



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

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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 fluorescence microscopy using staining and auto-fluorescence 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 fluorescence 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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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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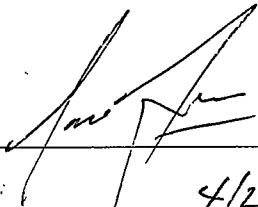
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ABSTRACT

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 fluorescence microscopy using staining and auto-fluorescence 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 fluorescence 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-biofilms.

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, chlorine, 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. Recent 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 [$\text{ML}^{-2}\text{T}^{-1}$]

R_x = net rate of microbial mass production per biofilm volume [$\text{ML}^{-3}\text{T}^{-1}$]

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.

$$(Eq2) \quad j_X = U_F C_X$$

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

The velocity U_F can be calculated as follows:

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

A_b = biofilm area [L^2]

v = 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:

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

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:

$$(Eq5) \quad \epsilon_1 \frac{\partial C_s}{\partial t} = - \frac{\partial j_s}{\partial z} + R_s$$

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:

$$(Eq6) \quad J_s = -F_d D \frac{\partial C_s}{\partial z}$$

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 biofilms 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 growing biofilms 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

Scientists 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 microalgae 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.

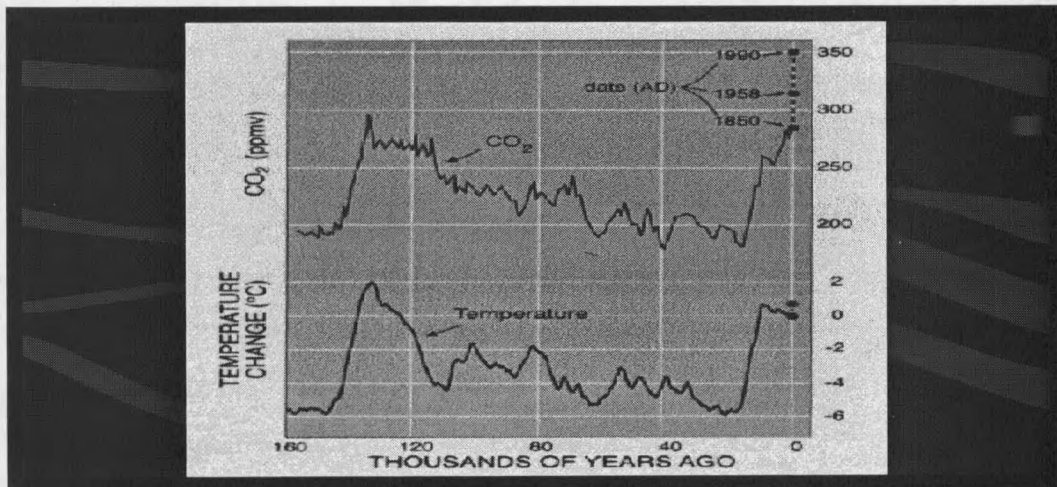


Figure 1. Global $[\text{CO}_2]$ and Temperature

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 CO₂, 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 NO₂ 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 CO₂, Terrestrial Sequestration- enhancing photosynthetic carbon fixation by expanding the terrestrial biosphere, Geologic

Sequestration-using geological formations like aquifers and coal beds to sequester concentrated CO₂ (i.e. trapping CO₂ in the same manner natural gas is contained in aquifers), and finally Advanced Biological Sequestration- using new technological advances in bioprocesses to convert CO₂ 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 CO₂ 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 Microalgae

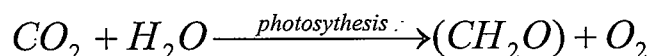
In recent years, microalgae have gained importance in the field of biotechnology. The special characteristics of microalgal metabolism can be utilized to develop new production or environmental technologies. These unicellular organisms only need inexpensive substrates such as solar light and CO₂ to grow. In effect microalgae 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, microalgae 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 microalga producing energy-rich compounds.

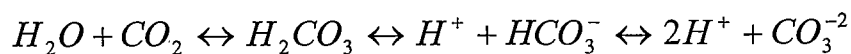
Photosynthetic Biological Sequestration

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. Microalga 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 be used to reduce CO_2 emissions from those respected facilities.

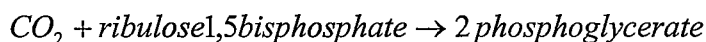
Microalgae and cyanobacteria are groups of microorganisms that have the capability for photosynthesis using water as the main reducing agent. The simplified chemical reaction is shown below.



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.



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.



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 CO₂ 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 times 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 to large scale bacterial contamination. These organisms also need to withstand direct aeration by flue gas; the micro-algae have to be tolerant to high CO₂ and HCO₃ 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 CO₂ in flue gas streams without the costs of separation, CO₂ capture and compression.

Selection of Organisms

Very little has been done on algal adhesion to surface. As we are 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

radiate and branch from the center of a roughly spherical colony. Each of the individual cells are about 5 to 10 microns and colony sizes vary from 25 to 1000 microns (Fukuda et al., 2001). These organisms are found abundantly in nature, particularly in fresh and brackish water.

The colonial micro-alga, *Botryococcus braunii*, was selected because of its ability to convert carbon dioxide from flue gas to a crackable hydrocarbon. This organism not only has the capacity to remove carbon dioxide from a gas stream as a carbon-sequestration alternative (main carbon source), but also produces an energy rich compound. The byproduct can possibly help offset the cost of sequestering carbon dioxide from flue gas. The advantages of using micro-algae over terrestrial plants in fixing carbon dioxide apply in this case.

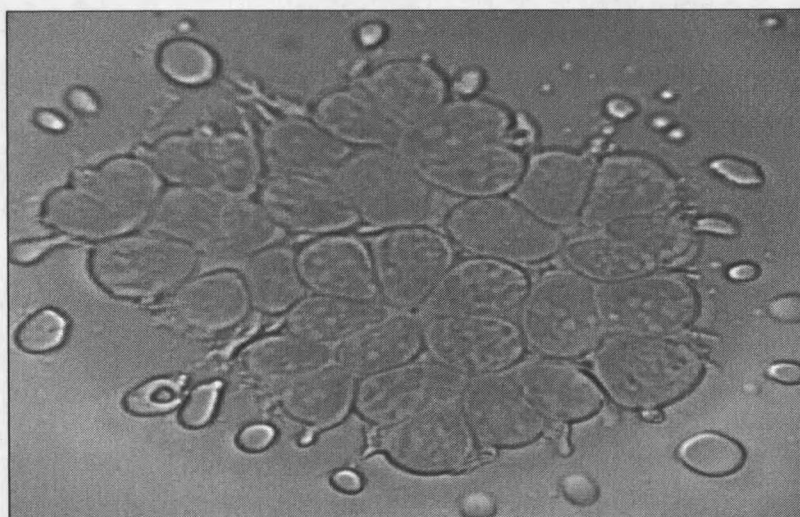


Image 1: *Botryococcus braunii* Colony at 60x and Dispersing Oil Droplets

Carbon dioxide concentrations have a profound influence on the growth of this organism. According to experiments done by Wolf & Momomura, 0.3% CO₂ enriched cultures showed a minimum mass doubling time of 40 hours compared to 6 days in cultures grown in ambient air (Chirac et al., 1985). Studies have shown that *Botryococcus braunii* has the ability to grow in pH 7 to 10, and plain aeration up to 15% CO₂ enriched aeration (Akin et al., 1993). The CO₂ and pH tolerance of this species will be very useful in the removal of CO₂ from flue gases.

Botryococcus braunii has been studied extensively as a means to produce a recyclable fuel source. Many other micro-algae contain fatty acids with carbon chains that are up to 22 carbon atoms; however the hydrocarbons made by *Botryococcus braunii* typically have chains that are greater than 30. Collectively these hydrocarbons are called botryococcenes (structure of a typical C₃₄H₅₈ botryococcene and isobotryococcene are shown in Figure 2 and 3) (Wolf et al., 1985). These energy rich compounds accumulate to very high levels in the micro-algae. An interesting feature of this micro-alga is that the hydrocarbon content is dependent on the physiological status of the colony. The green active state is characterized by the fact that hydrocarbons form close to 17% of the dry weight, while the brown resting state (large amounts of carotenoid pigments) is known to accumulate hydrocarbons which have up to 75% of the total dry weight (Wolf et al., 1985). Oils from this organism can be easily extracted and cracked. These products are known to be low in aromatic hydrocarbons and could even be used as blend stock for aviation fuels (Hillen et al., 1982). From an historical point of view, Sumatran crude oils

from the Ordovician period contained 1.4% botryococane (a hydrogenated derivative of botryococcene) (Wolf et al., 1985). This indicates that *Botryococcus braunii* may have contributed to our present fossil fuel reserves.

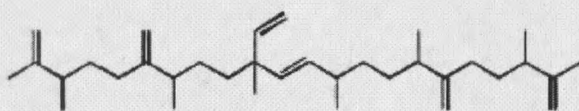


Figure 2. botryococcene ($C_{34}H_{58}$)

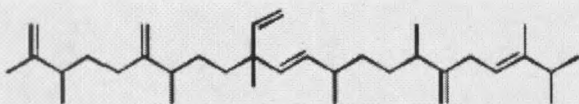


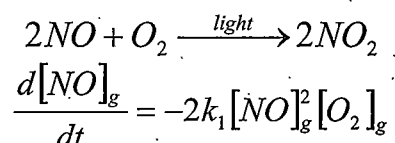
Figure 3. isobotryococcene ($C_{34}H_{58}$)

Dunaliella tertiolecta

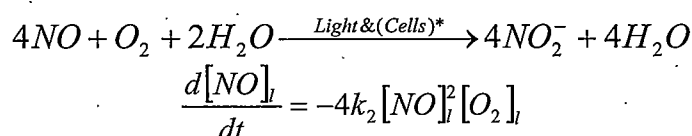
The micro-algae, - *Dunaliella tertiolecta*, was selected for its ability to remove nitrogen oxides from flue gas. Nitrogen is a very important macro-nutrient which is crucial for micro-algal growth. *Dunaliella tertiolecta* has the ability to use both organic and inorganic nitrogen sources. By using a low-nitrogen medium aerated with NO_x rich flue gas, the organism can be forced to use utilize inorganic nitrogen sources. In fact, some studies suggest marine algae might only use amino acid or other organic sources as nitrogen source under conditions where inorganic nitrogen is limiting (Hellio et al., 1998). Studies done in Japan also show that *Dunaliella tertiolecta* is capable of removing nitrogen oxide at wide ranges of NO concentrations. Even though the exact mechanism is not known, this micro-alga uses NO as a nitrogen source. In addition, studies have shown that *Dunaliella tertiolecta* cells grown in NO_x rich medium use less energy for growth than cells grown in NH⁴⁺ or urea rich solutions(Hellio et al., 1998). It

is hypothesized that in the presence of oxygen and light the poorly soluble nitrogen oxides are oxidized to form the highly soluble nitrogen dioxides. The proposed mechanisms are illustrated below (Nagase et al., 1997).

Mechanism (1)



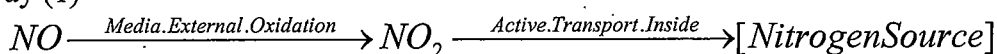
Mechanism (2)



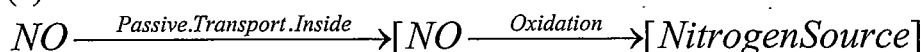
Mechanism (1) shows the oxidation of NO in the gas phase and Mechanism (2) shows the proposed reaction in an aqueous setting. Since we are conceiving of a reactor system in which flue gas is bubbled through an aqueous system, the retention time of the gas bubbles in the medium becomes an important parameter. Studies have shown that the retention times of bubbles in reactors of practical dimensions are very short. This means that the oxidation of NO in the gas phase (Mechanism 1) would be negligible under our proposed experimental conditions. The effect of dissolved oxygen concentrations in the reaction mechanism is still unclear. Under dark conditions, higher concentrations of aqueous oxygen increased the rate of NO removal. On the other hand, in dark conditions where O_2 was not added, the algae ceased to remove NO. A possible explanation is that, in the presence of light, the required O_2 was supplied via the light reaction of algal photosynthesis.

The proposed mechanism for removal of NO by *Dunaliella tertiolecta* can be summarized in a few steps. First, NO in the inlet flue gas is dissolved in an aqueous medium. The dissolution of NO from the gas phase is probably the rate limiting step. It is also likely, that NO sequestration by *Dunaliella tertiolecta* follows first order kinetics with respect to the amount of NO supplied (assuming the rate limiting step is also 1st order). So, the dissolution of NO can be enhanced by increasing the gas liquid contact area of the reactor system. Finally the dissolved NO is assimilated by the micro-algae and used as a nitrogen source. We can assume this is done via two possible pathways: (1) aqueous NO is oxidized to NO₂ or NO₃ in the media (out side the algal cells) and NO₂ and NO₃ are then taken up by the cell and (2) dissolved NO diffuses directly into the cells and is oxidized inside the cells.

Pathway (1)



Pathway (2)



Supporting pathway (2), recent research done by Nagase et al. suggests that dissolved nitric oxide was indeed directly taken up into cells through passive diffusion (Nagase and Yoshihara, 2001). NO is a small non-polar molecule, like O₂, it is likely that NO molecules can pass through the cell membrane at ease. Charged molecules such as NO₂ and NO₃ have to be transported inside the cell through labor intensive channels. In the same study mentioned above, H. Nagase reported that NO was preferentially utilized as a nitrogen source for cell growth rather than NO₂ or NO₃. In both cases (internally or

externally oxidized) aqueous NO is oxidized by a reaction with dissolved O₂, which is either supplied by photosynthesis or by dissolution from the gas phase.

This section will illustrate the characteristics of *Dunaliella tertiolecta* and why it might be an appropriate organism of the removal of nitrogen oxides. *Dunaliella tertiolecta* (Chlorophyceae) is a nano-flagellated unicellular marine alga which is about 6 to 12 microns in length. Another important feature is that it does not possess a rigid cell wall, but a fluid cell envelope. Since it lacks a rigid wall, it can rapidly respond to osmotic changes by rapidly altering cell volume. An efficient ion-transport machinery in the plasma membrane controls osmotic pressure by regulating intercellular glycerol levels (Tsukahara et al., 1999). Glycerol is used as an osmolyte or a compatible solute. *Dunaliella tertiolecta* can withstand a three to four fold change in osmotic pressure due to internal glycerol regulation.

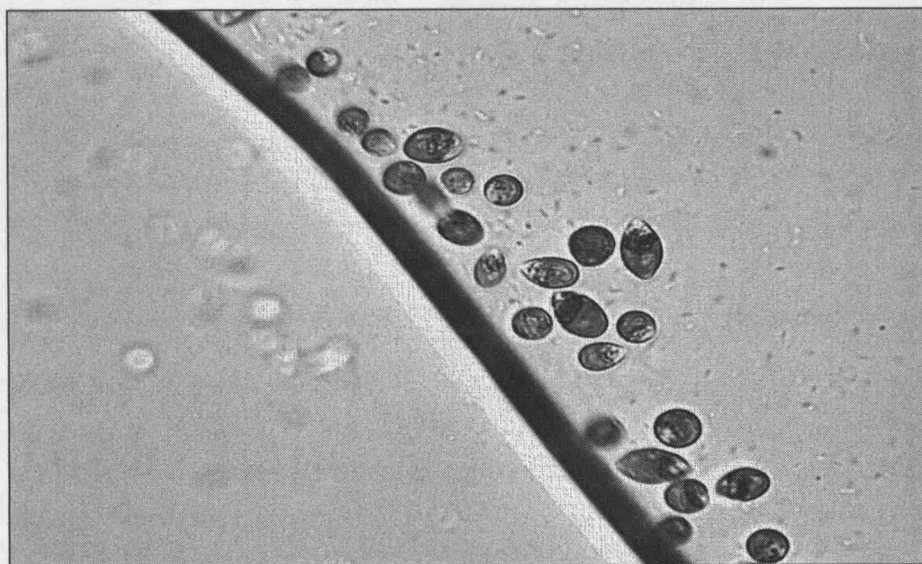


Image 2: *Dunaliella tertiolecta* at the Air/Media Interface (60x)

Dunaliella tertiolecta is an organism that is physiologically flexible and can grow in harsh conditions. Another characteristic is the ability of the organism to survive in a wide pH range. Since the high levels of CO₂ in flue gas cause the lowering of pH in the media, the tolerance for low pH is a prerequisite in choosing an ideal microbial candidate. Certain strains of *Dunaliella tertiolecta* can survive pH ranging from 10 to 4 and can effectively grow in temperatures ranging from 15 to 33 degrees Celsius (Yoshihara et al., 1996). This robust organism produces high amounts of carotenoids (α -carotene and β -carotene) which protect its vital organelles from photo degradation. Nevertheless, *Dunaliella tertiolecta* shows a high degree of photo-adaptation to UV radiation compared to other diatoms and Chlorophytes (Janssen et al., 2001). Another encouraging fact is that, technology for mass cultivation of *Dunaliella tertiolecta* has already been commercialized. Companies have mass produced this organism to obtain β -carotene, which accounts for more than 10% weight of a single cell. The ability of *Dunaliella tertiolecta* to remove NO_x and its capacity to survive in extreme environments makes it an ideal candidate to use in flue gas bio-remediation.

EXPERIMENTAL METHOD AND MATERIALS

Organisms

Two strains of green micro-algae were used in the experimentation: *Dunaliella tertiolecta* (LB 999 Butcher culture from Culture Collection of Algae at the University of Texas at Austin) and *Botryococcus braunii* (LB 572 Kutz culture from Culture Collection of Algae at the University of Texas at Austin).

A number of different growth medium were tested for optimal growth of these two organisms; the compositions are listed in Tables 2-8. Of the media tested, 'fresh water' Asm-1 produced the best growth for *Botryococcus braunii*. The growth solutions that supported thickest cultures of *Dunaliella tertiolecta* were F/2-Si (75%SW) and 'salt water' Asm-1. F/2 media general composition is given in Table 2, based on specific solution composition listed in Tables 3-5.

Table 2: F/2 Media Composition

Chemicals	Stock Solution (g/L)	Quantity (ml)
NaNO ₃	75	1
NaH ₂ PO ₄ .H ₂ O	5	1
F/2 Trace Metal Solution	(see below)	1
F/2 Vitamin Solution	(see below)	0.5
Soil Water Extract	(see below)	1.2

*Make up final volume with a 75% sea water solution

*Maintain pH at 7.5-8.0 using NaOH

*autoclave solution for 20 minutes

Table 3: F/2 Trace Metal Solution

Chemicals	Stock Solution (g/L)	Quantity
FeCl ₃ ·6H ₂ O	-----	3.15g
CuSO ₄ ·5H ₂ O	9.8	4.36g
Na ₂ MoO ₄ ·2H ₂ O	6.3	1.0ml
ZnSO ₄ ·5H ₂ O	22.0	1.0ml
CoCl ₂ ·6H ₂ O	10.0	1.0ml
MnCl ₂ ·4H ₂ O	180.0	1.0ml

Table 4: F/2 Vitamin Solution

Chemicals	Stock Solution	Quantity
Vitamin B12 (Cyanocobalamin)	5mg/5ml	0.1ml
Biotin	1mg/10ml	1.0ml
Thiamine HCl	-----	20mg

Table 5: Soil Water Extract

Chemicals	Quantity (g/L)
Volcanic Soil	20
MgCO ₃	1.0
CaCO ₃	2.0

*Solution must be filtered in a sterile manner after autoclaving

Salt Water Asm1 media general composition is given in Table 6, based on specific solution composition listed in Tables 7-8. To make fresh water Asm-1 Media, follow the same directions used to make 'Salt Water' Asm-1 Media replacing the 75% sea water with Nanopure water.

Table 6: 'salt water' Asm-1 Media Composition

Chemicals	Stock Solution (g/L)	Quantity (ml)
Macro-Nutrient Solution (10x)	(see below)	100
Micro-Nutrient Solution (10x)	(see below)	100
Soil water Extract	(see table 4)	35
75% Sea Water	-----	725

*75% Sea Water is made by mixing 750ml of natural sea water with 250ml of nanopure water

*Maintain pH at 7.5-8.0 using NaOH

*autoclave solution for 20 minutes

Table 7: Asm-1 Macro-Nutrient solution (10x)

Chemicals	Quantity (g/L)
NaNO ₃	1.6998
MgSO ₄	0.2407
MgCl ₂	0.1904
CaCl ₂	0.2220
K ₂ HPO ₄	0.1742
Na ₂ HPO ₄	0.1420

*add nanopure water to equal 1 L of solution

*autoclave solution for 20 minutes

Table 8: Asm-1 Micro-Nutrient solution (10x)

Chemicals	Quantity (mg/L)
FeCl ₃	6.4
H ₃ BO ₃	24.70
MnCl ₂	8.8
ZnCl ₂	4.4
CoCl ₂	0.1
CuCl ₂	1.1*10 ⁻⁶
Na ₂ EDTA	74.4

*add nanopure water to equal 1 L of solution

*autoclave solution for 20 minutes

Initial growth & stock culture preparation

The *Dunaliella tertiolecta* was grown in a flask with 200 ml of Asm-1 'salt water' media given in Tables 2-5. The continuously stirred flask was aerated with air through a bubbling stone. Air was allowed to escape through a 0.2 μ m filter at the top of the flask. The growth culture was illuminated with natural light during day time and artificial light during night time. Four ml of initial inoculum was grown in 200 mls of media for 30 days. The *Dunaliella tertiolecta* grew effectively to produce a light green suspension, and a visually higher concentration located along the liquid level of the container.

The *Botryococcus braunii* was acquired already partially grown from a serum bottle prepared at INEEL. Ten ml of inoculum was taken from the serum bottle mentioned above and added to a container filled with 30 ml of Asm-1 'fresh water' Media. The inoculated solution was then grown in a naturally illuminated condition for 60 days.

Methods of Analysis

Viable Cell Counts

Viable cell counts using the plating method proved ineffective in counting micro-algae. This was due to the inability of both *Dunaliella tertiolecta* and *Botryococcus braunii* to grow on solid agar. The rare sighting of isolated colonies on the agar plates were identified as colonies of associated bacteria and bacterial contamination.

