of biofilm thickness due to attachment [LT^{-1}]
U_d= decrease of biofilm thickness due to detachment [LT^{-1}]

Similar to Eq1 the mass balance for dissolved substrate in the biofilm is modeled as the following:

$$(\text{Eq5}) \quad \varepsilon_1 \frac{\partial C_S}{\partial t} = -\frac{\partial J_S}{\partial z} + R_S$$

C_S= concentration of the dissolved substrate S [ML^{-3}]
J_S= mass flux of substrate perpendicular to the solid surface per unit biofilm area [$ML^{-2}T^{-1}$]
R_S= net rate of substrate utilization per unit biofilm volume [$ML^{-3}T^{-1}$]
ϵ_1= volume fraction of the aqueous phase of the biofilm

The mass flux of the substrate in the biofilm is described using Fickian diffusion:

$$\text{(Eq6)} \quad J_S = -F_d D \frac{\partial C_S}{\partial z}$$

F_d= ratio of the diffusivity within the biofilm with the diffusivity in pure water
D= diffusivity on pure water [L^2T^{-1}]

The equations 1 through 6 enable simple basic modeling of the development of biofilm thickness and the dynamics and spatial distribution of microbial species and substrates in a mixed culture biofilm (Fruhen et al., 1991). Additional terms would have to be expressed in these equations when dealing with other important parameters i.e. reactions which are dependent on light, such as like photosynthesis.

Biofilms- Desirable and Undesirable Aspects

In many instances, biofilms are a nuisance and lead to undesirable consequences. When a layer of living microorganisms and their decomposition products deposits on the surface in contact with the liquid media, the word "biofouling" comes to mind. Biofouling caused by biofilm accumulation, is a major problem in many industries. In the food processing industry, biofilm formation has lead to spoilage of poultry (Kumar et al., 1999). Biofilm formation causes serious problems in industrial flow systems; in heat exchangers, biofilms are known to increase the resistance of both flow and heat transfer. Flow-impeding microbial growth also can increase the corrosion rate at the surface, leading to energy and product losses.

Biofilm formation is not always an adverse occurrence. Biofilms have been successfully used to maintain water quality. Microbial bioflims in trickling filters are

used to trap organic nutrients thereby reducing the organic content of waste water. They may also aid in the biodegradation of many toxic compounds and minimize accumulation of pollutants and help in environmental clean-up.

Biofilms represent a natural way of immobilizing cells. Immobilized microorganisms have been successfully utilized in reactors to improve productivity and stability of fermentation processes. Biofilm-aided immobilization has been applied to industrial processes involving acetic acid, ethanol, and polysaccharides (Nicolella et al., 2000). The application of biofilms in alternate fossil fuel production and in waste gas removal will be discussed in coming sections.

Biofilms in Bio-reactors

As mentioned in the previous section, novel methods have been used to incorporate biofilms in bioreactors. Biofilm reactors are beneficial in situations where reactor capacity using freely suspended organisms is limited by biomass concentration or hydraulic residence time (Nicolella et al., 2000). Slow growing organisms used in industries (e.g. algae, nitrifying bacteria, methanogenic bacteria) need very lengthy residence times to metabolize substrates. In this circumstance, biofilm reactors are able to retain the biomass within its confines. Biofilm reactors are not particularly useful when dealing with fast growing organisms because sufficient amounts of biomass can be produced quickly. In these situations, substrates are consumed with relatively small residence times and retention may not be required. Biofilm reactors are useful in systems where substrate feed streams are too dilute to bring about adequate increases in biomass. With dilute feed streams sufficient amounts of biomass must be retained within the

reactor to achieve continuous conversion. The major advantage of using biofilm reactors is that large volumetric conversion can be achieved without the need for separating the biomass or treating the effluent.