Total Cell Counts

Direct cell counts were conducted using a Hemocytometer. The ruling on the hemocytometer slide covered 9 square millimeters. The boundary lines of the Neubauer ruling divided the 9 square millimeter area into 9 separate squares of 1 square millimeter each. The center square millimeter was further ruled into 25 groups of 16 small squares. A sample of 1 microliter was then taken from a well stirred batch culture using a 1-10 ml size pipette. After the cover glass was placed on the main slide, the sample was inserted into a v-shaped groove between the cover glass the main slide. The hemocytometer was then viewed under a light microscope at 10x magnification. The number of cells in each square were counted and recorded. Knowing that the space between the cover glass and the main slide was 0.10 mm, the volume over each square could be calculated. The cell count was determined using the calculation:

$$\text{Number of cells per mm}^3 = (\text{Number of cells per mm}^2) * (\text{dilution}) * (10)$$

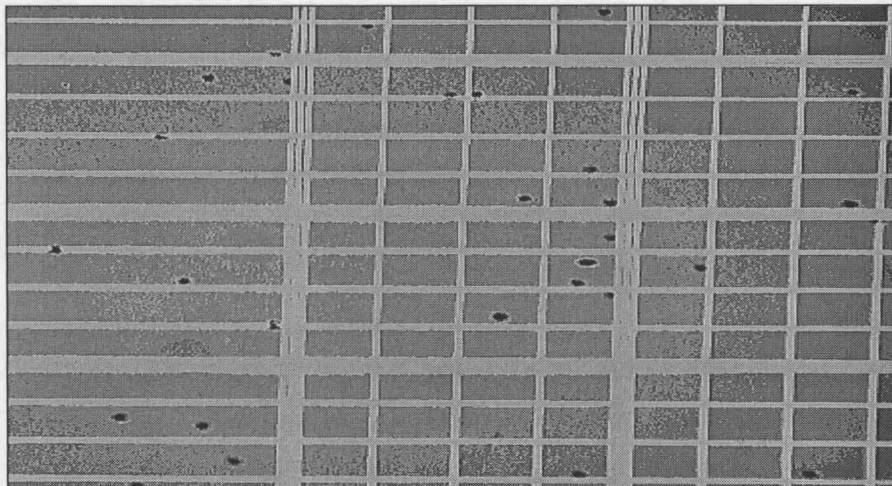


Image 3: A Sample View at 10x Magnification of a Hemocytometer with Micro-algae

The hemocytometer proved effective in counting cells of *Dunaliella tertiolecta*, but ineffective when used on *Botryococcus braunii*. It was very difficult to identify individual cells within cluster-looking colonies of *Botryococcus braunii*. Reliable cell counts were not obtained for *Botryococcus braunii*.

Experiment 1: Wet Slide Image Analysis

In the duration of this research, many different methods and medias were employed to grow both *Dunaliella tertiolecta* and *Botryococcus braunii*. This proved to be an ideal opportunity to study the morphological changes that occurred as these cultures aged; as both species of green algae have very slow growth rates compared to bacteria. Periodically, some samples were taken from growth containers, put on wet slides and viewed under the microscope. In instances where microbial adherence and colony formation were observed, digital photographs were taken. Some wet slide procedures even involved scraping samples from the interior of glass containers. This procedure proved effective in exposing microbial structures that were attached to the inner walls of the growth vessels. Microscope and staining procedures to view associations are described later. Some of these wet slide images are displayed in the section Results and Discussion.

Experiment 2: Parallel Plate Flow-Cell

Flow Cell and Reactor System

A parallel plate flow cell was used to study the attachment and detachment phenomena of *Dunaliella tertiolecta*, *Botryococcus braunii* and associated organisms. The parallel plate flow cells used in this study were identical to the flow cells used in previous experiments with bone composite coupons (Pasmore). Figures 4 and 5 show the basic components of a parallel plate flow cell. The bottom plate contains a sunken well where a coupon can be inserted. The top plate of the rectangular flow conduit is made up of a glass cover slip (43 X 61) mm. The glass cover slip serves as an observation window and also enables light to penetrate to the photosynthetic cells attached to the coupon surface. The dimensions of the groove and coupon spacing are given in Tables 9-10.

Table 9. Dimensions of Flow Cell Groove

Length of Flow Cell groove	5.28 cm
Width of Flow Cell groove	1.19 cm
Height (depth) Flow Cell groove	0.165 cm

Table 10. Dimension of Coupons and Flow Channels

	<u>Teflon</u>	<u>Steel</u>	<u>Aluminum</u>	<u>Tubing</u>
Length of Coupons	3.68 cm	3.68 cm	3.75 cm	na
Height of Coupon	0.145 cm	0.147 cm	0.147 cm	na
Height of Flow Channel	0.02 cm	0.018 cm	0.018 cm	na
Cross sectional area of Flow Channel	0.024 cm ²	0.021 cm ²	0.021 cm ²	0.018 cm ²

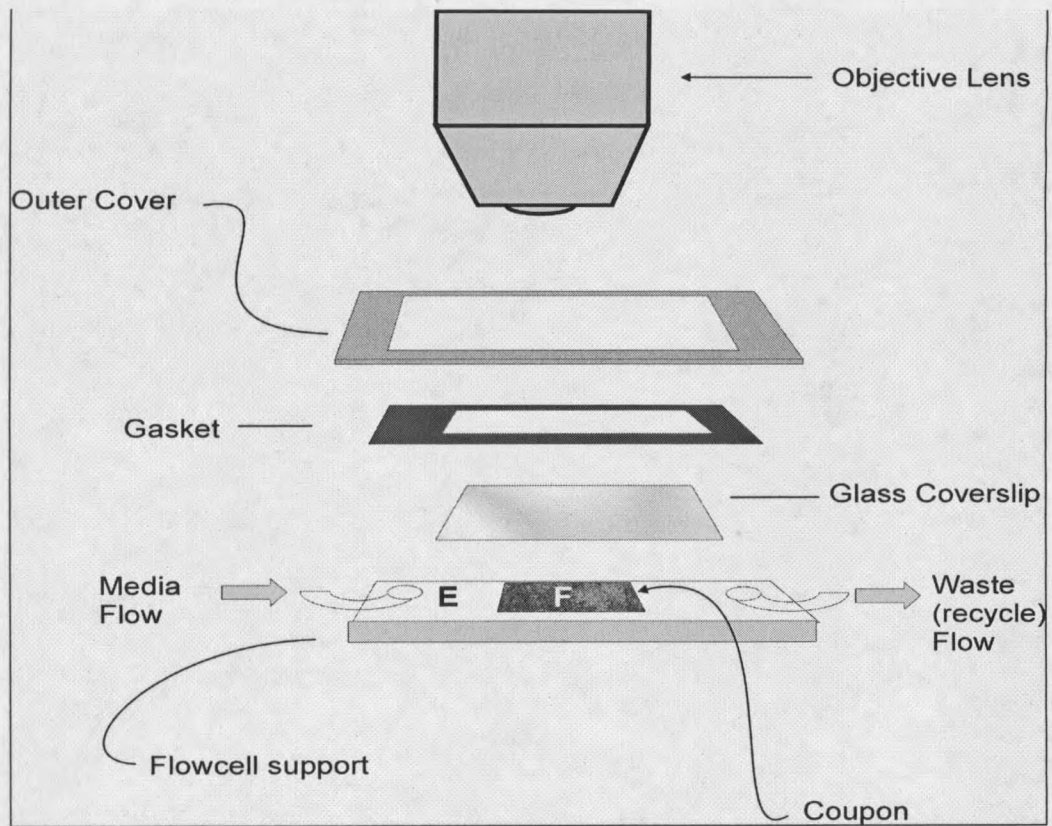


Figure 4. Parallel Plate Flow Cell with Coupon

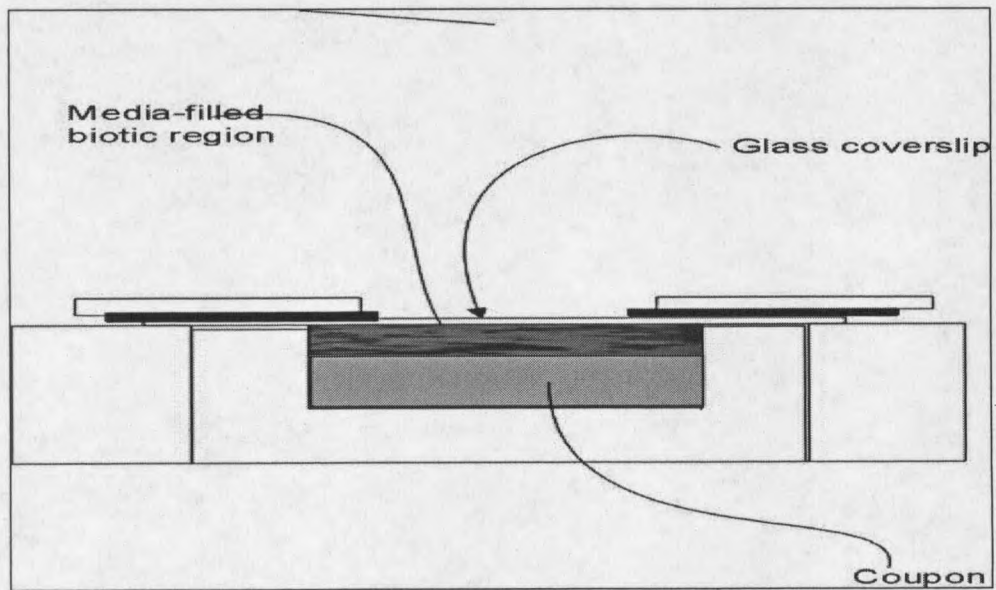


Figure 5. Parallel Plate Flow Cell Cross-section

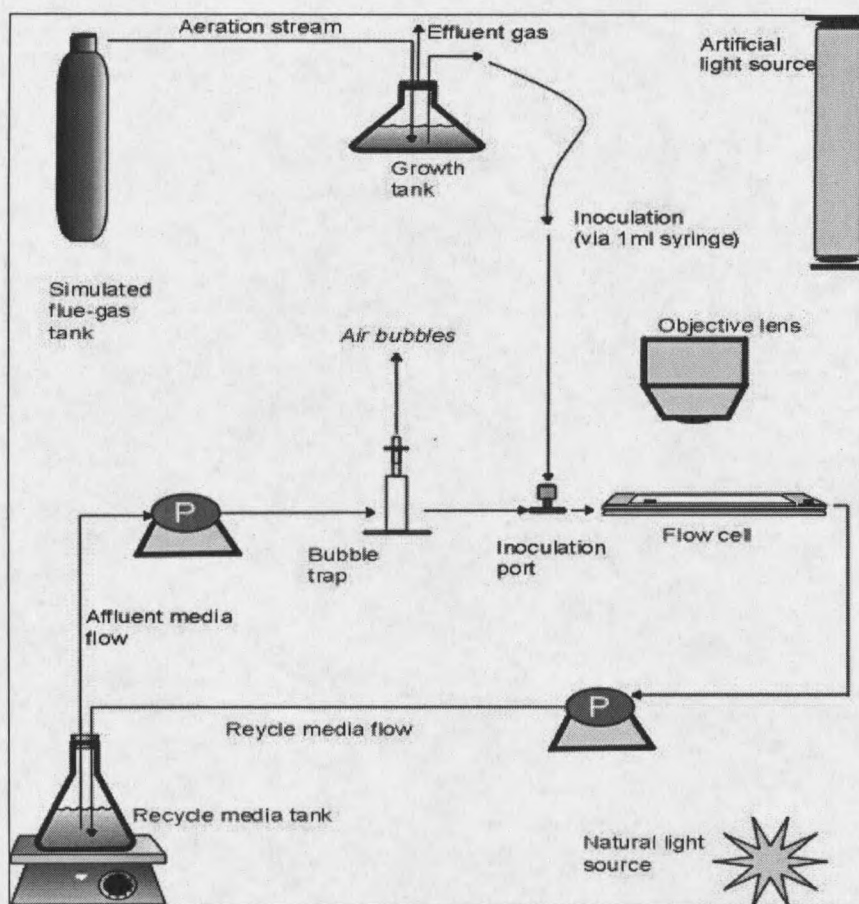


Figure 6. Complete Experimental System for the Parallel Plate Flow Cell

The whole reactor setup is shown in Figure 6. The system contains of a flue-gas tank (Air Liquide specialty Gases), growth tank, illumination bulb, stir plate, recycle/mixing tank, bubble trap, inoculation port, flow cell, peristaltic pumps (Cole Parmer, Masterflex L/S), and assorted tubing (Norprene size 14) and connectors. A bubble trap (Figure 7) was used to prevent air bubbles from disrupting biofilm formation. The entire system was put together on a mobile setting so that it could be moved to and from the Nikon microscopes.

Coupons

Three different types of surfaces were chosen to investigate attachment phenomena. These surfaces were: 316 stainless steel, Teflon, and Aluminum. The coupons were machined to fit inside a parallel plate flow cell as described in Figure 4. After the coupons were machine cut, they were polished using a silicon carbide sand paper (grit size 10 microns). Polishing provided all three coupons with similar surface roughness and thickness values. The exact dimensions of the coupons are given in Table 10. The polished coupons were stored in a sterile nanopure water solution to prevent surface conditioning agents from attaching.

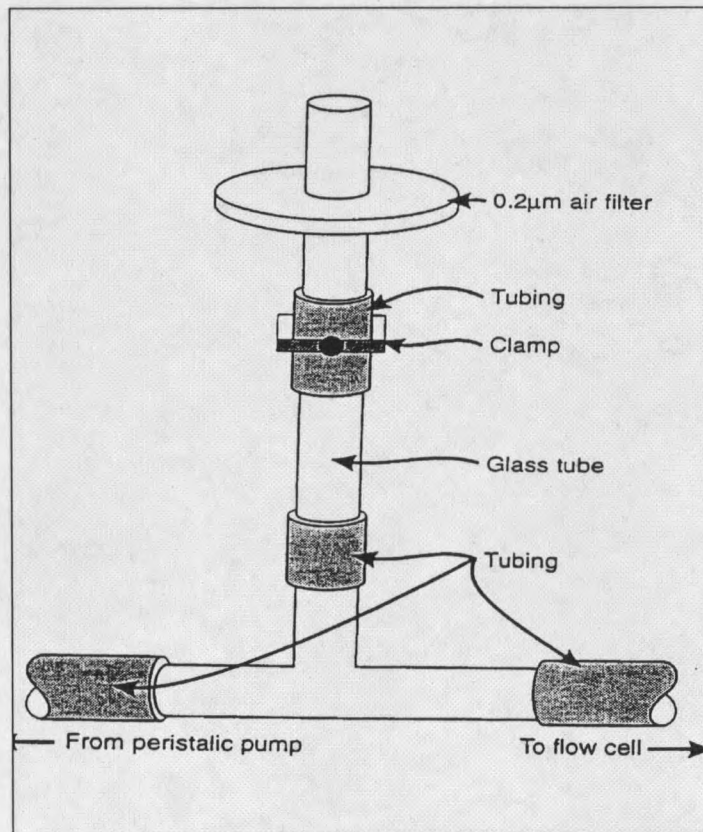


Figure 7. Bubble Trap (Scheuerman, 1996)

Flow Cell Hydrodynamics

The Reynolds number is normally used to classify flow conditions in closed conduits as laminar or turbulent. A Reynolds number smaller than 2100 (in linear flow) is referred to as laminar flow. For values larger than 2100, flow is said to be turbulent. The Reynolds number is mainly a function of mean bulk water velocity and the geometry of the flow system. The Reynolds number of the flow channel can be calculated using a wetted perimeter (W_p) to adjust for the rectangular conduit. The definition used for the wetted perimeter is shown in equation 7. The equations that were used for the calculations to quantify the system are given below.

Wetted Perimeter:

$$\text{Equation 7: } W_p = 2w + 2h$$

w = width of flow channel

h = height of flow channel (between the surface of the coupon and the glass cover slip)

Hydraulic Diameter:

$$\text{Equation 8: } D^o = 4 \frac{A_{cs}}{W_p}$$

A_{cs} = cross sectional area of flow channel

W_p = wetted perimeter

Reynolds Number:

$$\text{Equation 9: } N_{RE} = \frac{u_{avg} D^o \rho}{\mu}$$

u_{avg} = average linear flow velocity

ρ = fluid density

D^o = diameter of fluid vessel

μ = viscosity of fluid

Maximum Velocity (Stoodley, 2002):

$$\text{Equation 10: } u_{\max} = \frac{3}{2}(u_{\text{avg}})$$

Wall Shear Stress:

$$\text{Equation 11: } \tau_w = \frac{4\mu(u_{\max})}{D}$$

τ_w = shear stress for laminar flow

First-Order Kinetic Model of Detachment (Duddrige et al., 1982):

$$\text{Equation 12: } \frac{dN}{dt} = [\mu_s - \psi(\tau_w)]N$$

$\Psi(\tau_w)$ = removal rate constant (laminar flow)

N = number of attached cells

μ_s = specific growth rate of attached cells

Table 11. Hydrodynamic Characteristics of Flow Channels

	<u>Teflon</u>	<u>Steel</u>	<u>Aluminum</u>	<u>Tubing</u>
Bulk fluid flow rate through flow cell	0.3 ml/min	0.3 ml/min	0.3 ml/min	0.3 ml/min
Bulk fluid mean linear velocity	0.21 cm/sec	0.24 cm/sec	0.24 cm/sec	0.27 cm/sec
Reynolds number of flow through the flow cell	0.92	0.92	0.92	4.6
Wall Shear of flow through the flow cell	0.028 N/m ²	0.036 N/m ²	0.036 N/m ²	0.013 N/m ²

Shear stress is known to be a factor effecting both attachment and detachment.

According to research preformed on bacterial cells, a general increase in shear stress has a negative effect on the extent of attachment (Mueller, 1990). The Reynolds numbers and shear stress values of the flow channels for the three systems with the different

coupons, were very similar in magnitude (see Table 11). This suggests that hydrodynamic factors can be ignored when comparing attachment data between these three different surfaces.

Preparation

The whole flow system described in Figure 4 was sterilized in an auto-clave and allowed to dry. (The coupons were inserted inside the flow cell prior to sterilization) Once the system was dry, 150 ml of Asm-1 'salt water' medium was added to the recycle tank in a bio-hood. The calibrated peristaltic pumps were adjusted to a flow rate of one ml/min allowing the media to circulate through the system for 12 hours. Then, the whole system was checked for leaks and cover slip cracks.

Inoculation

The flow was turned off when the system was ready for inoculation. Prior to inoculation, the inlet stream leading to the inoculation port was blocked so the injected inoculum would proceed down stream into the flow cell. Four tenths (0.4) ml of inoculum was taken from the aerated growth tank and injected (1 ml disposable syringe) into the inoculation port. After inoculation, the effluent stream leaving the flow cell was also blocked. By blocking the effluent stream the micro-algae were confined to an area within the flow cell. The cells were allowed to attach and multiply under a no-flow condition for another 5 days. Next, both pumps were activated at a flow rate of 0.3 ml/min. All experiments were run at room temperature.

Sampling and Image Acquisition

Before sampling, the entire system was moved to the microscope room. To prevent alteration in flow pattern while moving, both inlet and outlet streams of the flow cell were clamped. The flow chamber was then mounted onto the microscope stage and the field of view was established. The surface of the coupons at the bottom of the flow cells were viewed under a Nikon Eclipses 800 microscope equipped with an Hg bulb. Earlier experiments indicated both *Dunaliella tertiolecta* and *Botryococcus braunii* auto-fluoresced when exposed to an excitation wavelength of 596 nanometers (emission wavelength 615). This provided a means to take samples of non-transparent surfaces without adding any harmful tags or dyes. The magnifications used were between 10X and 60X. Transmitted light was used for sampling the transparent surface of Teflon. An imaging tool, MetaView, was used to acquire and produce 24-bit images of the coupon surface. The regions of the coupon that harbored the most cells were sampled. The x and y coordinates of the specific regions were recorded so it was possible to return to that particular coordinate in subsequent sampling sessions. Sampling was done once prior to the initiation of flow, and at regular intervals afterwards. The images taken of the coupon surface from each sampling session were collected and analyzed.

Experiment 3: Staining Image Analysis

After completing all the necessary sampling sessions, the flow channel was stained with two different dyes to observe biofilm formation and bacterial cohabitation. The blue fluorescent DAPI nucleic acid stain with a maximum excitation wavelength of 358 nm and a maximum emission wavelength of 461 nm was used in this study. The

molecular structure of the stain is given below in Figure 8. DAPI stains both dsDNA and RNA of cells without staining the cytoplasm. This particular stain was chosen because its blue fluorescence stands out in contrast to the red auto-fluorescence of the algal cells.

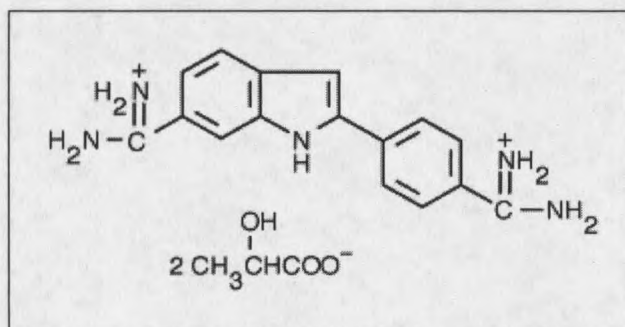


Figure 8. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Probes.com, 2003)

Staining Procedures

The tubing directly upstream of the inoculation port and directly downstream of the flow-cell were clamped shut. Then, the clamped section which includes both the inoculation port and the flow-cell were disconnected from the system. The stream leading away from the flow cell was unclamped so that any solution injected through the inoculation port would proceed through the flow channel. Next, a 1ml syringe was used to inject 0.8ml of DAPI solution (5 mg/ml) through the flow cell (see Figure 9). The open end was closed and the stain was allowed to fixate for 15 minutes. After fixation, the stream was reopened and 3ml of fresh media was injected through the inoculation port. The media was used to washout unbound DAPI particles that would otherwise produce blurred images. Most unattached cells were also washed out in this process.

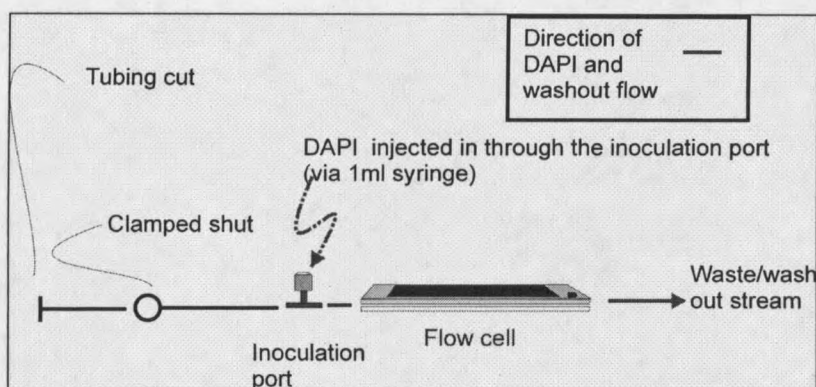


Figure 9. Staining Diagram

The DAPI stained flow cell was viewed under a mercury-arc lamp. Both 10x and 60x magnifications were used, and at 60x the water immersion lens produced clearer images. By using the wavelengths between 596-615 nm for auto-florescence and wavelengths of 358-461 nm for DAPI, two distinct images of the same surface were formed. The two images were combined using the color-combine function built in to the MetaView soft ware. In certain flow-cell runs, the dye rhodamine B was also used to identify matrix formation. The staining procedure for rhodamine B was almost identical to the procedure used for DAPI. The flow-cell was initially fixed with same volume of rhodamine B (as used for DAPI), but 10 times as much fresh media was used in the washout step. The rhodamine B dye was prevalent when viewed at wavelengths between of 500-600 nm. The images, the observations and the analysis are revealed in the Results and Discussion.

Experiment 4: Capillary Tube Flow Attachment Observations

The general procedure for the observation experiment was very similar to the methods described above. The main difference is a simple 3 mm by 3 mm square glass tubing (Fredrick & Dimmock) was used to replace the parallel plate flow cell. In addition a number of flow breaks and filters were used to confine algal growth to a particular area of the flow system. Unlike the parallel plate flow cell experiment where algal and bacterial cells were recycled with the media, in this experiment the recycled media was continuously filtered. By utilizing the (Whatman PolycapTM AS) aqueous solution filter we were able to provide a sterile media stream into the 3 mm by 3 mm capillary tube. Another alteration was that the recycle tank was directly aerated with simulated flue gas (15% CO₂, 2.5% O₂, 500 ppm NO, balance N₂). The basic experimental layout is shown in Figure 10.

Preparation

The recycle/aeration tank was aseptically filled with 400 ml of F/2 media (described in the media section) in a bio-hood. The volume through the system outside the recycle/aeration tank was calculated to be 90 ml (volume of tubing, filter, flow breaks, pumps, glass capillary and inoculation port). The whole reactor system then was moved to a shaded area close to a window. The location was such that it prevents direct sunlight from hitting the reactor while allowing plenty of indirect sunlight. During night time no extra light source was added to maintain a day/night cycle.

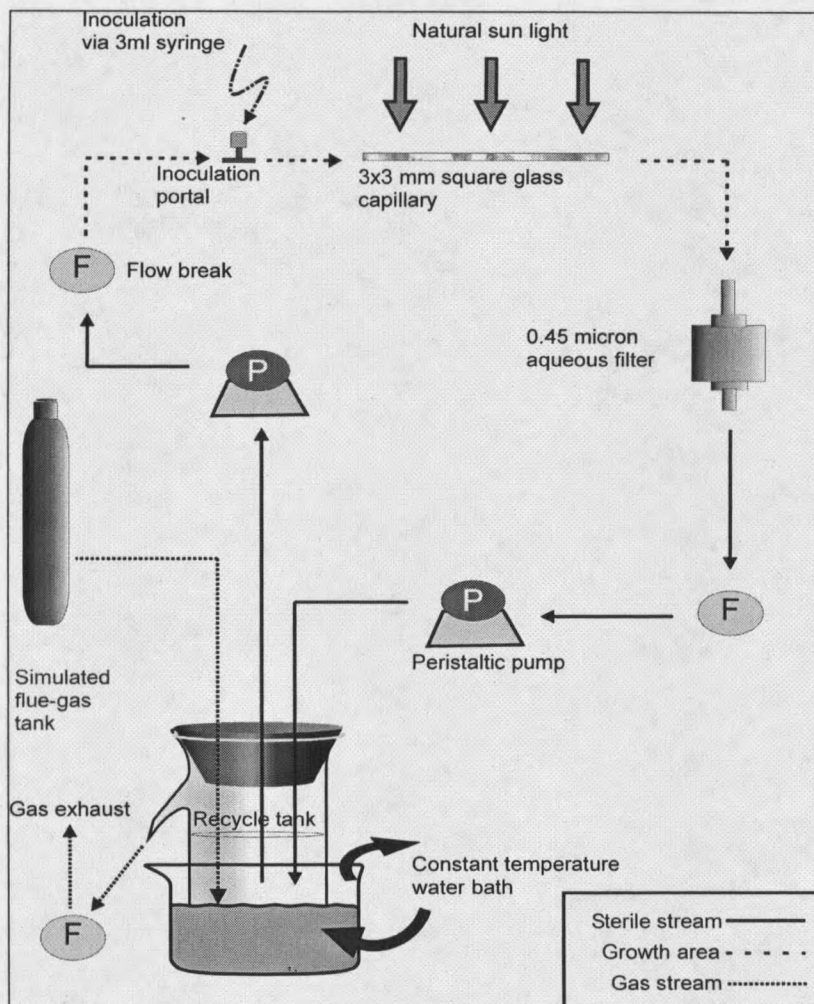


Figure 10. Complete 3 x 3 mm Capillary Flow Cell Schematic

Prior to initiation of flow, the pumps were tested and calibrated. The two peristaltic pumps were then activated at a low flow rate of 0.3 ml/min. Directly afterwards, the aeration gas flow was started with a tank pressure of 1500 psi and an inlet gas pressure of 2 psi. The simulated flue gas started to vigorously bubble through the media via a diffusing stone. To insure that the media throughout the system was saturated with the dissolved gases, the gases were circulated with the initial flow rate for about 24 hours.

Inoculation

Two ml of inoculum was taken from the growth tank using a long needle attached to a 3 ml syringe. The long needle was replaced with a sharper needle needed to puncture the septum of the inoculation port. One ml of inoculum was then inserted into the inoculation port (the remaining 1 ml was in the syringe was reserved for observation under the microscope using a wet slide). When the inoculum (green tinted plume) reached the effluent end of the glass capillary, the tubes connecting both sides of the capillary were clamped shut. Directly afterwards, both the gas input and the media flow were turned off.

The organisms were maintained within the glass capillary under a no-flow condition for 7 days. This allows time for algal cells to attach to the glass substratum (unhindered by flow). The stagnant flow period would also give enough time for the organisms to recover from the shock created by the new media. Since the organisms were originally grown in the same container for over 4 weeks, the salt content of the growth tank must have increased drastically due to evaporation. Upon inoculation, the organisms need to adapt to the lower ionic strength of the media in the capillary tube.

Sampling

The sampling procedures were almost identical to the manner described in Experiment 1. The main difference was that all images were acquired using regular bright field microscopy. The Hg bulb was not used in this experiment.

Table 12. Hydrodynamic characteristics of 3x3 mm Capillary Tube

	<u>Capillary</u>
Bulk fluid flow rate through flow cell	0.3 ml/min
Bulk fluid mean linear velocity	0.06 cm/sec
Reynolds number of flow through the flow cell	1.9
Wall shear of flow through the flow cell	0.00133 N/m ²

RESULTS AND DISCUSSION

Wet Slide Analysis and Observations

Image 4 shows an aged culture of *Dunaliella tertiolecta* in saturated salt solution. This 20x bright field image (Image 4) shows cells of *Dunaliella tertiolecta* adhering to each other and salt crystals. Such behavior was typically seen in older growth containers where evaporation of the sea water medium was prevalent (Image 6). Just the presence of the salt crystals shows that these algal cells are capable of surviving in very high salt concentration. These organisms are known to regulate cell osmolarity in response to osmotic changes in its environment. Swelling or shrinking of the cytoplasm usually associated with osmotic changes was not observed in healthy cells of *Dunaliella tertiolecta*. In some cultures, a small number of cells were grossly enlarged and had a more spherical appearance (Image 5). When viewed under 60x magnification, these larger cells seem to be completely static. On the other hand, the majority of the population (consisting of smaller cells) showed signs of motility. A possible explanation is that these larger cells could be dead or damaged cells that are not able to regulate cell osmolarity, while presumably the smaller more active cells can. Image 5 shows an example of both types of cells under a 60x magnification.

Attachment tendency or cell clustering of *Dunaliella tertiolecta* was observed to be more common in older, nutrient-depleted samples. In younger, nutrient-rich cultures, these flagellated *Dunaliella tertiolecta* were seen to be much more mobile and independent. By looking at the Images 6 and 7, one can see the difference in cell

association between a sample from an aged culture and a sample from a relatively fresh culture. Both images were taken at a 20x magnification.

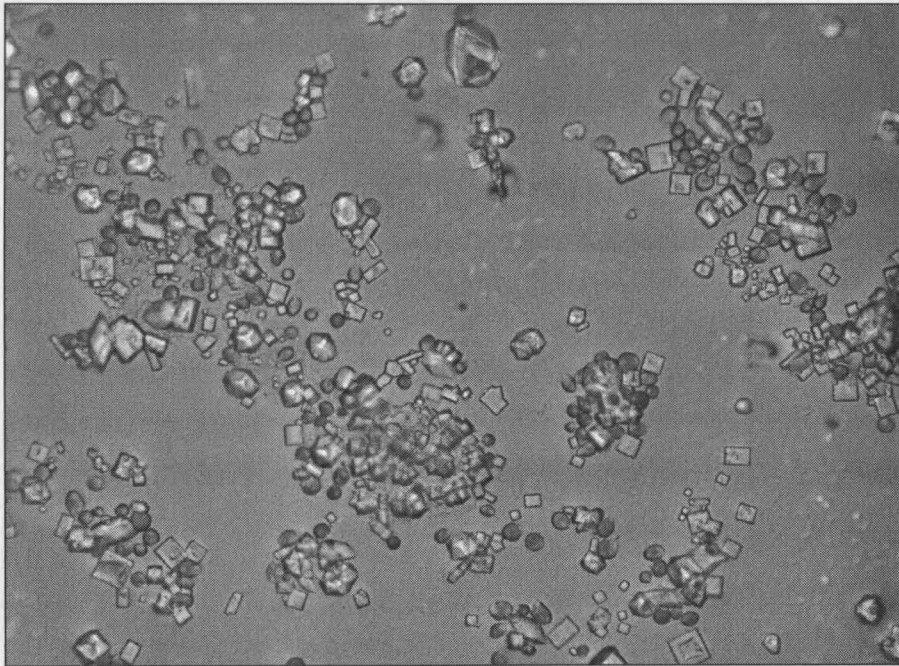


Image 4: Wet Slide of Aged Culture of *Dunaliella tertiolecta* (20X)

Images 8 and 9 show *Dunaliella tertiolecta* attached to solid particles suspended in the growth medium. Individual cells of *Dunaliella tertiolecta* were also seen attached to various particles submerged in the growth medium. In younger cultures attachment was limited to a very small minority of the cells in the container. Nevertheless, this shows that given the right conditions the highly mobile cells of *Dunaliella tertiolecta* are capable of attaching to solid surfaces.

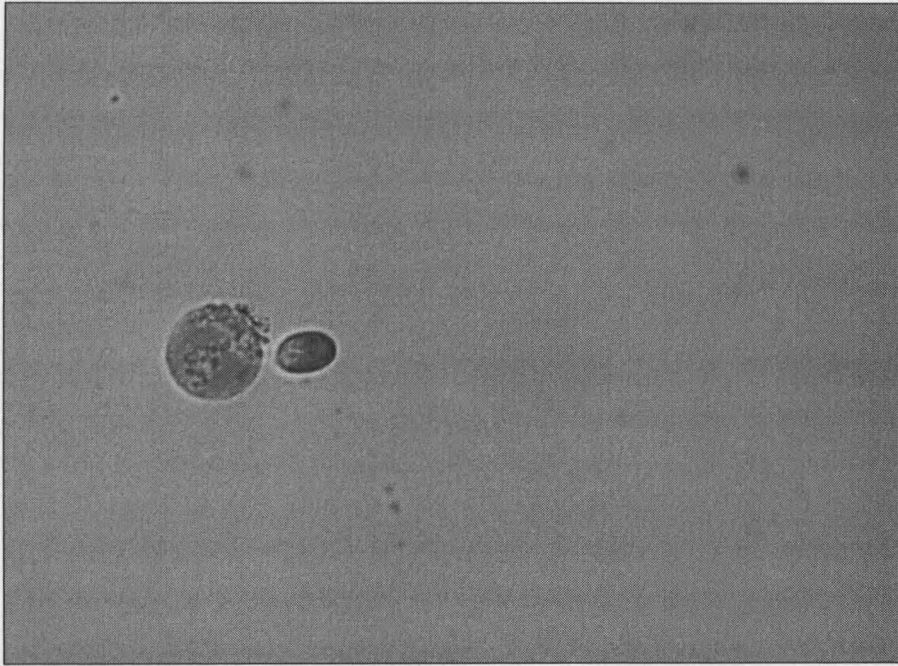


Image 5: *Dunaliella tertiolecta* Cell Types (60X)

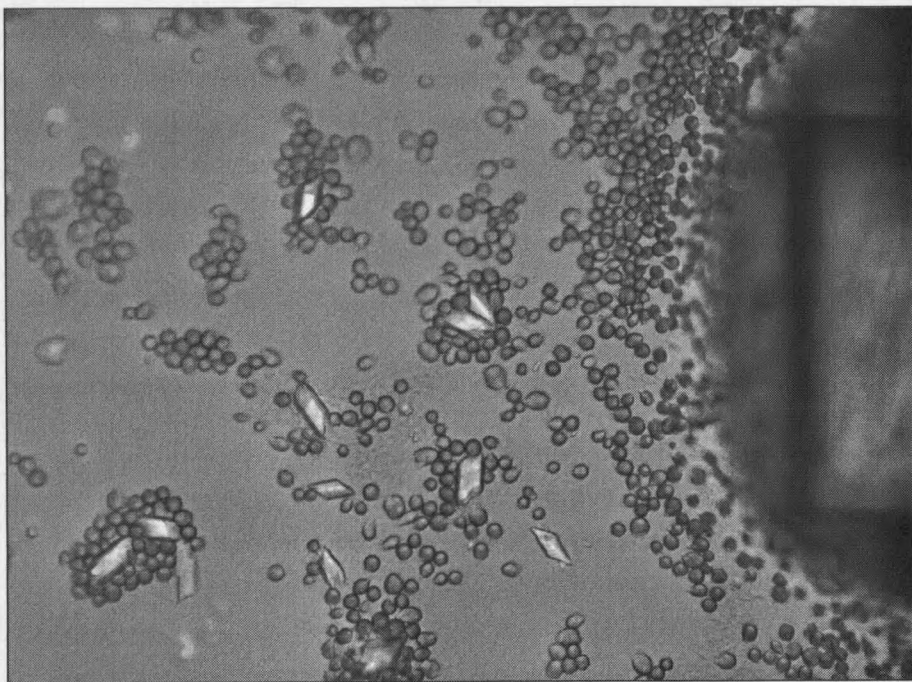


Image 6: *Dunaliella tertiolecta* Aged Culture (20X)

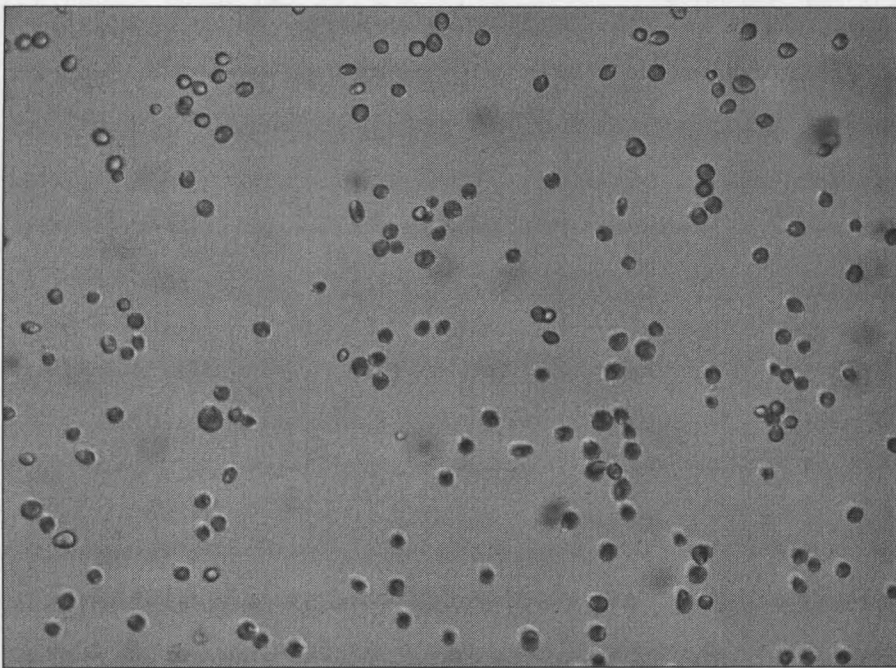


Image 7: *Dunaliella tertiolecta* "Fresh" Culture (20X)

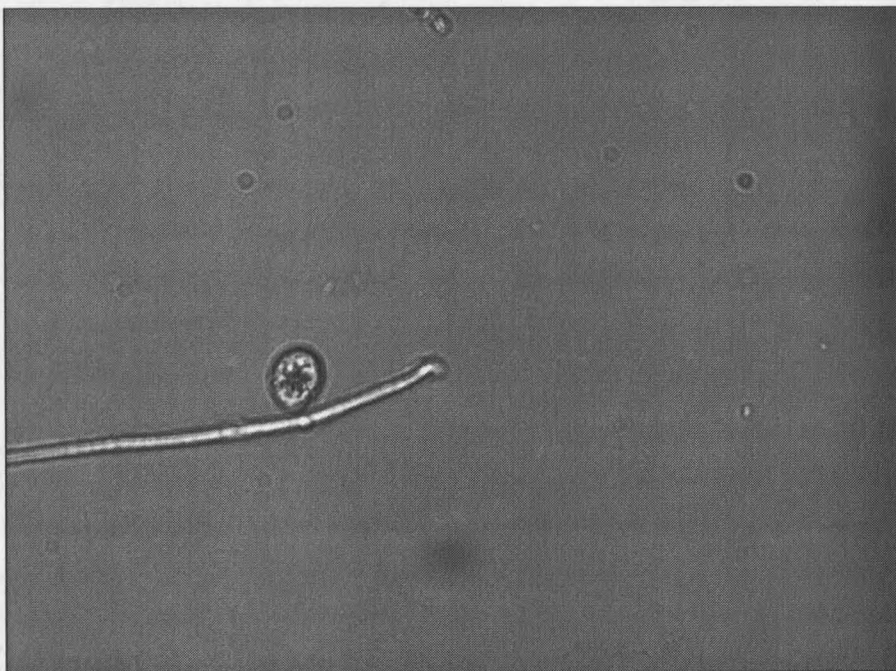


Image 8: *Dunaliella tertiolecta* Cell Attached to an Unknown Particle (60X)

Images 4 through 9 were *Dunaliella tertiolecta* samples on wet slides taken straight from the growth suspension. Other samples were taken by scraping the inner glass surfaces of growth containers. By using the scraping technique, a greater degree of attachment and biofilm formation were observed. Once again, the older and nutrient depleted cultures had a greater tendency of forming microbial mats. It was also noticed that cultures in containers that were continuously stirred (magnet) and aerated (air) displayed greater adherence of cells to glass than the ones which were not.

Image 10 is from a sample that was scraped off the inner glass surface of a growth vessel. The image shows evidence of the presence of some type of extra cellular matrix. Unlike the sides of Image 10 which appear to be relatively free of cellular substance, the space between individual cells in the middle contains a darker, more opaque substance.



Image 9: *Dunaliella tertiolecta* Attached Cells (60X)

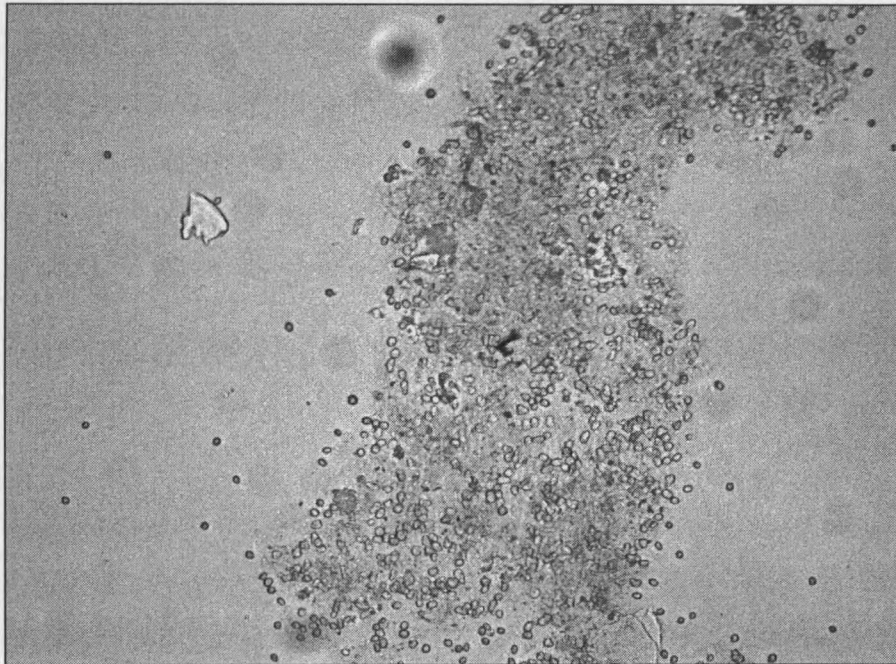


Image 10: *Dunaliella tertiolecta* Bioflim Type Structure (10X)

Image 11 displays this characteristic in a more apparent manner. The two red arrows point out the lateral edges of the matrix type substance. Along the edge few individual cells were seen detaching and dispersing away from the main cluster. Image 12 and 13 show 60x images of the same structure found in Image 10. Image 12 was taken at the outer border of the scraped sample, while Image 13 was taken from the interior of the structure.