Problems with Biofilm Reactors

Although biofilm reactors can overcome limitations dealing with low reactor biomass levels, a new problem arises. As the biofilm thickness within the reactor increases, resistance to mass transfer tends to become relevant. There is also a reduction in surface area available for substrate transport and reaction. In order to overcome this drawback, scientists are looking at ways of grow bioflims on the surfaces of small particles. The particle size can be optimized through a compromise between conversion rate and particle sedimentation rate. Biofilm structure (density, porosity, roughness, shape and thickness) will also affect hydrodynamics, mass transfer and conversion in a biofilm reactor. Particle based biofilms (where biofilms coat the surface of packing material) could be successfully used in a variety of reactor types (i.e. fluidized beds reactors, airlift suspension reactors, and up flow sludge blanket reactors) (Characklis et al., 1990).

Non-Bacterial Biofilms

Scientist and researchers commonly use bacteria to study the biofilm phenomenon. It is not correct to assume that biofilms are solely produced by bacteria. In the presence of light, biofilms can be composed of algal cells, in addition to other microorganisms such as bacteria and fungi and micro invertebrates (Jarvie et al., 2002). Algal biofilms are sometimes referred to as algal mats when floating in water. Microbial

mats are structurally coherent macroscopic accumulation of microorganisms forming laminated structures rich in organic content on solid surfaces and sediments (Wiggli et al., 1999). Algal biofilms are found in almost every type of submerged aquatic environment, e.g. in thermal springs, hyper saline basins, bottom of ponds and in tidal regions. In intertidal systems, algal biofilms act as organic sponges which bind and concentrate organic molecules and ions. Free metal ions (Cd^{2+}, Cu^{2+}, Cr^{3+}, Pb^{2+}) and some toxic lipid soluble metals are absorbed by algal EPS. The EPS formed by micro-algae and diatoms on intertidal mudflats play an important role in stabilizing sediments against resuspension (Decho, 2000). EPS bound sediments serve as a sink for heavy metal contaminants. In the typhoon shelters of Honk Kong, artificial dispersion of the sediments has caused a substantial increase in toxicity levels (Wong and Cheung, 1999).

Environmental Significance of Green House Gas Emissions

Flue Gas and the Environment

From the times of the Industrial Revolution, the concentration of CO_2 in the atmosphere has been increasing steadily. The elevation in CO_2 levels is mainly attributed to the human consumption of fossil fuels. In the past 60 years the anthropogenic CO_2 emitted to the atmosphere has swelled from 280 parts per million (pre-industrial) to 360 parts per million (1998) (Yoshihara et al., 1996). Prediction of fossil fuel use in the next century indicates an unrelenting increase in carbon emission and rising CO_2 concentrations in the atmosphere. An intergovernmental panel study shows that global carbon emission will increase from about 7.4 billion tons of atmospheric carbon (GtC)

per year in 1997 to approximately 26 GtC/year by 2100 (DOE, 1997). Even though there is still much debate on the effects of elevated CO_2 levels on the global environment, many researchers agree the increase in CO_2 levels are associated with global warming. Figure 1 shows a pattern relating atmospheric CO_2 levels to global temperature.