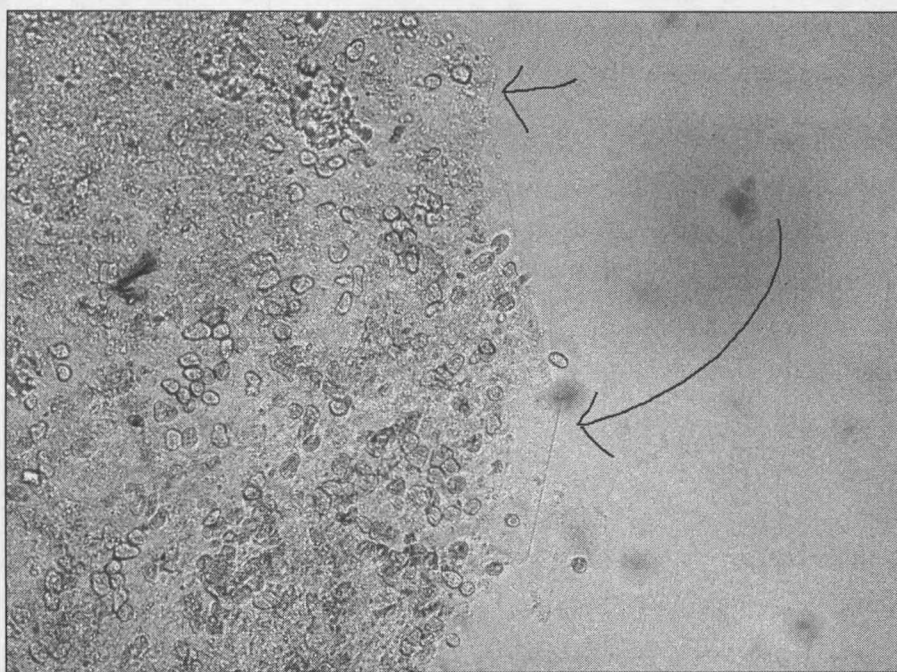


Image 11: *Dunaliella tertiolecta* Biofilm Type Structure (20X)

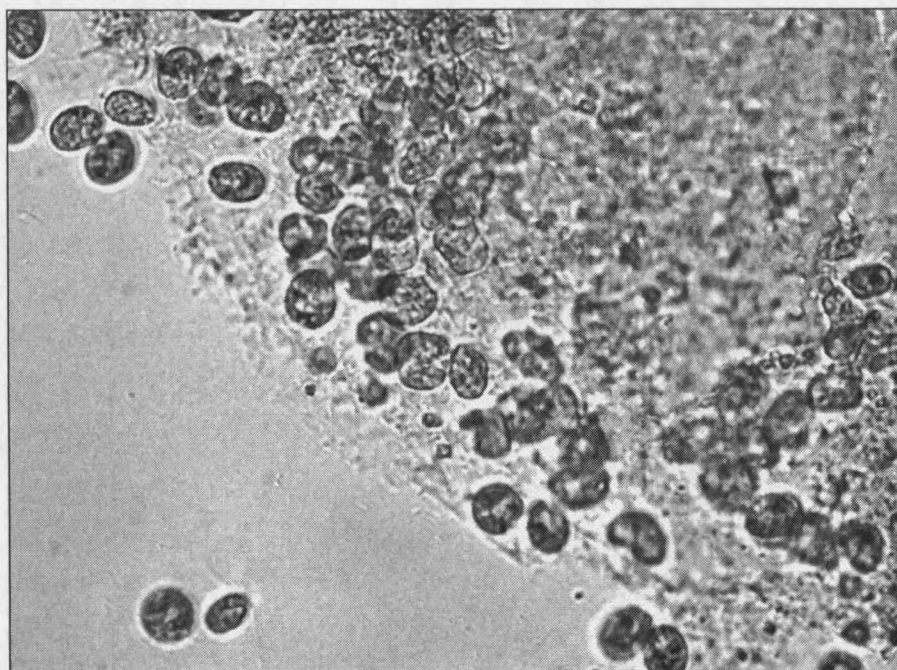


Image 12: *Dunaliella tertiolecta* Edge of Biofilm Type Structure (60X)

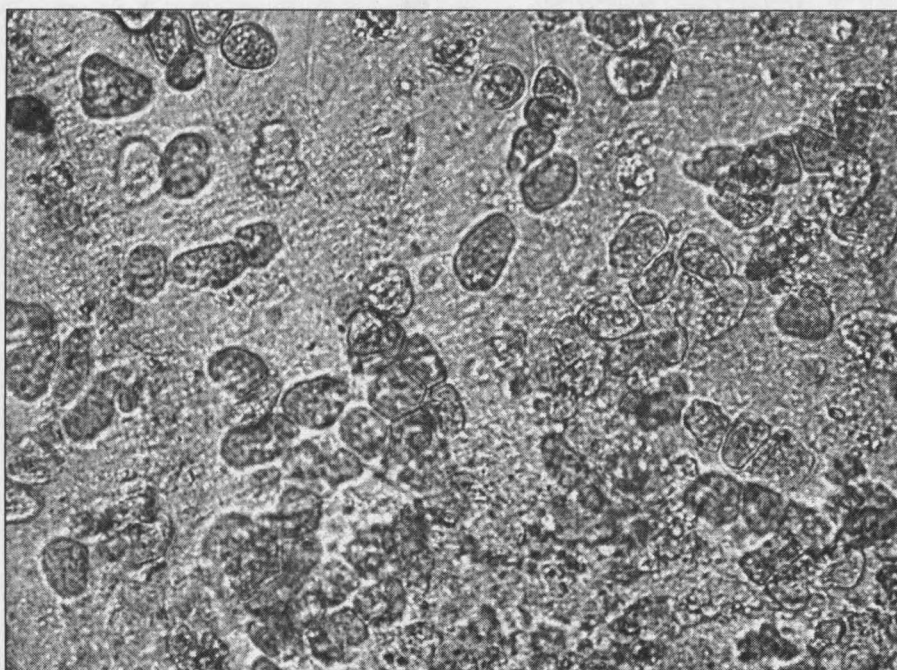


Image 13: *Dunaliella tertiolecta* Interior of Biofilm Type Structure (60X)

Florescence microscopy was also used to observe biofilm formation. Using a mercury bulb at wavelengths between 596 nm and 615 nm, only the cellular components of the micro-algal cells could be viewed. By combining a bright light photo and a florescent photo of the same structure, we were able to demonstrate the existence of supporting matrix. Image 14a is a bright field photo of a scraped sample. Image 14b is a florescent photograph of the same sample view at wavelengths between 596-615 nm. Images 14a and 14b were then combined by using the color combine function in MetaView to create Image 14c. In Image 14c, auto-florescence is shown in red against the blue background. The gray areas where the red shade is absent appears to be a coherent matrix. The same procedure was used to acquire Images 15a through 15c. The chemical components of this matrix type structure are unknown.

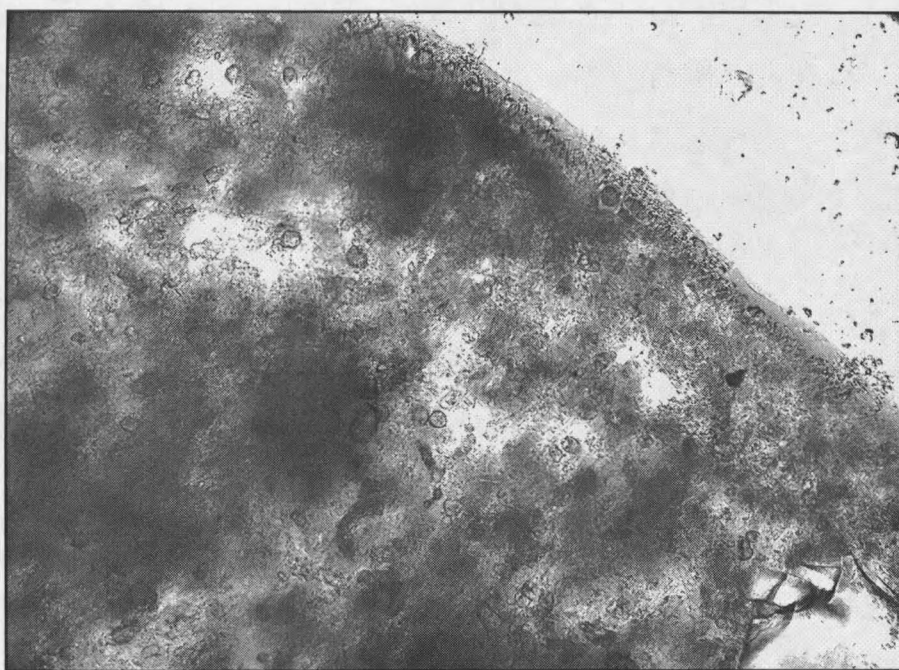


Image 14a: *Dunaliella tertiolecta* Bright field View (10X)



Image 14b: *Dunaliella tertiolecta* Auto-florescent View (10X)

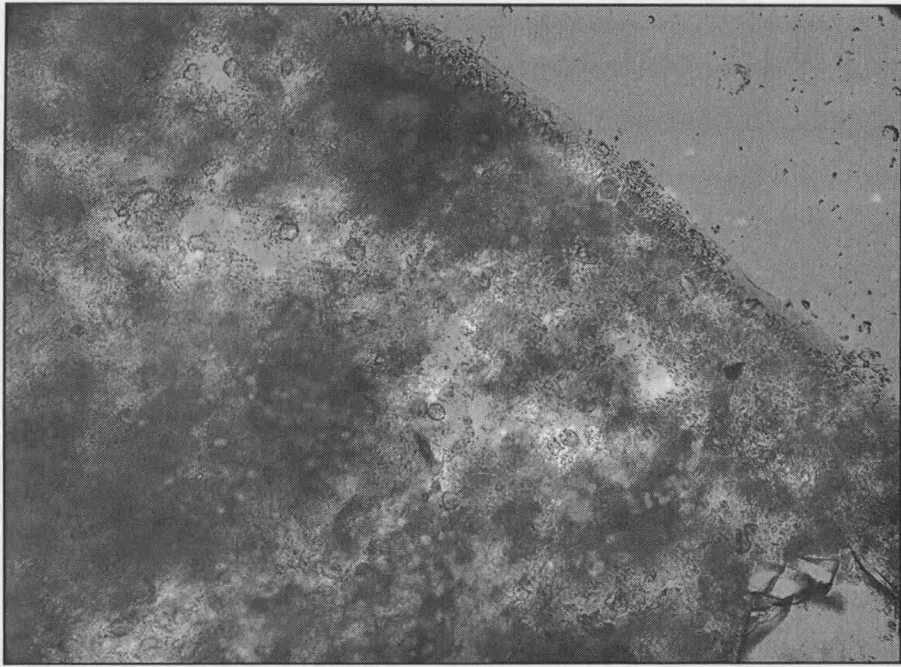


Image 14c: *Dunaliella tertiolecta* Color Combined View (10X)

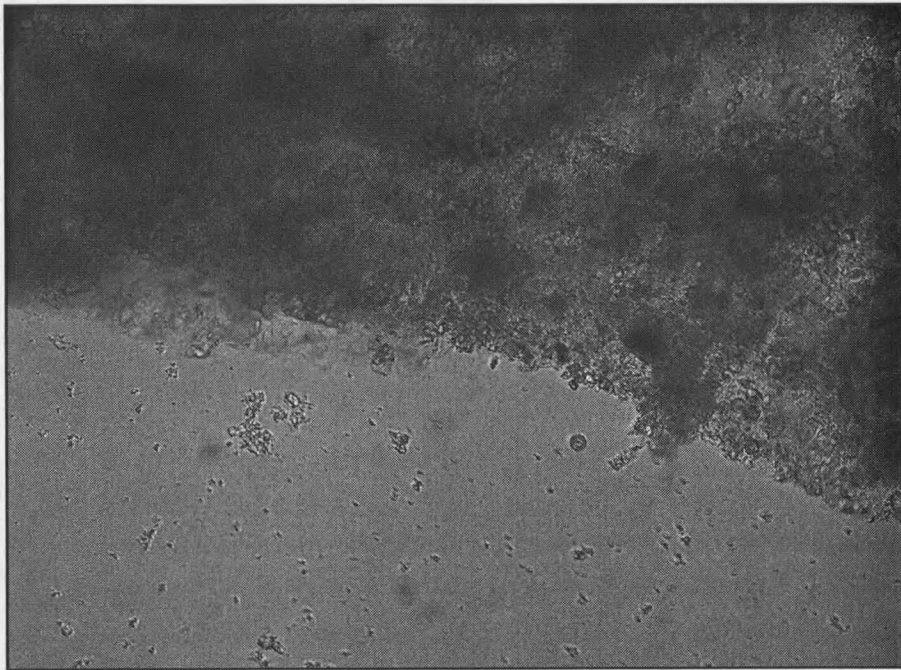


Image 15a: *Dunaliella tertiolecta* Bright field View (10X)



Image 15b: *Dunaliella tertiolecta* Auto-fluorescent View (10X)

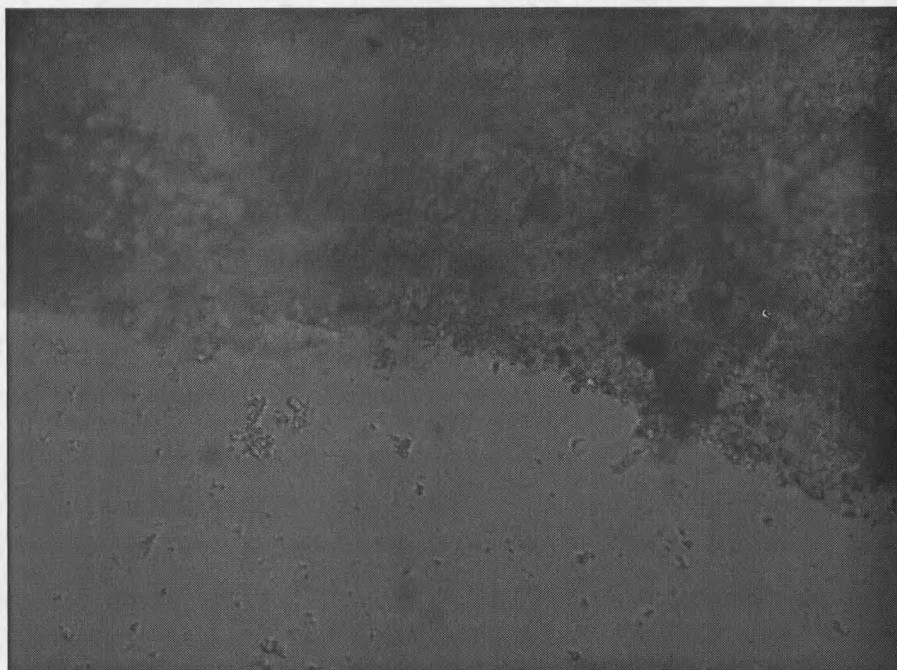


Image 15c: *Dunaliella tertiolecta* Color Combined View (10X)

To further investigate the composition of the matrix, the wet slides of scraped samples were stained with DAPI. DAPI is widely used to stain nucleic acids and can be viewed by confocal light at 358 and 461 nm. The slide shown in Image 16a-16c was stained with DAPI and viewed at range 358-461 nm, and then the same slide was viewed at 582-600 nm to observe auto-florescence. Images 16a-16c were all taken at 60X magnification; Image 16a shows auto-florescence, Image 16b shows the DAPI stain; and Image 16c is the color combination of both previous images.

With a combination of DAPI images with auto-florescent images the presence of extra-cellular non-algal DNA can be ascertained. Although DAPI will stain both bacterial (prokaryotic) and algal (eukaryotic) nucleic acids, the DAPI concentration and stain incubation times did not allow the stain to penetrate the algal plasma membrane. The stain only stained nucleic acids outside the plasma membranes. This leads to an intriguing discovery. DAPI images showed the presence of a large number of associated organisms. These organisms, presumably bacterial, appeared to be in a state of co-habitation with the micro-algae. In Image 16c and Image 17c the algal cells are colored red (auto-florescence); the bacterial cells are shown in blue (DAPI). The blue color may also represent some algal nucleic acid particles that are present outside the algal plasma membrane. The supporting structure gluing the whole 'biofilm' together could be of bacterial and algal origin.



Image 16a: *Dunaliella tertiolecta* Auto-Florescent View (60X)



Image 16b: *Dunaliella tertiolecta* DAPI (60X)



Image 16c: *Dunaliella tertiolecta* Color Combined View (60X)



Image 17a: *Dunaliella tertiolecta* Auto-fluorescence (60X)



Image 17b: *Dunaliella tertiolecta* DAPI (60X)



Image 17c: *Dunaliella tertiolecta* Color Combined View (60X)

Wet slide image analysis was primarily done on *Dunaliella tertiolecta* samples. Growth cultures of *Botryococcus braunii* didn't show much variation from a younger container to aged container. *Botryococcus braunii* was also extremely slow growing and sufficient sampling volumes were not present for this type of observation. The few, observed samples showed consistent colony formation (Images 18-20). Wet slide images showed the existence of hydrophobic substances, presumably hydrocarbons, around larger colonies (Image 1). *Botryococcus braunii* did not show any signs of attachment to solid surface on these wet slides.

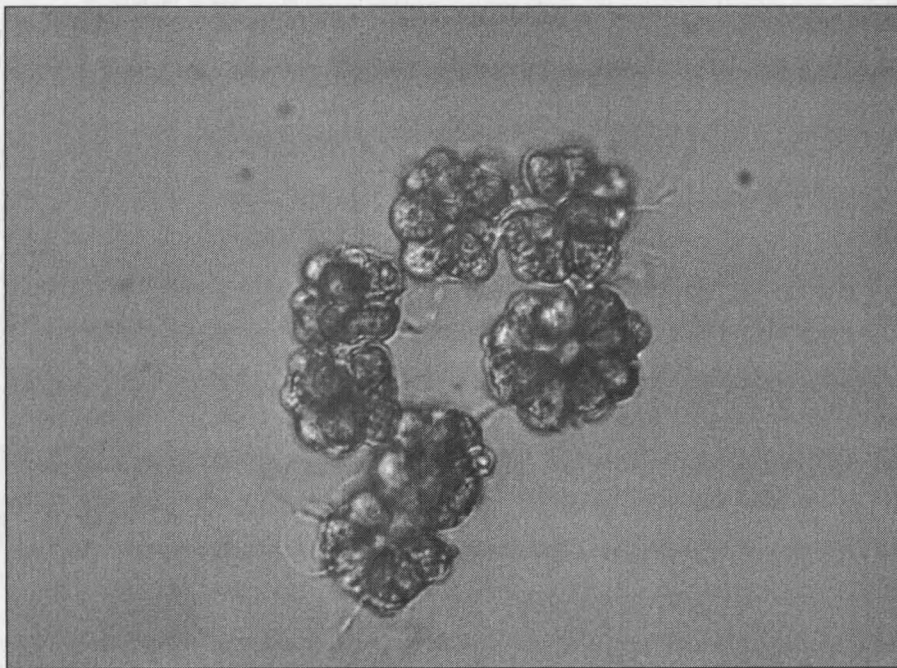


Image 18: *Botryococcus braunii* Loosely Connected Colonies (60X)

Images 20a through 20c illustrate the auto-florescence capability of *Botryococcus braunii*. Individual cells within the colonies can be clearly identified using auto-florescence. Image 20c is a color combination of the bright field and auto-florescent images.

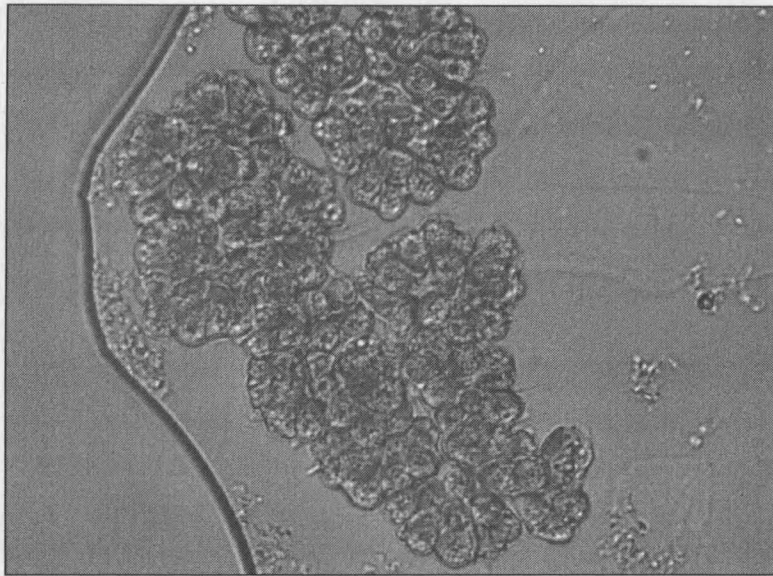


Image 19: *Botryococcus braunii* Large Colonies (60X)

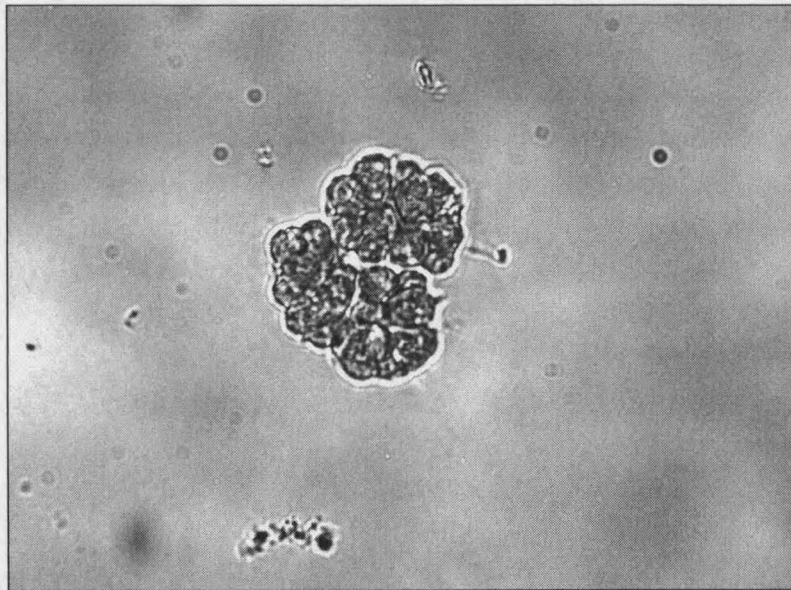


Image 20a: *Botryococcus braunii* Bright field View (60X)



Image 20b: *Botryococcus braunii* Auto-florescence (60X)

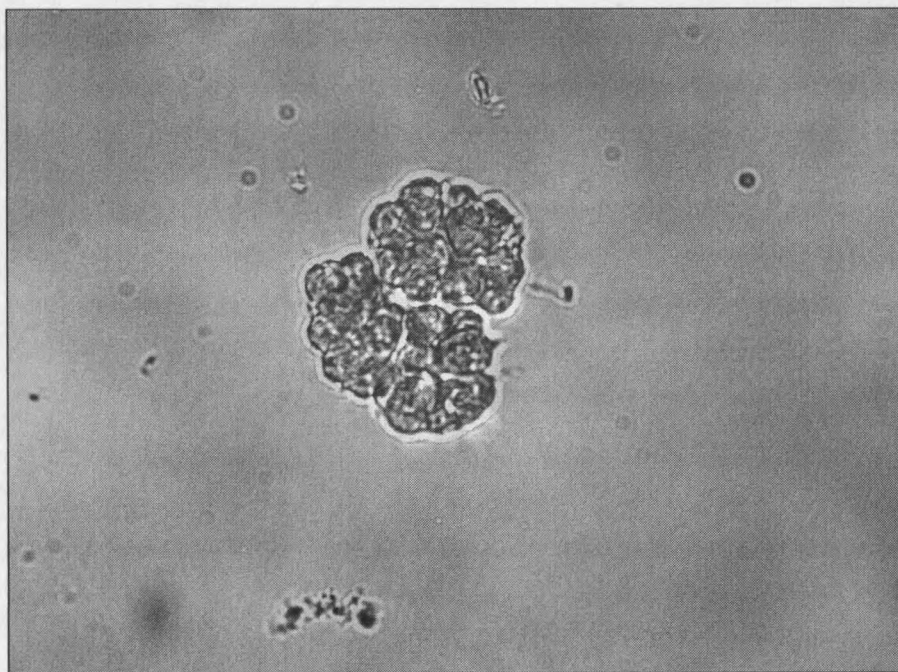


Image 20c: *Botryococcus braunii* Color Combined View (60X)

Capillary Flow-Cell Attachment Observations

The 3 by 3 mm capillary flow-cell experiments were carried out to make observations of micro-algal attachment under flow conditions. Some semi-quantitative results are possible, and several valuable observations were made. All images were taken using the 10x and 20x objective lenses. Higher magnifications could not be used because the capillary was too thick to accommodate the larger dimensions of the higher objective lens.

Right after the inoculation, the once clear glass capillary acquired a slightly green tint. The inner bottom surface was modestly greener than the rest of the flow chamber. This indicates an increase in cell density at the floor of the capillary, probably due to the gravitational sedimentation of cells. Image 21 is a bright field view of the capillary floor at a 20x magnification. The red circles were drawn around cells that seem to be immobile and adhering to the glass surface. On the other hand, the cells that are blurred and elongated in appearance were in rapid motion. Image 22 is the very same region of the capillary, but taken at a 10x magnification. The photo shows that about 5 to 10 % of the cells in view give the impression that they are indeed adhering to the bottom of the tube. When the flow-cell was slightly agitated, a majority of the cells circled in red remained fixed and did not shift. Both images 21 and 22 were taken 2 days after inoculation, before the initiation of flow. Image 23 was taken using tritc filter at 20X magnification. The smaller bright spot indicate cells that are adhering to the glass and the longer, more blurred speckles are cells in motion.

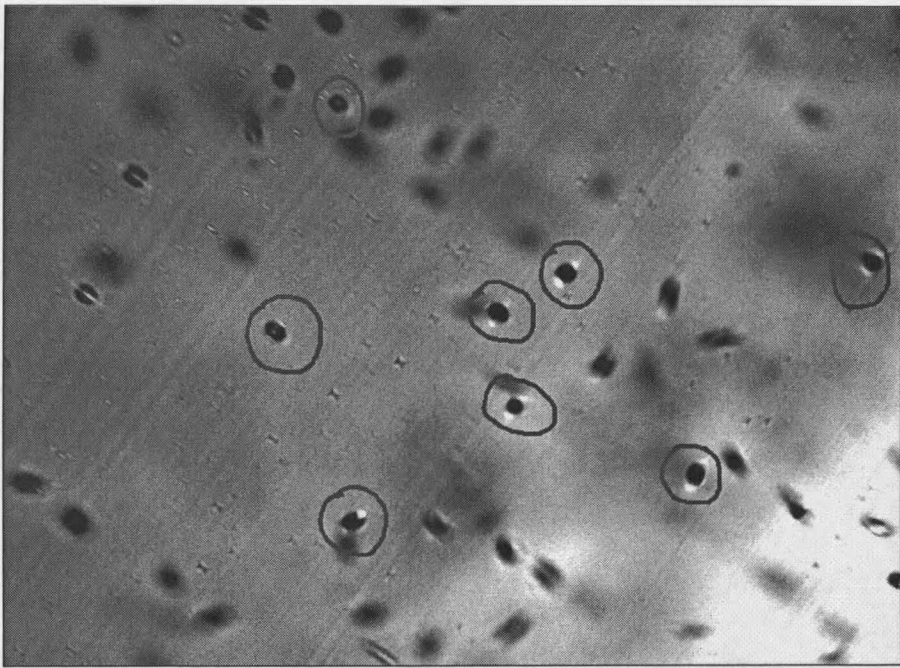


Image 21: *Dunaliella tertiolecta* Bottom Surface (20X)

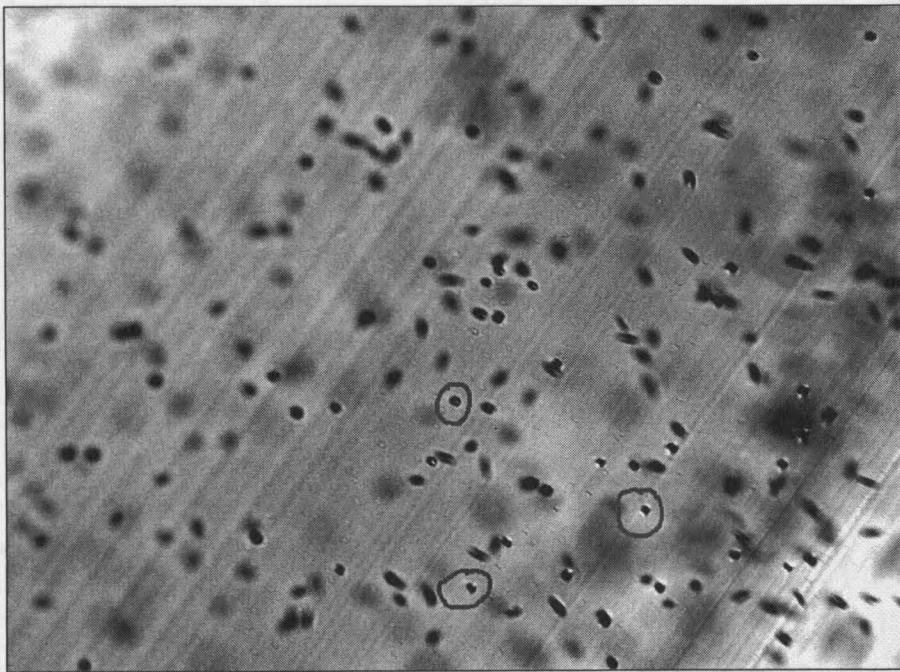


Image 22: *Dunaliella tertiolecta* Bottom Surface (10X)

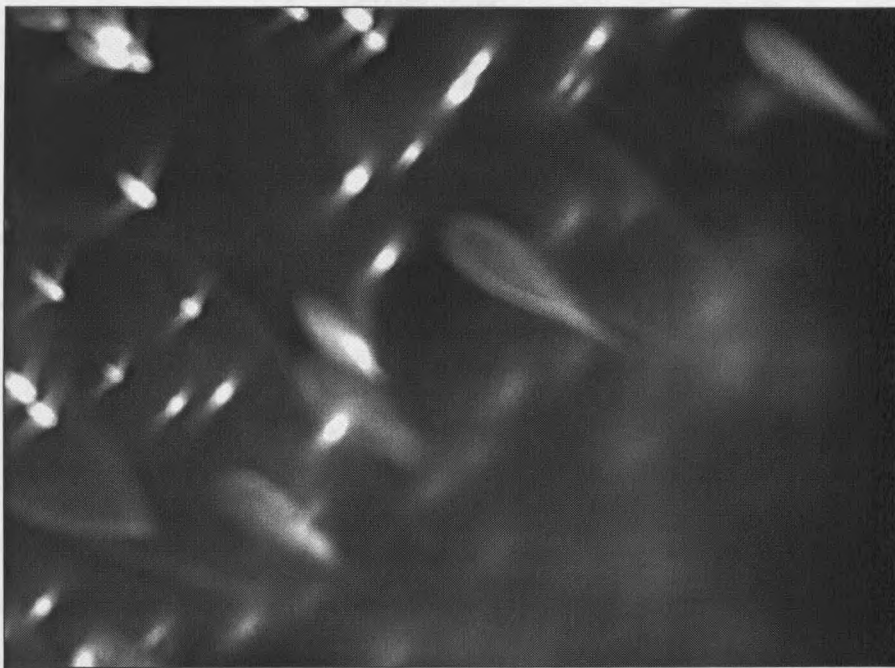


Image 23: *Dunaliella tertiolecta* Tritc Filter View of Bottom Surface (20X)

Images 24 and 25 are bright field photographs taken at 10x magnification. These pictures were taken 2 days after the capillary column was inoculated. At this stage flow had not been initiated and the inoculum was confined within the glass capillary. They show dark accumulations of cells located in the bottom corner of the capillary tube. When this flow cell was lightly agitated these cluster were dislodged from their original positions, demonstrating that they were not attached to the glass surface. Unlike the large clusters, some individual cells were seen adhering to the floor of the capillary tube. The *Dunaliella tertiolecta* cells that were not attached, nor part of the larger clusters, were seen moving rapidly in a random manner. Cells that were ether attached or part of a cluster seem to have lost, or temporarily lost, the ability for self propelled motion. Surface attachment or cluster formation could possibly be associated with a phenotypic change.

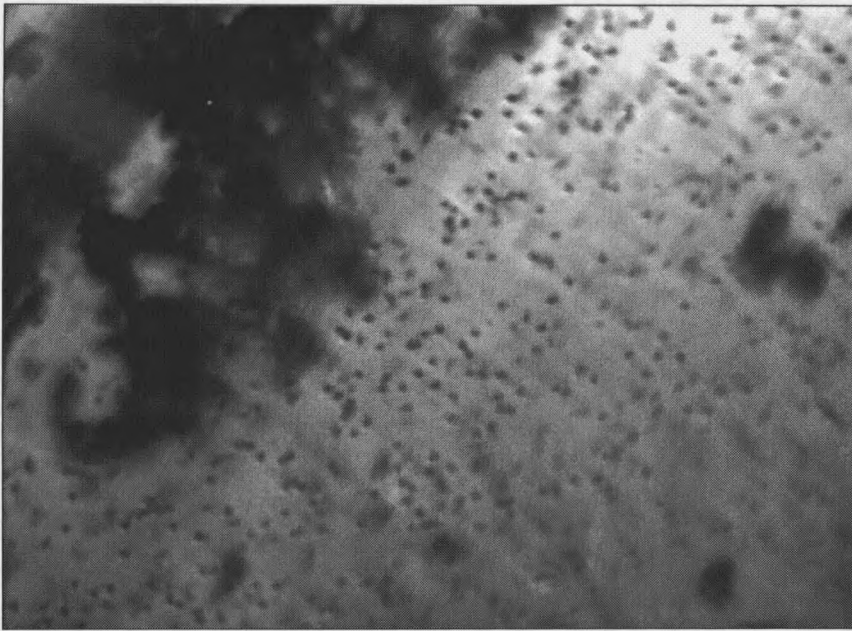


Image 24: *Dunaliella tertiolecta* Bottom Corner (10X)

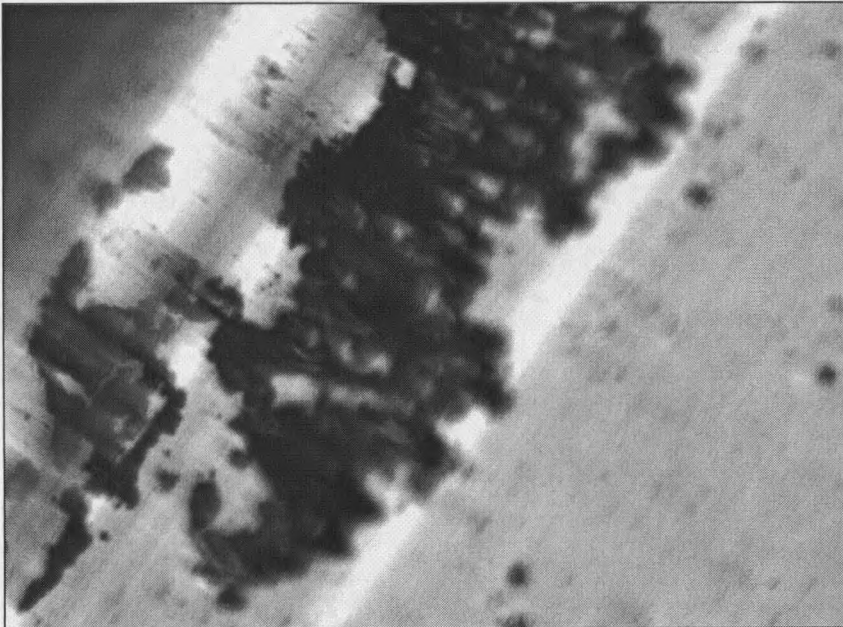


Image 25: *Dunaliella tertiolecta* Bottom Corner (10X)

After flow was initiated, photographs of the interior of the capillary tube were periodically taken. The imposed flow rate through the system was kept constant at 0.3 ml/min. The linear average velocity through the capillary duct was 0.056 cm/sec. The Reynolds number of flow through the square conduit was calculated to be 1.852, indicating laminar flow. Using the corresponding laminar flow equations the shear stress within the capillary was calculated to be 0.00133 N/m^2 .

As media was pumped through the capillary, large portion of cells were washed out of the duct. After the wash out phase, the algae in very small cell clusters were seen adhering to the glass walls. Following 2 days of constant flow, the flow was then halted for another 2 days. Images 26 and 27 were taken 2 days after flow was stopped this second time (11 days after inoculation). Image 27 show cells firmly attached to the bottom glass floor. Both Images 26 and 27 show a degree of clustering. Clustering of cells could possibly be due to cell division and multiplication. It is highly likely that these clusters were produced by a single 'mother' cell initially attaching to the surface. Consistent with the 'mother cell' hypothesis, the observation of the spacing of cells indicates algal growth and multiplication taking place in a lateral manner. At this stage, growth of attached cells is presumed to be dominantly parallel to the glass surface. Perhaps, growth leading away from (perpendicular) to the substratum could be observed once a complete mono-layer covers the surface.

Images 28 and 29 show side views of the bottom surface of the capillary tube. These photos were taken after: (1) 7 days incubation in capillary, (2) one day of flow and (3) two days without flow. By this time all most all planktonic cells had been washed

down the system. Both photographs clearly show a dense green mono or bilayer formation.

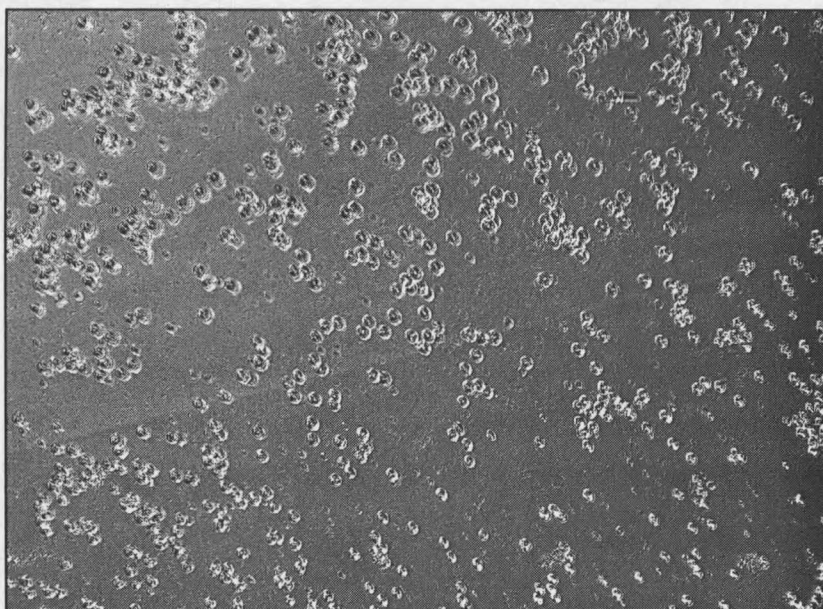


Image 26: *Dunaliella tertiolecta* Bottom Surface After Flow (20X)



Image 27: *Dunaliella tertiolecta* Bottom Surface After Flow (20X)

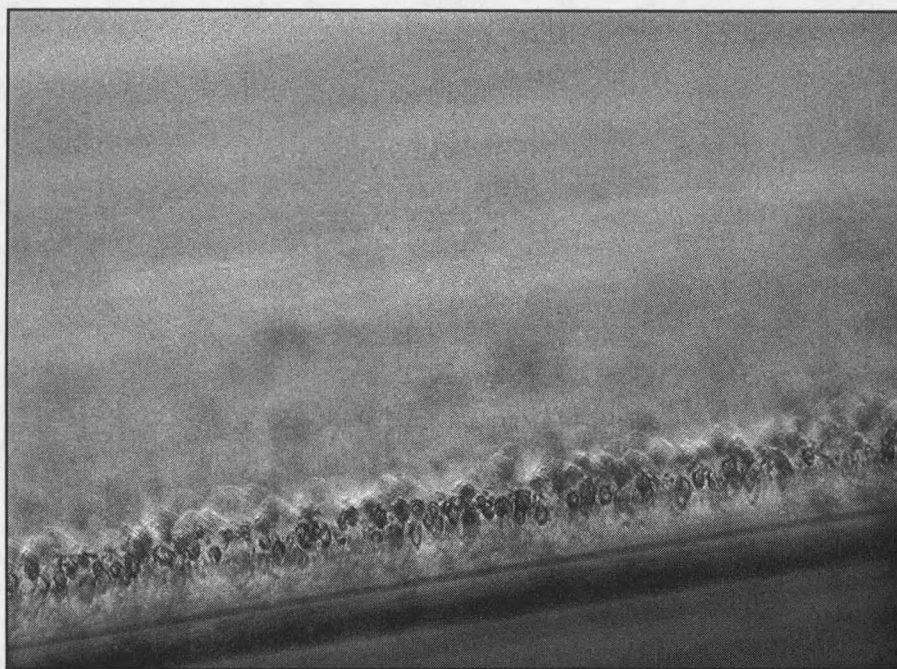


Image 28: *Dunaliella tertiolecta* Mono-layer After Flow (20X)

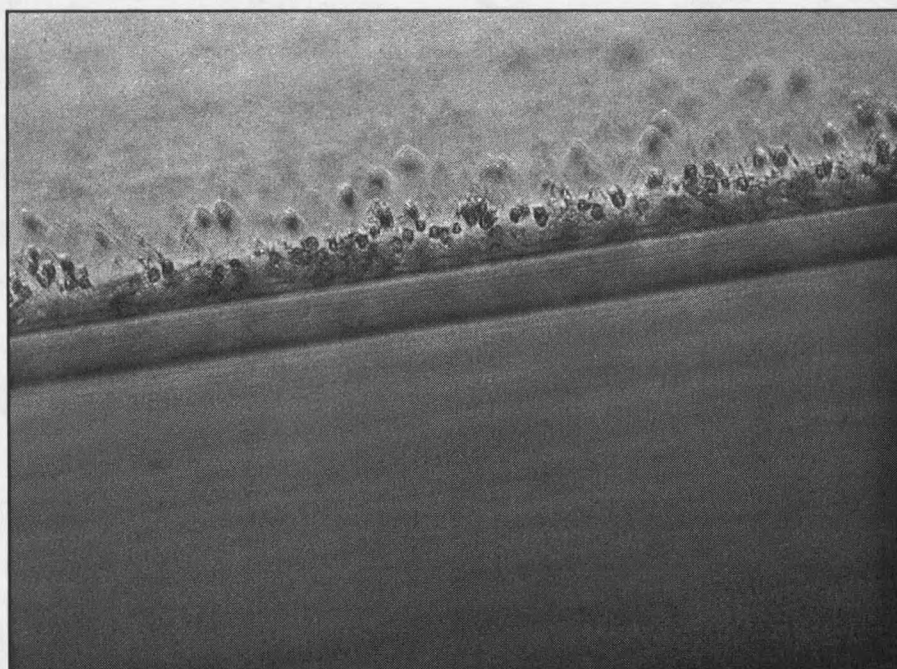


Image 29: *Dunaliella tertiolecta* Mono-layer After Flow (20X)



Image 30: *Dunaliella tertiolecta* Mono-layer After Flow (20X)

The couple of key observations were made from the 3 mm by 3 mm capillary tube experiments on *Dunaliella tertiolecta*. Clear evidence of attachment on glass surfaces was observed. The experiments confirmed that mono and bi-cellular layers could be grown under conditions of laminar flow. A thicker inoculum and a greater experimental duration could possibly produce thicker biofilms. One still has to keep in mind that these

organisms are quite large in size, and thicker layers of cell would greatly reduce light and nutrient penetration to the interior.

One capillary was inoculated with *Botryococcus braunii* and observed for surface attachment. Because the inoculum was not very dense it was very difficult to find large colonies within the capillary. Image 31 show an instance where a colony was seen adhering to the glass surface. The lower part of the structure had a brownish green color, indicating that it indeed was an algal colony, not an artifact. The spherical bubble seen in the center could be ether air, or oil embedded in the colony. This image was the only instance observed that indicate some type *Botryococcus braunii* of attachment. Thus there was no strong, conclusive evidence in this experiment suggesting a tendency of *Botryococcus braunii* to attach to the glass surfaces.

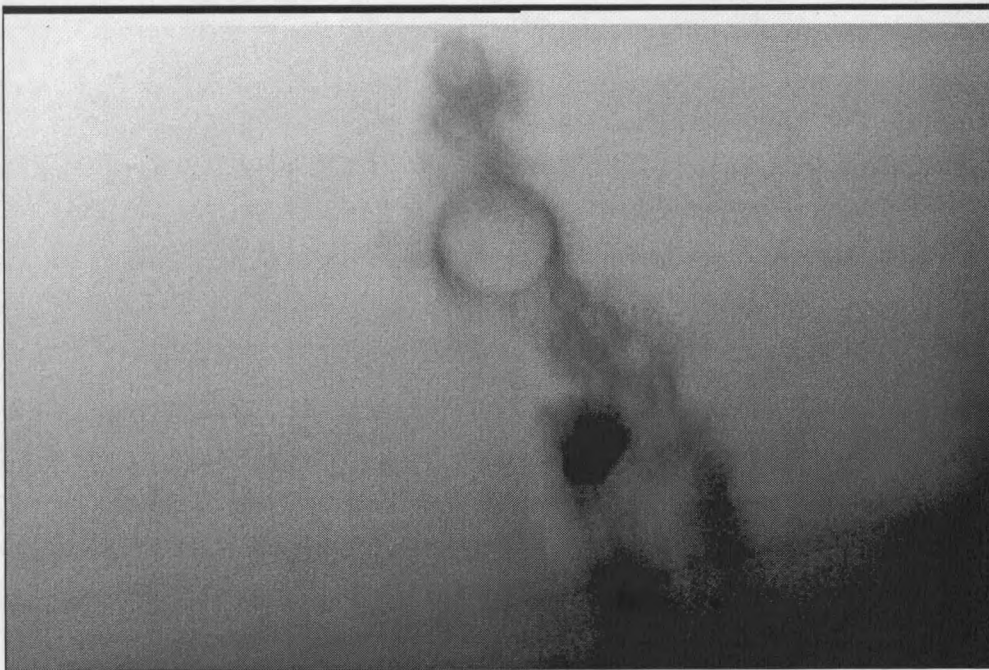


Image 31: *Botryococcus braunii* flow-cell bottom (20X)

Parallel Plate Flow Cell Results *Dunaliella tertiolecta*

Dunaliella tertiolecta on Aluminum

The attachment of cells to an aluminum coupon inside a flow cell was analyzed. Four different locations of the coupon surface that appeared to accommodate the greatest number of attached cells were chosen for sampling. The sampling area in each one of those locations was 0.65 mm^2 . Figures 11 through 14 show the number of attached cells as a function of time for each of these locations after starting flow.