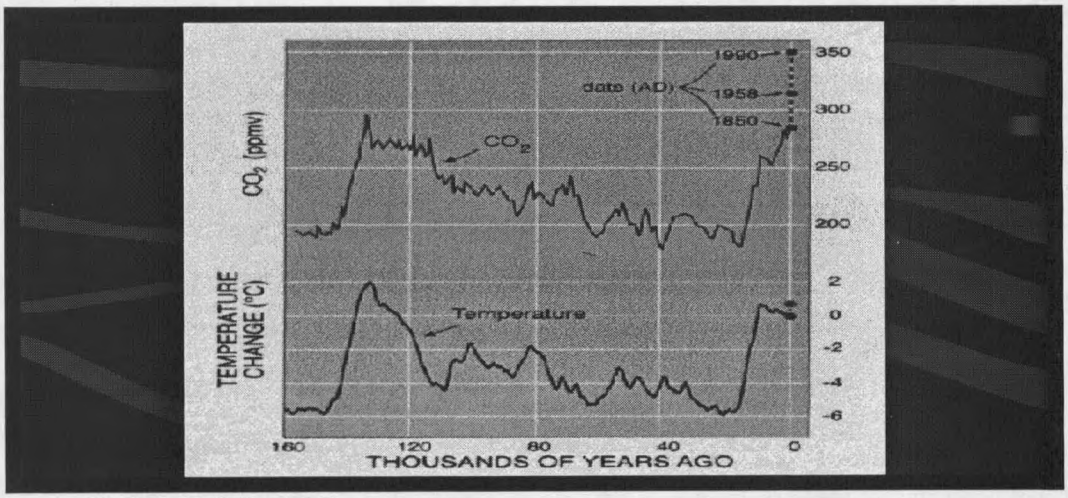

Figure 1. Global [CO_2] and Temperature

Therefore, the CO_2 emissions due to fossil fuel combustion should not go unchallenged because the long term survival of our species would be at risk. Carbon sequestration, trapping and storing carbon released from global energy usage, could be a major force in reducing atmospheric CO_2 emissions from fossil fuel combustion.

In many industrial countries, a majority of the fossil-fuel-related CO_2 emissions come from electric power plants (DOE, 1997). Apart from CO_2, fossil fuel flue gas contains elevated amounts nitrogen oxides. NO and NO_2 are formed in high-temperature combustion reaction and are collectively called NO_x. These gases are harmful to humans when inhaled and detrimental to the environment. The potential health effects include asthma and may also increase the effect of airborne allergens (Folinbee 1992). NO_2 is the

major component of ground-level ozone. Unlike the ozone layer in the higher atmosphere, which provides a shield against ultraviolet rays, ground level ozone causes oxidant air pollution. Sulfur-containing gases along with oxides of nitrogen constitute the main precursors to acid rain. Disruption of the natural ecosystem caused by acid rain has resulted in major ecological damage to forests and lakes in North America and Europe. Like CO_2, nitrogen oxides also contribute to global warming. Localized concentrations of NO_x as low as 0.1 parts per million (ppm) contribute to photochemical smog. Because NO_2 is difficult to collect straight for the atmosphere, it has to be efficiently sequestered from flue gas before emission. The major components of flue gases from thermal coal-fired power plants are as follows (Matsumoto et al., 1996):

Table 1. Typical Flue-gas Composition

O_2	CO_2	SO_x	NO_x	N_2 & inert gases
1.3%	11%	50 ppm	70 ppm	the remaining

Proposed Sequestration Methods

In the last decade, a few carbon sequestration methods have been investigated for feasibility. The main ones are: Separation of Carbon Dioxide-using existing separation methods (i.e. low-temperature distillation), Ocean Sequestration- using the ocean as a potential sink for injecting existing CO_2, Terrestrial Sequestration- enhancing photosynthetic carbon fixation by expanding the terrestrial biosphere, Geologic

Sequestration-using geological formations like aquifers and coal beds to sequester concentrated CO_2 (i.e. trapping CO_2 in the same manner natural gas is contained in aquifers), and finally Advanced Biological Sequestration- using new technological advances in bioprocesses to convert CO_2 into organic matter (i.e. designing bioprocesses involving newly discovered carbon fixing organisms).

Advanced biological sequestration using photosynthesis has distinct advantages. Photosynthesis is a well-known process. It is responsible for the nearly all the CO_2 fixation taking place in nature. Utilizing this method doesn't involve the incurring cost of separation, capture and compression. In many instances, these biological reactions result in the production of commercially useful organic compounds. Although there is much to be learned about natural processes, their inherent advantages provide the incentive for focused research.

Sequestration Utilizing Microalgea

In resent years, microalgea have gained importance in the field of biotechnology. The special characteristics of microalgal metabolism can be utilized to develop new production or environmental technologies. These unicellular organisms only need inexpensive substrates such as solar light and CO_2 to grow. In effect miroalgae can be used as cheap and efficient biocatalysts to produce high-value compounds (chemicals, vitamins, carotenoids, pigments, polysaccharides or hydrocarbons) (Vilchez et al., 1997). On the other hand, microalgea can be employed to eliminate unwanted chemicals, especially nitrogenous, phosphorus or sulfur compounds. Humanity has not yet tapped into the use of photosynthesis as a sufficient energy source. Photosynthesis

fixes almost 10^{11} tons of carbon and $2*10^{10}$ tons of nitrogen every year (Vega et al., 1991). It is interesting to envision efficient photo bioreactors filled with microalgea producing energy-rich compounds.