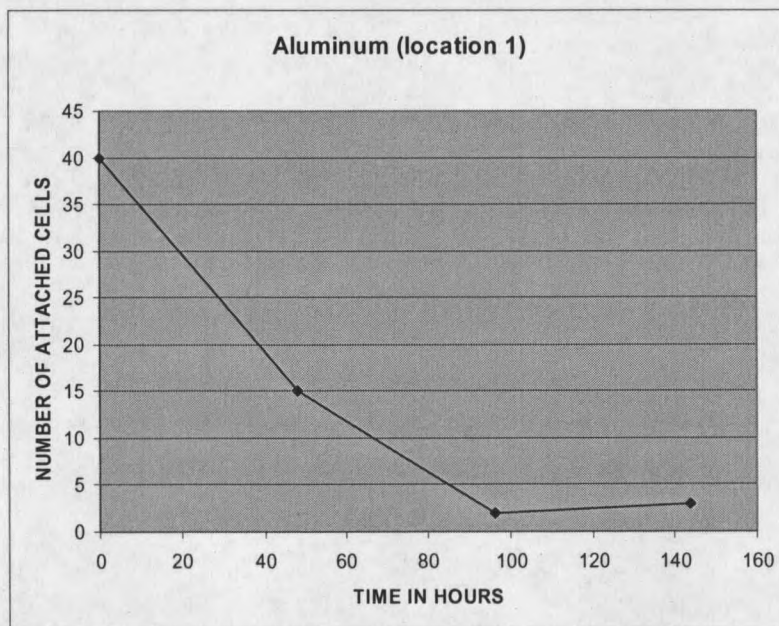


Figure 11: *Dunaliella tertiolecta* on Aluminum Coupon (location 1). Number of cells attached to a 0.65 mm^2 sampling area of an aluminum coupon, under flow conditions.

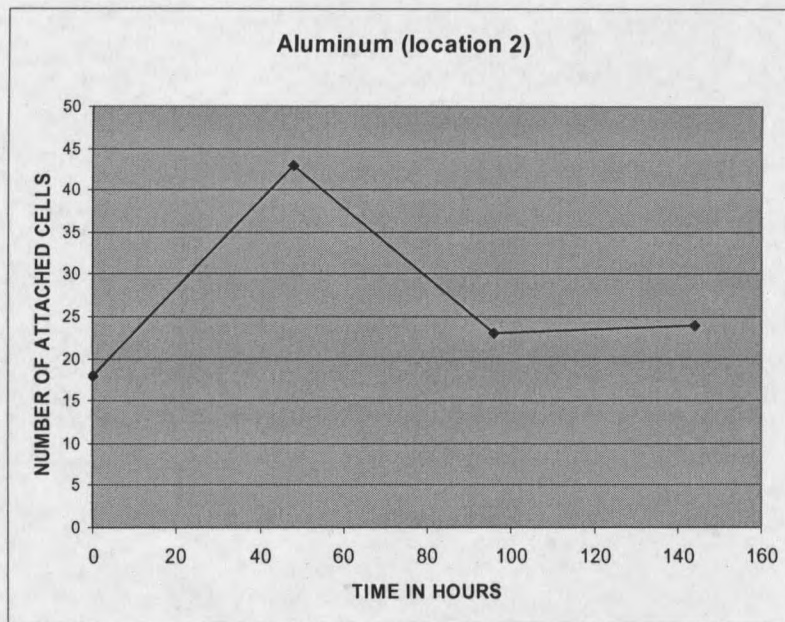


Figure 12: *Dunaliella tertiolecta* on Aluminum Coupon (location 2). Number of cells attached to a 0.65 mm² sampling area of an aluminum coupon, under flow conditions.

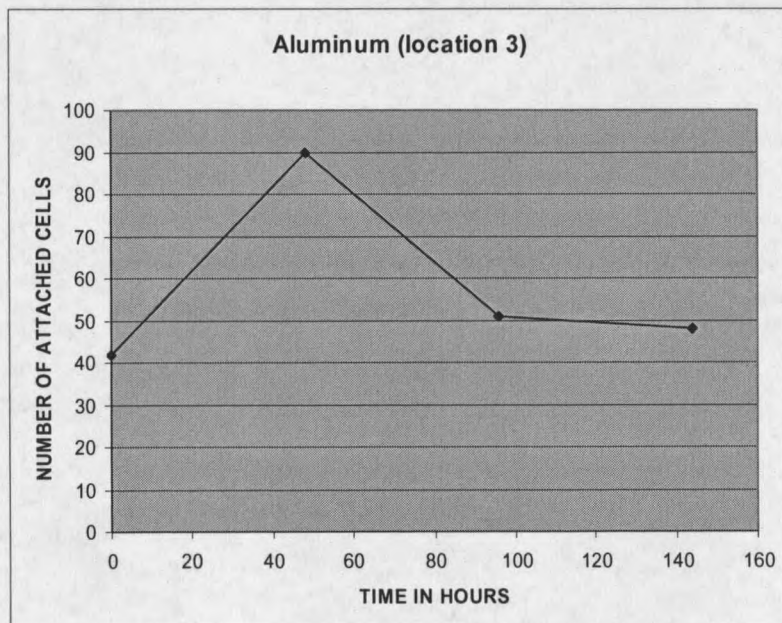


Figure 13: *Dunaliella tertiolecta* on Aluminum Coupon (location 3). Number of cells attached to a 0.65 mm² sampling area of an aluminum coupon, under flow conditions.

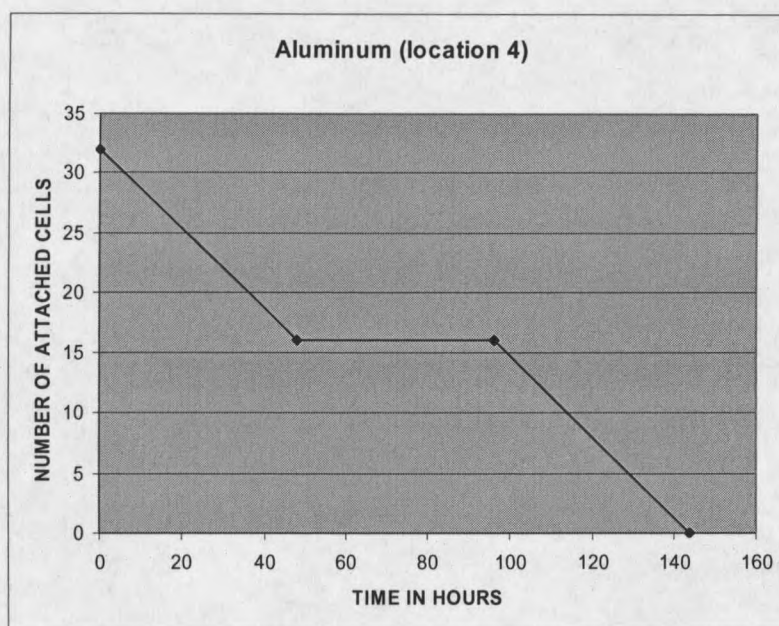


Figure 14: *Dunaliella tertiolecta* on Aluminum Coupon (location 4). Number of cells attached to a 0.65 mm^2 sampling area of an aluminum coupon, under flow conditions.

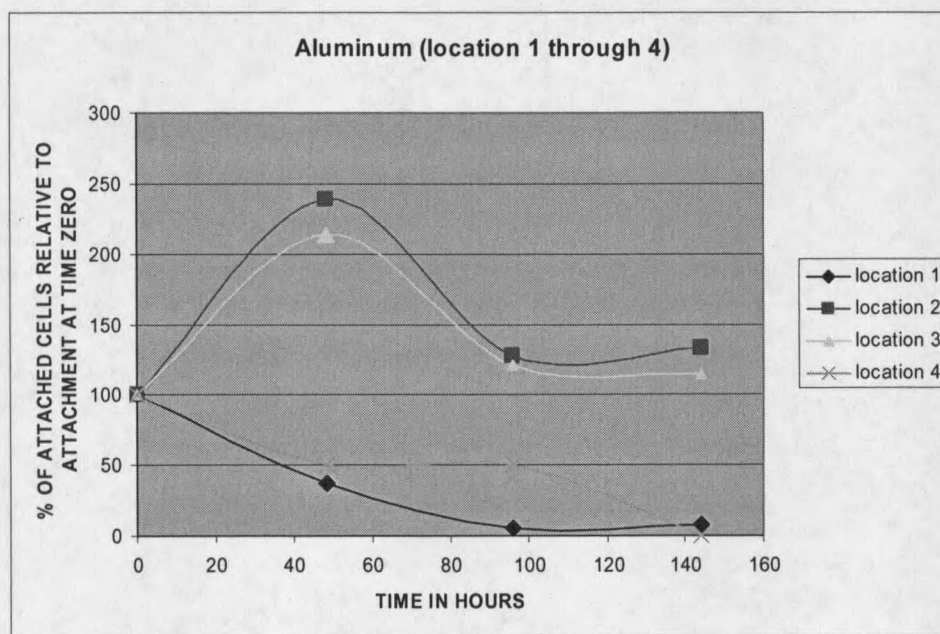


Figure 15: *Dunaliella tertiolecta* on Aluminum Coupon. Comparison of location 1 through 4 on the aluminum coupon

The aluminum coupon experiment produced interesting results. In locations (1) and (4) the number of adhering cell dropped substantially in the first 48 hours after flow was activated. In both those locations about 50% of the originally attached cells either detached or were eroded by the flow induced shear. After the heavy loss of adhering cells in the first 48 hours, the number of adhering cells gradually decreased, approaching zero in 140 hours. The detachment data from the other two locations, (2) and (3) yielded different results. In location (2) and (3) the number of attached cells actually increased in the first 48 minutes of flow. In both these locations the number of cells fastened to the surface essentially doubled in quantity. During the next few time-steps (48 to 144 hours) the number of adhering cells in location (2) and (3) reduced in a similar manner to the results seen in the first 48 to 96 hours on location (1) and (4).

An increase in the initial net cell adherence could be due to new attachment and cell replication. Cell multiplication could be excluded because of the fact that *Dunaliella tertiolecta* had relatively low growth rates (in the media & conditions used in this experiment) and insufficient growth to make an impact considering the duration of the experiment. The early increase in attached cells in location (2) and (3) could possibly be explained by examining the surface features of the coupon. Even before flow was administered, the attachment of cells to the surface varied from location to location. The reason that some locations had high adsorption rates during the first 48 hours of flow could be attributed to the presence of 'preferred sites of attachment'. In flow studies using *Pseudomonas aeruginosa*, Mueller suggested the notion that there might be certain sites on the surface where cells absorbed faster (Vanhaeke et al., 1990). Also, under flow conditions these micro-algal cells might have the ability to detach/erode from one

location and reattach to a more suitable location further down stream. The quantity of adhering cells probably continued to increase until these 'preferred sites of attachment' were mostly occupied. A possible scenario could be, once the sites were full, that weakly adhering cells were the first to get washed out down stream. The detachment rate started to fall soon afterwards because the remaining cells displayed a greater degree of adherence. According to the results there seems to be evidence of both reversible and irreversible forms of attachment. In location (2) and (3) the number of attached cells remained relatively constant from 96 minutes onwards. This could possibly indicate the presence of a few cells that are irreversibly attached to the surface. Experiments could be run for a longer duration to further investigate attachment.

The areas that harbored large numbers of attached cells could have different surface roughness values. The variation in surface roughness from location to location could have happened due to various means including heterogeneous deposition of media components, bacterial film formation, or surface reactions (i.e. oxidation). Surface roughness is known to influence bacterial accumulation on a substratum (Vanhaecke et al., 1990). Attachment studies on bacteria suggest that small cracks and grooves protect adhering microbes from shear stress and provide a greater surface area for attachment. Images of the aluminum surface taken after this experiment illustrated its heterogeneous nature of the surface (Image 32). Roughness might play an important role on the surface adherence of *Dunaliella tertiolecta*. Interestingly, in certain areas that harbored large amounts of cells, a misty or turbid substance was seen auto-florescing.

The algal cells were bright red in color, while this unknown substance was a yellowish orange (viewed with filter, excitation 596 nm and emission 615 nm).

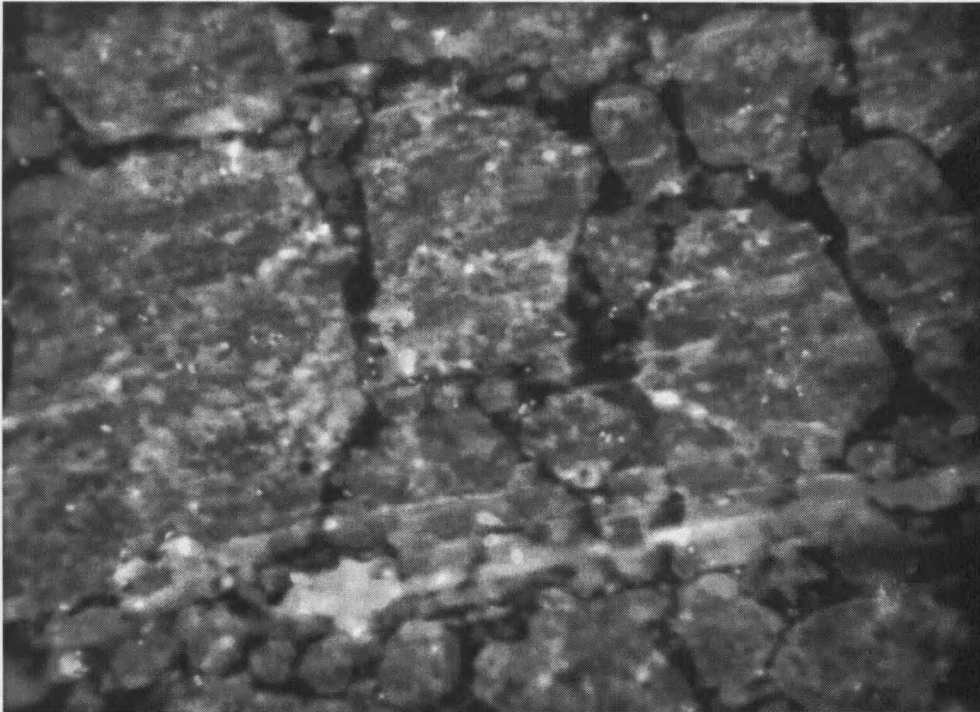


Image 32: (60x) Aluminum Surface Stained with DAPI After the Experiment

Dunaliella tertiolecta on Teflon

The attachment data of cells on the Teflon coupon was next analyzed. Four different locations of the coupon surface that appeared to accommodate the greatest number of attached cells were chosen for sampling. The sampling area in each one of those locations was again 0.65 mm^2 . Figures 16 through 19 show the number of attached cells as a function of time.

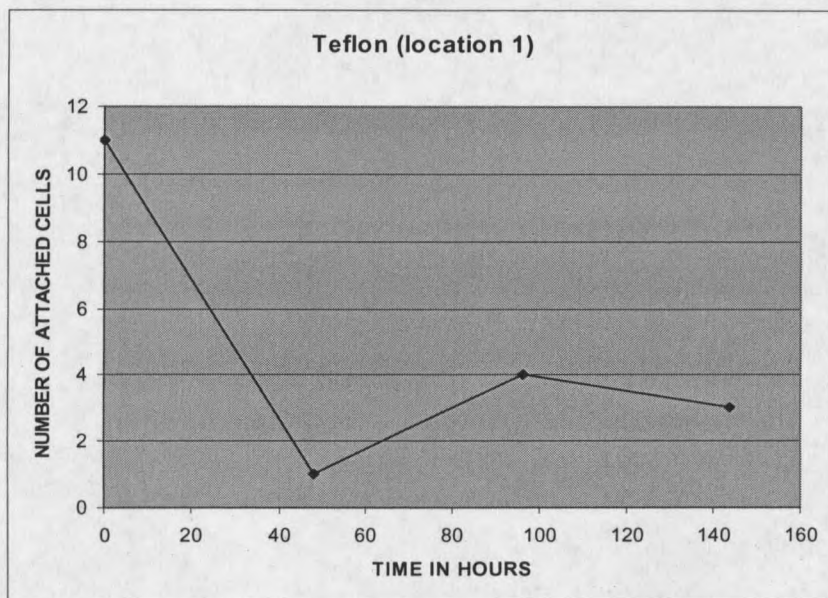


Figure 16: *Dunaliella tertiolecta* on Teflon Coupon (location 1). Number of cells attached to a 0.65 mm^2 sampling area of a Teflon coupon, under flow conditions.

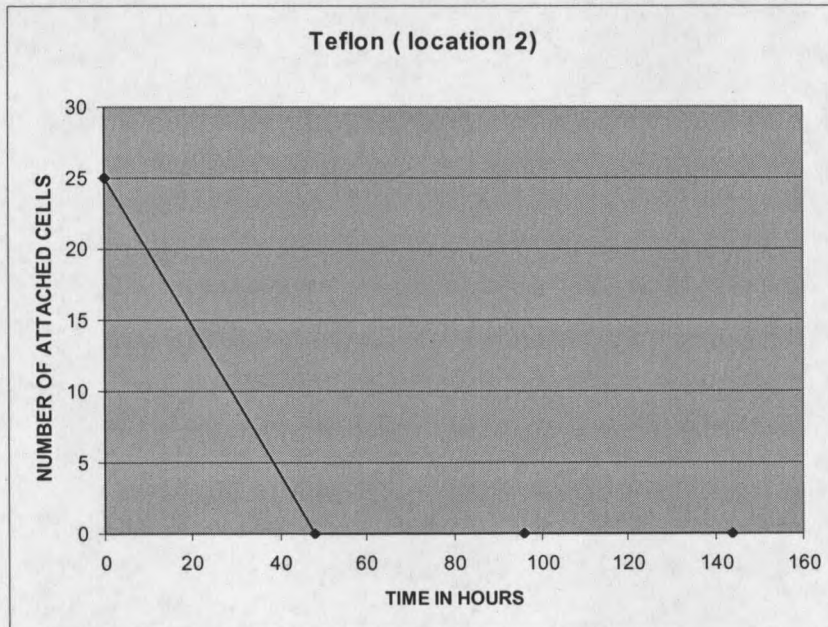


Figure 17: *Dunaliella tertiolecta* on Teflon Coupon (location 2). Number of cells attached to a 0.65 mm^2 sampling area of a Teflon coupon, under flow conditions.

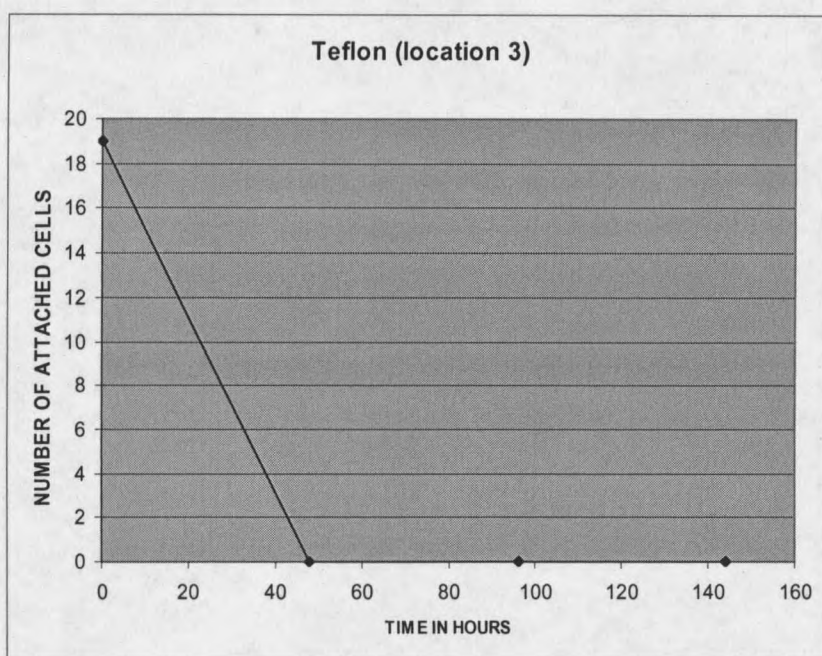


Figure 18: *Dunaliella tertiolecta* on Teflon Coupon (location 3). Number of cells attached to a 0.65 mm^2 sampling area of a Teflon coupon, under flow conditions.

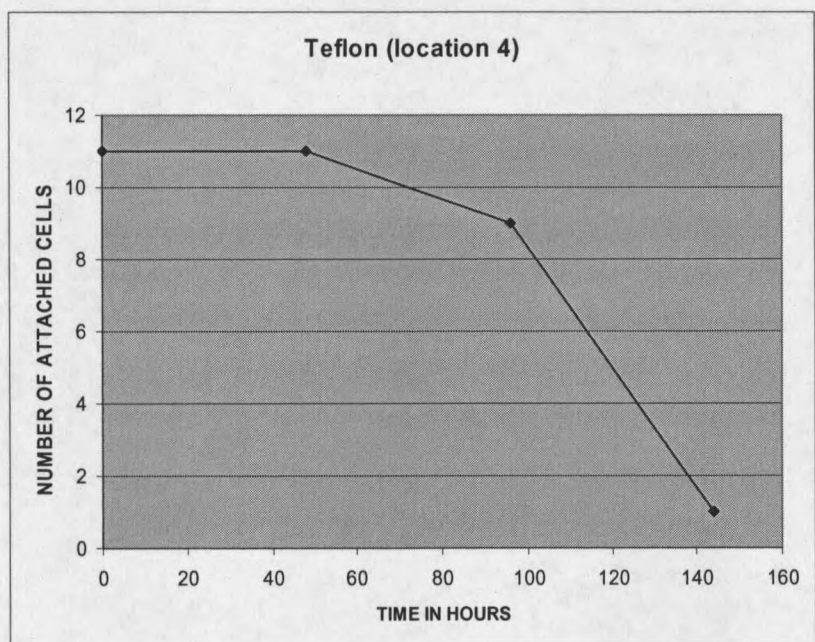


Figure 19: *Dunaliella tertiolecta* on Teflon (location 4) Coupon. Number of cells attached to a 0.65 mm^2 sampling area of a Teflon coupon, under flow conditions.

The ultra smooth surface of Teflon showed a smaller number of cells initially attaching to the surface prior to flow. Once flow started, three of the four locations sampled produced a quick wash out of most all previously adhering cells. The surface of Teflon is known to be very smooth and highly hydrophobic in nature. Studies on bacterial attachment suggest that surfaces that are hydrophobic favor cell attachment (Lopez-Cortes, 1998). Although there are several forces mediating attachment of cells to surfaces, hydrophobic forces are probably the most important. Data from location (2) and (3) may imply that very low surface roughness could negate the attachment enhancing effect of surface hydrophobicity (Bar-Or, 1990). It is also important to note that *Dunaliella tertiolecta* exhibits a slightly negative charge on the outer surface of the cell that could possibly resist interaction with a hydrophobic surface (Svetlicic et al., 2000).

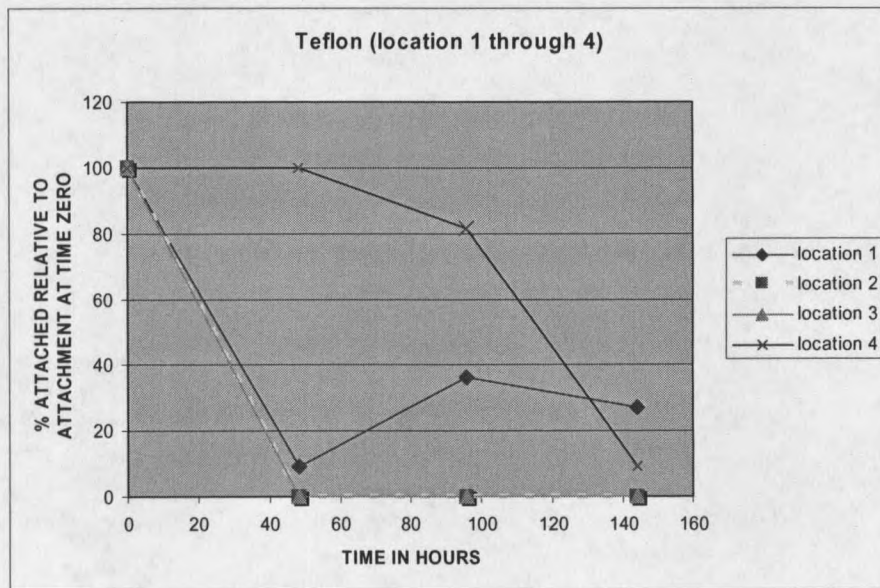


Figure 20: *Dunaliella tertiolecta* on Teflon Coupon. Comparison of locations 1 through 4 on the Teflon coupon

Overall, cells of *Dunaliella tertiolecta* appear to adhere to Teflon weakly. According to data from 3 of the 4 locations, flow induced shear was able to erode virtually all the attached cells relatively quickly. The turbid substance previously noted around locations on the aluminum coupon with high cell density was not present on Teflon.

Dunaliella tertiolecta on Steel

Initial attachment to the steel surface prior to flow activation was higher than in the case of both aluminum and Teflon. Unfortunately, only one of the sampled locations produced viable data. Due to unknown circumstances most areas exhibiting a high density of cells were quickly and completely washed out with the onset of flow. [This was probably due to an event where an air bubble wiped out all algal cells from a large track of the coupon surface.] Once again, the steel data shows a large reduction (about 70%) of the number of adhering cells within the first 48 hours. The general trend of detachment rate resembling first order was noticed from 48 to 144 hours after flow activation. The data indicates agreement with the notion that weakly attached cells wash out relatively quickly and strongly adhering cells tend to gradually erode away in an increasing slower manner. A relatively small quantity of the turbid substance noticed in the aluminum coupon was also present on steel.

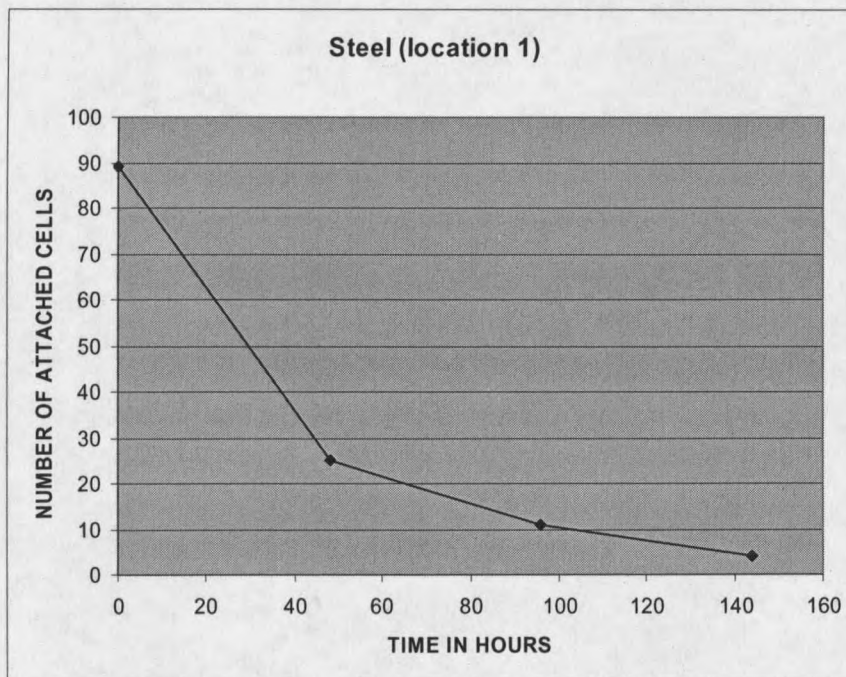


Figure 21: *Dunaliella tertiolecta* on Steel Coupon. Number of cells attached to a 0.65 mm^2 sampling area of a steel coupon, under flow conditions

Comparison: Adhesion on Surfaces

Diagrams comparing detachment data from all three coupon types are shown in Figures 22 and 23. Initial attachment data prior to flow indicated that the surfaces of aluminum and steel were more conducive to attachment than was Teflon. Activation of flow immediately saw the detachment of a majority of attached cells on Teflon, and on steel because of an unknown event. It is concluded that the majority cells on Teflon seem to be adhering weakly. The general trend of a flattening (slowly decreasing) detachment rate was noticed on both steel and Teflon, indicating something like a first order rate. On Teflon, 3 of the 4 sampled locations indicated a quick, near-complete initial washout. Teflon also yielded the lowest tendency for attachment, consistent with this quick washout. Aluminum was the surface that retained the greatest number of adhering cells

for the duration of the experiment. The surface properties of the aluminum coupon seemed to induce strong adhesion. Aluminum was the only coupon of which there was any evidence of reattachment of cells that might have detached further upstream.

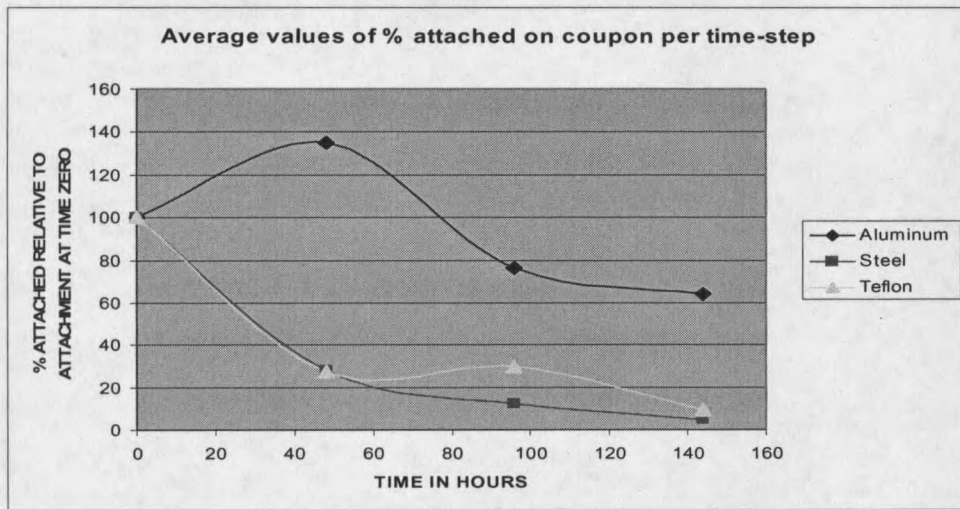


Figure 22: *Dunaliella tertiolecta* Normalized Comparison of Detachment on the 3 Coupons. Comparison of detachment data from all three coupons normalized relative to the number of attached cells prior to flow activation.

There is also a high likelihood that the aluminum coupon contained some irreversibly attached cells. Even after 144 hours of flow, 2 locations on the aluminum coupon had over 100% of the initial number of attached cells, while the other two coupons virtually lost all the attached cells. For Teflon and steel coupons, this phenomenon was not observed. The turbid substance present around cells attached to both steel and aluminum could have had an effect on the surface adherence efficiency of *Dunaliella tertiolecta*, but this is speculation.

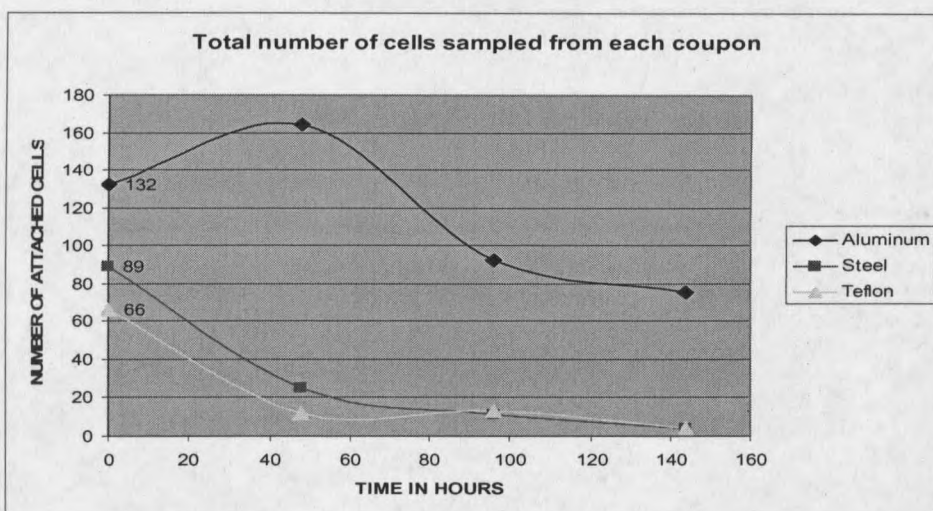


Figure 23: *Dunaliella tertiolecta* Comparison of Detachment Using All Raw Data Points.

Initial Detachment from Steel

Figure 24 shows the results of a separate experiment run on a steel coupon. This experiment was run to investigate the relative detachment of *Dunaliella tertiolecta* directly after the activation of flow. Samples were taken from time 0 (no flow), time 1 (flow activation), and at 1 minute intervals for 10 minutes, then at 5 minute intervals up to 20 minutes. The second datum point, at time 1, was taken just as flow was started. The exaggerated number was most likely caused by a sampling error. When flow was initiated there was an early washout of weakly adhering and non-adhering cell from all parts of the coupon. At this particular stage there was a clear difficulty in distinguishing the attached cells from the non-attached cells moving across the image field.

Disregarding time 1, during the initial 5 minutes there was a substantial drop in the number of adhering cells (from 169 to 107). In the next 5 minutes, the values stabilized around 100. At time 11 a stray bubble virtually wiped out a majority of adhering cells. The bubble event was clearly visible under the microscope. The general

trend was that the weakly adhering cells detach quickly (as seen in the first 5 minutes) and strongly adhering cells are slowly eroded away. If the bubble event had not occurred, the detachment rate probably would have slowly decreased and flattened.

Figure 25 shows data from another location on the same coupon mentioned above. Samples were taken in regular intervals from 25 minutes to 210 minutes after flow started. The data indicates a very slow decrease in attached cell numbers. At this stage, the cells seem to be adhering in a strong manner. Weakly adhering cells could well have been washed out before the first sample was taken from this particular location. The data is consistent with the general trend (very early washout of weakly attached cells and a slowly diminishing detachment rate of the remaining cells) discussed in the previous experiments.

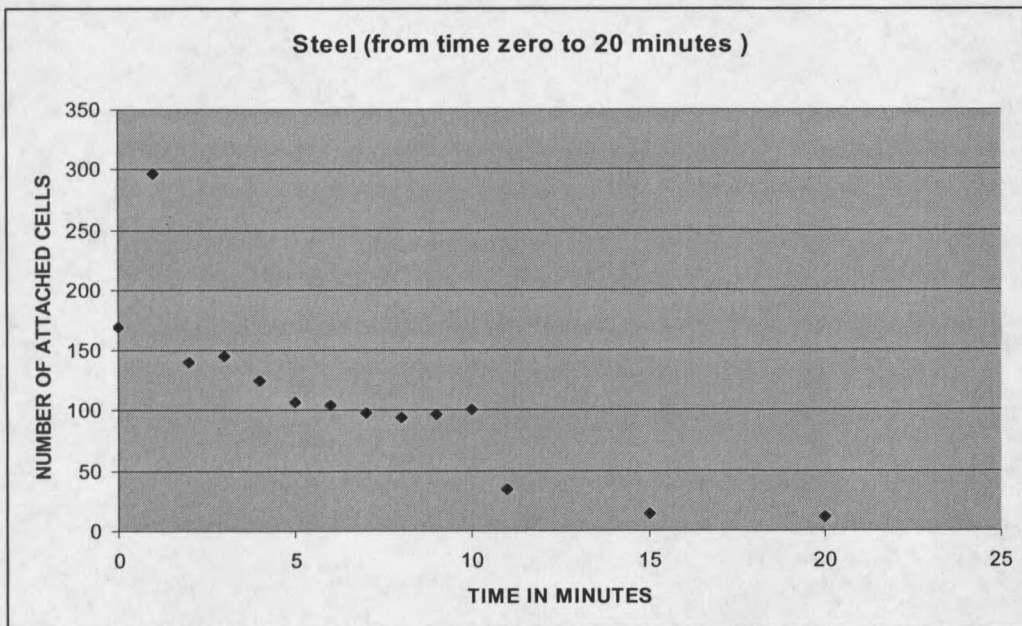


Figure 24: *Dunaliella tertiolecta* Minute by Minute Sampling on Steel. Detachment data from a steel coupon sampled minute by minute from 0 to 20 minutes after flow started

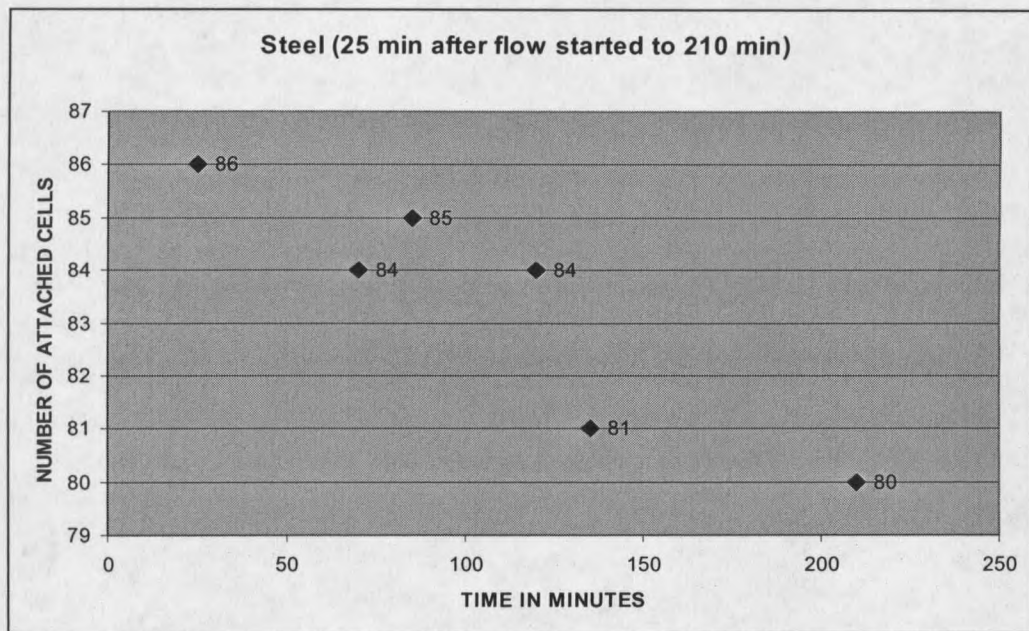


Figure 25: *Dunaliella tertiolecta* 25minutes to 210minutes Sampling on Steel. Detachment data from a location on a steel coupon starting at 25 minutes (after flow activation) and ending at 210 minutes.

Parallel Plate Flow Cell Results : *Botryococcus braunii*

Attachment of *Botryococcus braunii* on aluminum, Teflon and steel coupons were investigated using the parallel plate flow cell system. Viable results were obtained using the aluminum and the Teflon coupons, but not the steel coupon. The cover glass forming the ceiling of the flow chamber cracked on numerous occasions. This was probably due to a build up of pressure within the chamber; once flow started, large clusters of *Botryococcus braunii* appeared to accumulate and appear to have clogged the system's tubing, leading to the breakage of the cover slip. The botryococcene oil produced by the organisms might also play a role in clogging up the system. A backup flow cell was run

every time the cover slip broke; unfortunately the backup flow cell using the steel coupon also yielded no results.

Botryococcus braunii on Aluminum-

Attachment of *Botryococcus braunii* prior to initiation of flow could not be verified because the cells are already immobile in nature (as compared to *Dunaliella tertiolecta* in which the unattached cells were seen in a constant state of motion). It was not easy to determine if cells were just laying on the substratum or if they were indeed attached to the surface. Once flow was administered the extent of attachment could be observed. *Botryococcus braunii* were not present as single cells; they were congregated in very large, independent colonies. All images of the aluminum surface were taken using florescence microscopy. Images 33a through 33d show samples taken at a specific location (0.65 mm² sampling area) on the aluminum coupon at 24 hour increments. Image 33a is a picture taken before flow was initiated to the system. After every 24 hour increment, the images show a decreased number and the cell cluster structure seems to slowly breakdown. The first three pictures (Images 33a-33c) illustrate the loss of adhesion between some cells within the colony, but the colony itself seems to be attached to the substratum. It is speculated that during the last time step (between time 48 and time 72) there was a sloughing event where a large portion, if not the whole cluster, was washed out. The trend in most of the sampled locations was that the gradual erosion of cells from the periphery of the colony was followed by detachment of the colony itself. As time passes, there probably is a threshold where shear force breaks the bonds attaching the remaining colony to the substratum. Considering the duration of the

experiment, the slow rate of *Botryococcus braunii* cell growth and replication would not factor as a major variable.

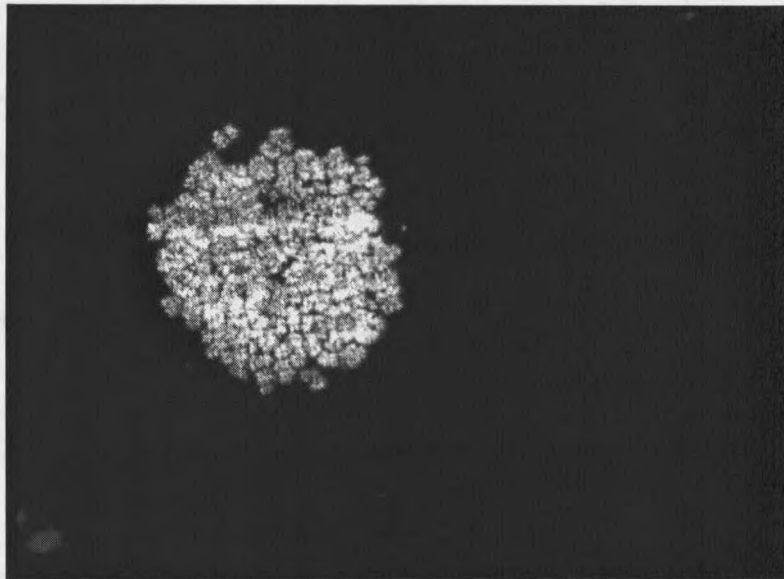


Image 33a: Time 0 'no flow' (10x) *Botryococcus braunii* on Aluminum # (1)

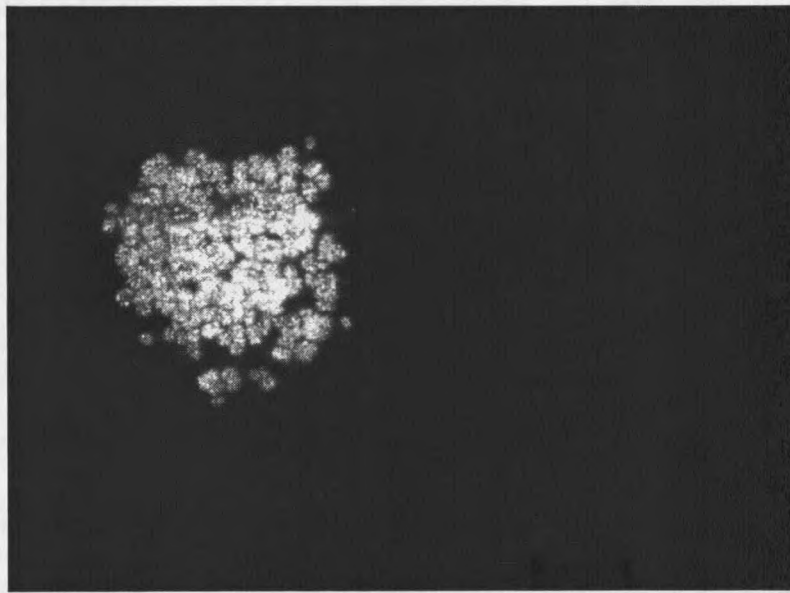


Image 33b: Time 24 hours 'flow' (10x) *Botryococcus braunii* on Aluminum # (1)



Image 33c: Time 48 hours 'flow' (10x) *Botryococcus braunii* on Aluminum # (1)



Image 33d: Time 72 hours 'flow' (10x) *Botryococcus braunii* on Aluminum # (1)

Botryococcus braunii on Teflon

The locations sampled on the Teflon coupon showed little evidence of surface attachment. All images of the Teflon coupon were taken using regular bright field microscopy. Image 34a and 34b are pictures of the same location; Image 34a was taken before flow, and Image 34b was taken 24 hours after flow started. Within the 24 hour period the cell clusters were completely washed out. The clusters apparently were weakly attached to the surface or just not attached at all. Images 35a (before flow) and 35b (after 25 hours of flow) were sampled at another location on the Teflon coupon. In 24 hours, a majority of cell clusters, present when Image 35a was taken, were washed out. The sole remaining cluster was also lost within the next 25 hour period (image not shown). The presence of this cluster in two consecutive sampling sessions was the only evidence suggesting the existence of adherence between *Botryococcus braunii* and the Teflon surface. There was also no indication of cell dispersion as seen on the aluminum experiment. According to our results, *Botryococcus braunii* adheres to aluminum more strongly than it adheres to Teflon.