Photosynthetic Biological Sequestration

Terrestrial plants need atmospheric concentrations of CO_2 for biomass production and are usually limited by water and sunlight, whereas growth of aquatic photosynthetic organisms is limited by the low rate of transport of CO_2 into the aquatic environment. Microalgea in particular have the ability to thrive in CO_2 rich solutions. Artificially increasing the transfer rate of CO_2 to the aqueous system has resulted in dramatic increases in microalgal productivity (Negoro et al., 1991). Microalgal bioprocesses which can directly utilize CO_2 in power station flue gas can used to reduce CO_2 emissions from those respected facilities.

Microalgae and cyanobacteria are groups of microorganisms that have the capability for photosynthesis using water as the main reducing agent. The simplified chemical reaction is show below.

$$CO_2 + H_2O \xrightarrow{photosythesis} (CH_2O) + O_2$$

CO_2 behaves as a unique gas in nature. In the aqueous phase, it is susceptible to nucleophilic attack by water, forming ions. The reactions of the aquatic carbon cycle are displayed below.

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{-2}$$

In the oceans, approximately 95% of the dissolved carbon is in the form of bicarbonate ion (Falkowski, 1997). In both terrestrial plants and micro-algae the carbon fixation is catalyzed by the enzyme RuBisCO. But, the enzyme is not capable of utilizing bicarbonate as a substrate. The photosynthetic carboxylation reaction, catalyzed by RuBisCO specifically uses CO_2 as a substrate in the following reaction.

$$CO_2 + ribulose1,5bisphosphate \rightarrow 2\,phosphoglycerate$$

Increasing evidence suggests that bicarbonate ions are transported into the cell to concentrate intracellular carbon levels. Then the cellular mechanism converts the bicarbonate to CO_2, thereby supplying RuBisCO with its substrate (Badgar et al., 1994). This enzyme is also involved in the reverse process call photorespiration; photorespiration reduces the net efficiencies of photosynthesis. Visible light (wavelength less than 700 nm) provides the energy need for the excitation process involved in photosynthesis. Visible light is abundant and makes up about 45% of the total incident radiation. The genetic predisposition of the enzyme RuBisCO seems to be the main factor limiting an increasing of photosynthetic efficiency. Photorespiration (performed by RuBisCO) causes the loss of about half of the prefixed carbon. In simple organisms like cyanobacteria and micro-algae, the gene for RuBisCO can be genetically engineered to reduce photorespiration.

There are some major advantages in using micro-algae for carbon fixation. While higher plants depend on passive diffusion to acquire CO_2, micro-algae possess a carbon-concentrating mechanism which elevates CO_2 around the photosynthetic active sites. Micro-algae also have much higher growth rates and need less space (compared to higher

plants). Higher growth rates mean that screening programs for development of suitable strains can be carried out very rapidly. These organisms can benefit from higher CO_2 concentration and can grow in conditions that are not suitable for plant growth. Certain micro-algae can achieve high growth rates in salinity 2 to 3 time's seawater (Nagase et al., 1997). Unicellular algae can grow in very low nutrient freshwater/seawater media. Because of the low availability of nutrients, the growth solutions are somewhat resistant large scale bacterial contamination. These organisms also need to withstand direct aeration by flue gas; the micro-algae have to be tolerant to high CO_2 and HCO_3 concentrations, low pH caused by NO_x and SO_x, and higher than ambient temperatures. Even though much further R&D is required, the underlining fact remains that micro algae have the potential to fix CO_2 in flue gas streams without the costs of separation, CO_2 capture and compression.

Selection of Organisms

Very little has been done on algal adhesion to surface. As we our interested in improving conversion of photosynthesis by algal species by forming attached biofilm systems, two organisms were selected which might be useful in this application.

Botryococcus braunii

Botryococcus braunii is a green micro-alga which generally exists as a colony of individual cells supported by a colonial matrix. Densely-packed conical cells seem to