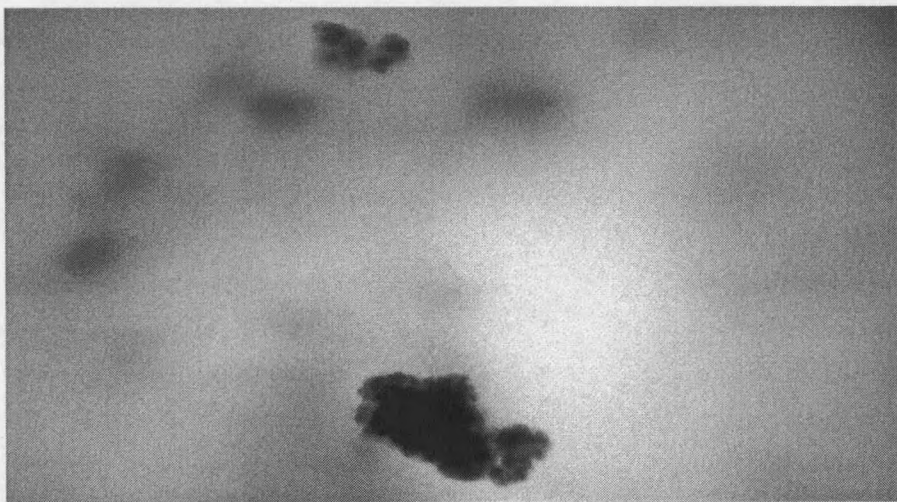


Image 34a: Day 1 'no flow' (10x) *Botryococcus braunii* on Teflon # (1)

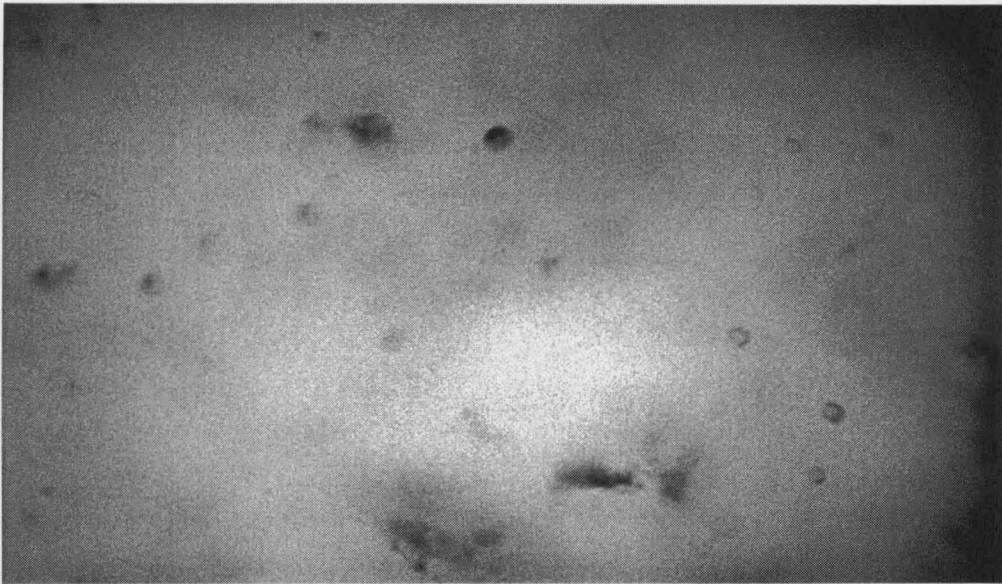


Image 34b: Day 2 'flow' (10x) *Botryococcus braunii* on Teflon # (1)

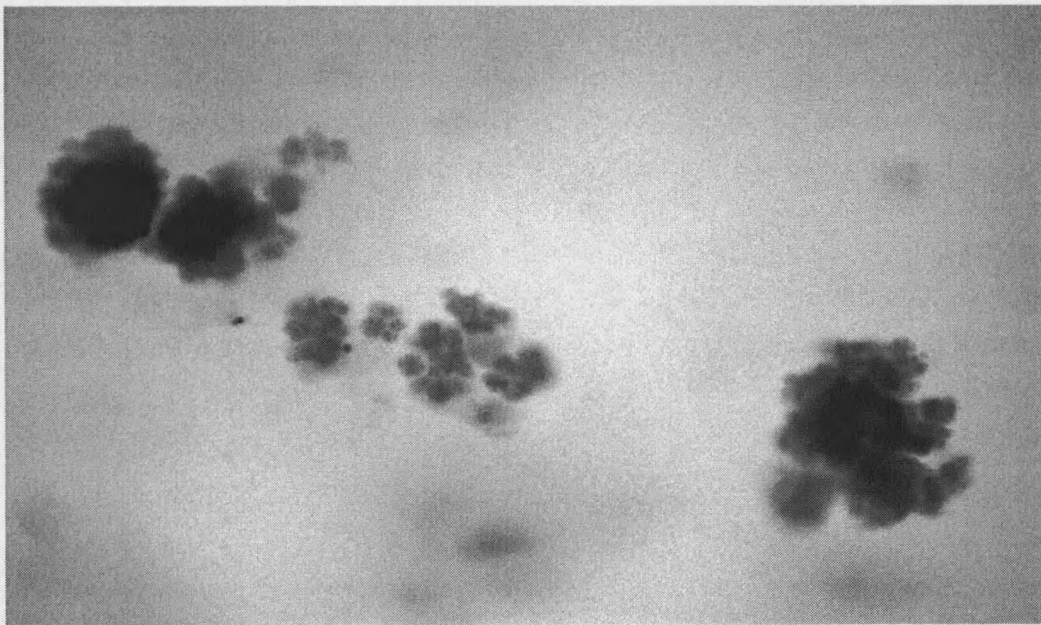


Image 35a: Day 1 'no flow' (10x) *Botryococcus braunii* on Teflon # (2)

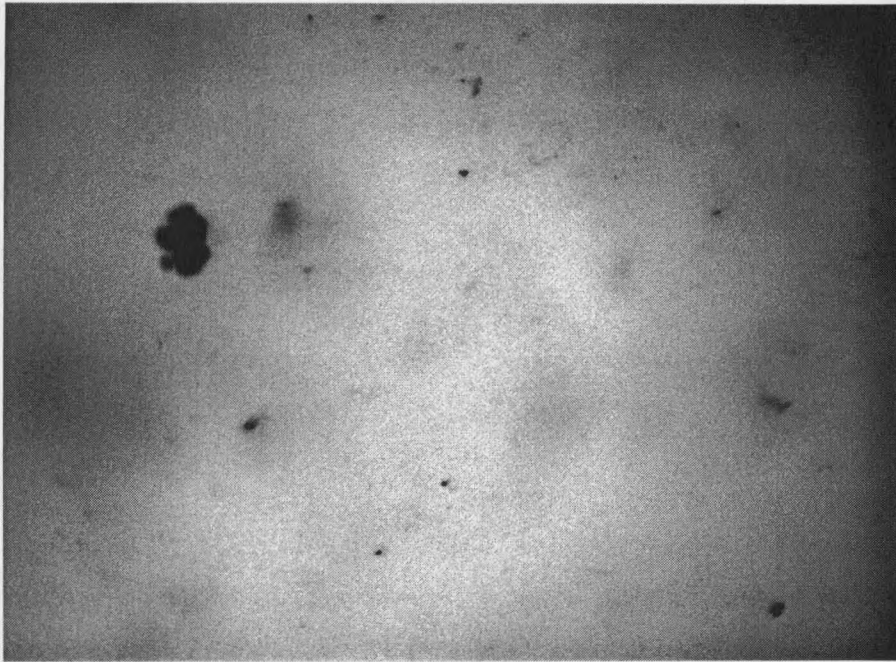


Image 35b: Day 2 'flow' (10x) *Botryococcus braunii* on Teflon # (2)

Image Analysis with Stain

After completion of the time-dependent experiments, the flow channel was stained with two different dyes to observe biofilm formation and bacterial cohabitation. The majority of the images shown in this section were stained with DAPI and a few were stained with both DAPI and rhodamine B. The stain analysis was carried out on flow cells containing *Dunaliella tertiolecta* on aluminum, steel and Teflon coupons; and on a flow-cell containing *Botryococcus braunii* adhering to aluminum.

Dunaliella tertiolecta on Aluminum-

Image 36 shows a region within a DAPI stained flow-cell containing an aluminum coupon. The concentrations and retention times used in the staining process allowed the DAPI stain to penetrate the cell membranes of some associated organisms while leaving

the cells of *Dunaliella tertiolecta* unstained. Algal auto-florescence was used to observe the *Dunaliella tertiolecta* by using a filter with the appropriate wavelengths. (Refer to Experimental Methods and Material sections for more details on staining procedures.) The red color is from auto-florescence of the algae and the blue comes from the organisms stained with DAPI. Image 37c is a (20X) picture which was formed by combining Image 37a (auto-florescence photograph) with Image 37b (DAPI image). Image 36 and 37c show that organisms stained by DAPI (blue) seem to lightly cover the whole surface of the coupon and form larger clusters on the periphery of the algal cells. These particular images and other images shown later indicate that these unknown organisms favor locations on the coupon in close proximity to the algal cells.

Images 38 through 40 show color combined images taken at 60X magnification. These images show in more detail the magnitude of microbial accumulation around the cells of *Dunaliella tertiolecta*. When viewed under the 60X objective lens, a few of the algal cells were seen twitching while being attached to the substratum. The twitching action indicates that some of these algal cells had survived the toxic effects of the DAPI stain. Even some of these twitching cells were encapsulated with dense populations of these unidentified microbes. These unknown organisms, presumably bacteria, may be cohabitating using metabolic products produced by these attached algal cells. Given the fact that media used in these experiments are very light in nutrient content, the bacteria might have to rely on the algae or algal byproducts to survive. On the other hand, certain bacteria are known to produce compounds that promote and enhance micro-algal growth (de-Bashan et al., 2002).

Images 41a through 41d are 20X photographs take of the same position on the coupon. Images 41a, 41b, and 41c are auto-florescence, DAPI, and rhodamine B pictures, respectively. Rhodamine B was used to stain particles that were neither auto-florescent nor stained by DAPI. The exact target of the rhodamine B dye is not known, but it has been used in the past to stain the extra-cellular matrix of biofilms. Image 41d which is a combination of all three Images 41a, 41b, and 41c, indicate the existence of some substances that were not stained by neither auto-florescence nor DAPI. Image 42, another color combined picture taken at a different location, also shows the existence of these substances (green color). This might indicate the presence of a polymeric matrix covering the surface of the substratum. The cells of *Dunaliella tertiolecta* may even be imbedded in a biofilm created by the bacteria. In aquatic alpine ecosystems, both the algae and associated bacteria contribute in forming the polymeric extracellular slime layer enclosing the microbial community (Anonymous, 2002). In theory, the mutualistic association may be explained through the major metabolic pathways present in algae and bacteria. CO₂, a likely byproduct of bacterial metabolism, could be utilized by the algae in photosynthesis. Also O₂ produced in algal photosynthesis could be used by the aerobic bacteria in cellular respiration.

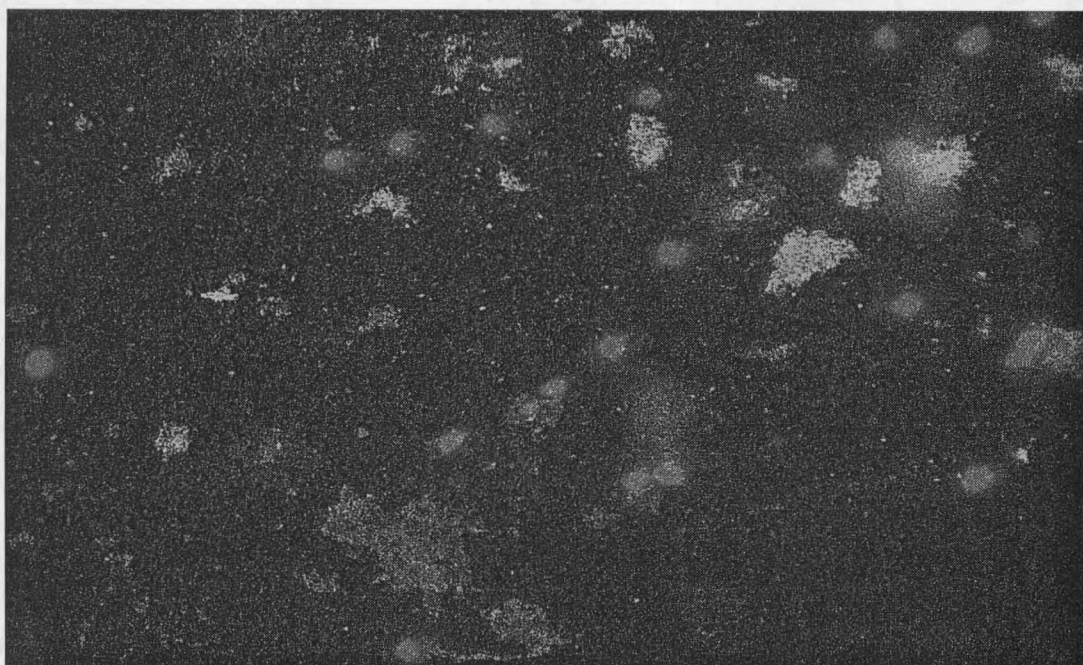


Image 36: (10X) Color Combined-view of DAPI and Auto-florescence *Dunaliella tertiolecta* & Associated Organisms on Aluminum



Image 37a: (20X) Auto-florescent – view of *Dunaliella tertiolecta* on Aluminum

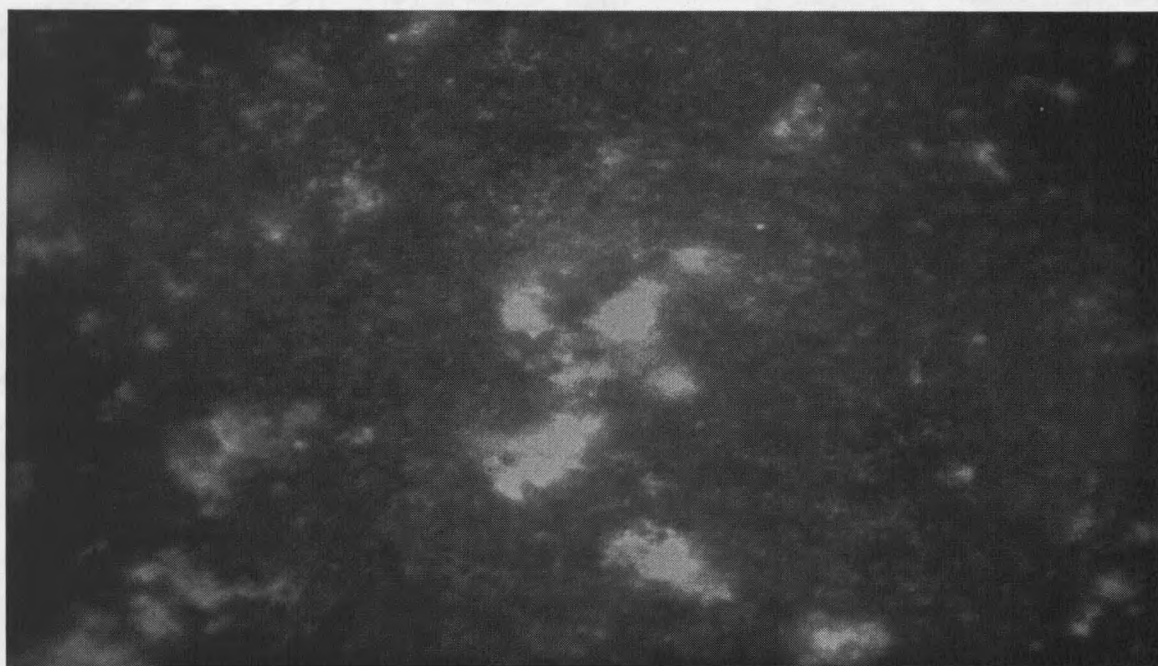


Image 37b: (20X) DAPI- view of *Dunaliella tertiolecta* & Associated Organisms on Aluminum



Image 37c: (20X) Color Combined-view of DAPI and Auto-fluorescence *Dunaliella tertiolecta* & Associated Organisms on Aluminum



Image 38: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated organisms on Aluminum



Image 39: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Aluminum

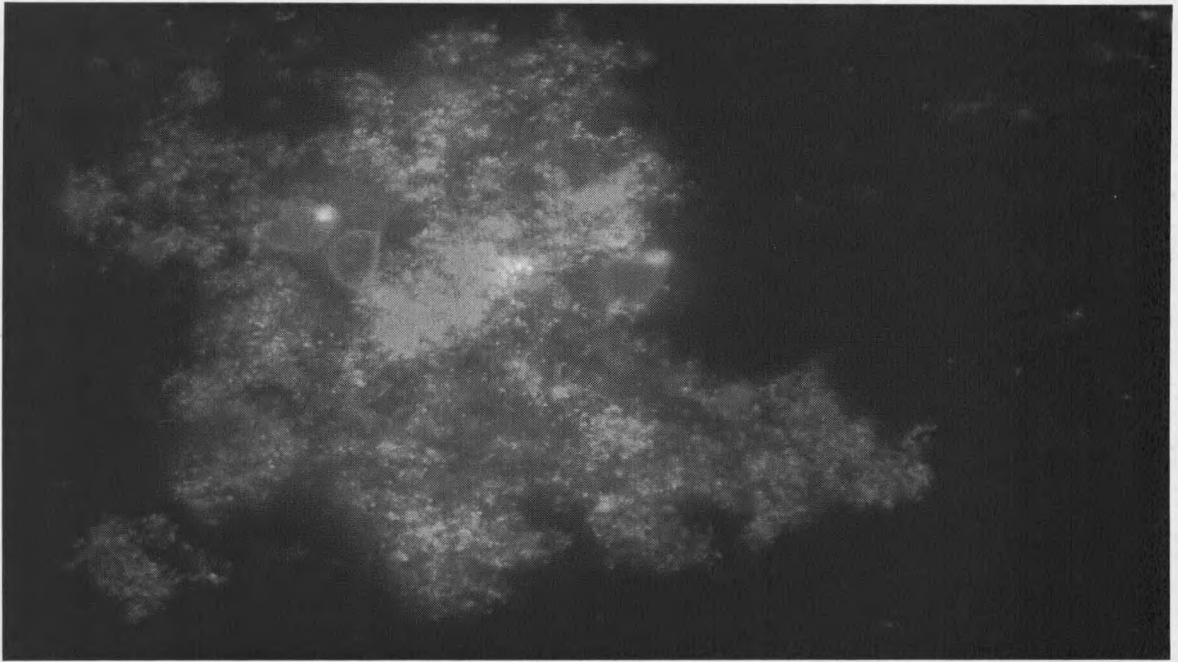


Image 40: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Aluminum

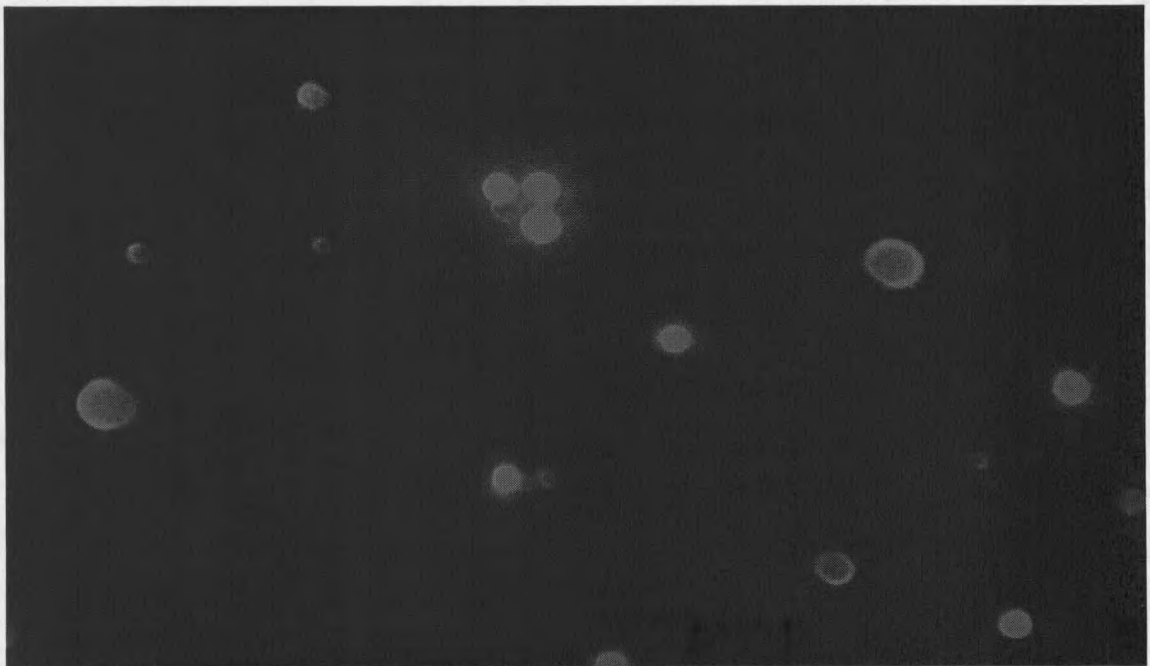


Image 41a: (20X) Auto-florescent – view of *Dunaliella tertiolecta* on Aluminum



Image 41b: (20X) DAPI – view of *Dunaliella tertiolecta* & Associated organisms on Aluminum

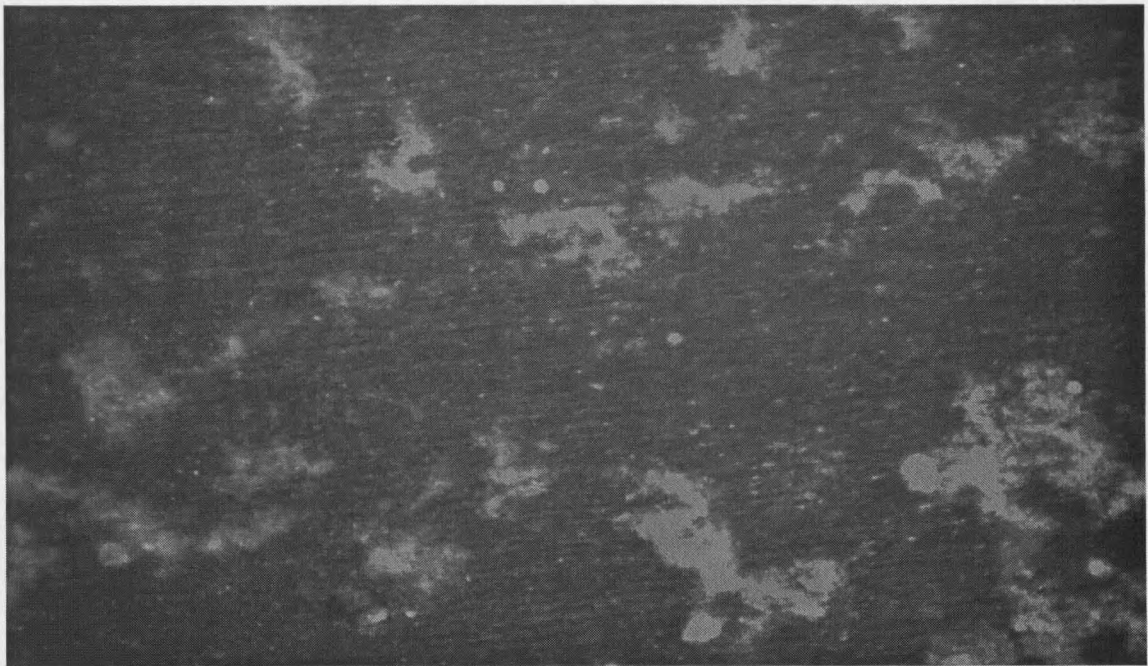


Image 41c: (20X) Rhodamine B-view of *Dunaliella tertiolecta* & Associated Organisms on Aluminum

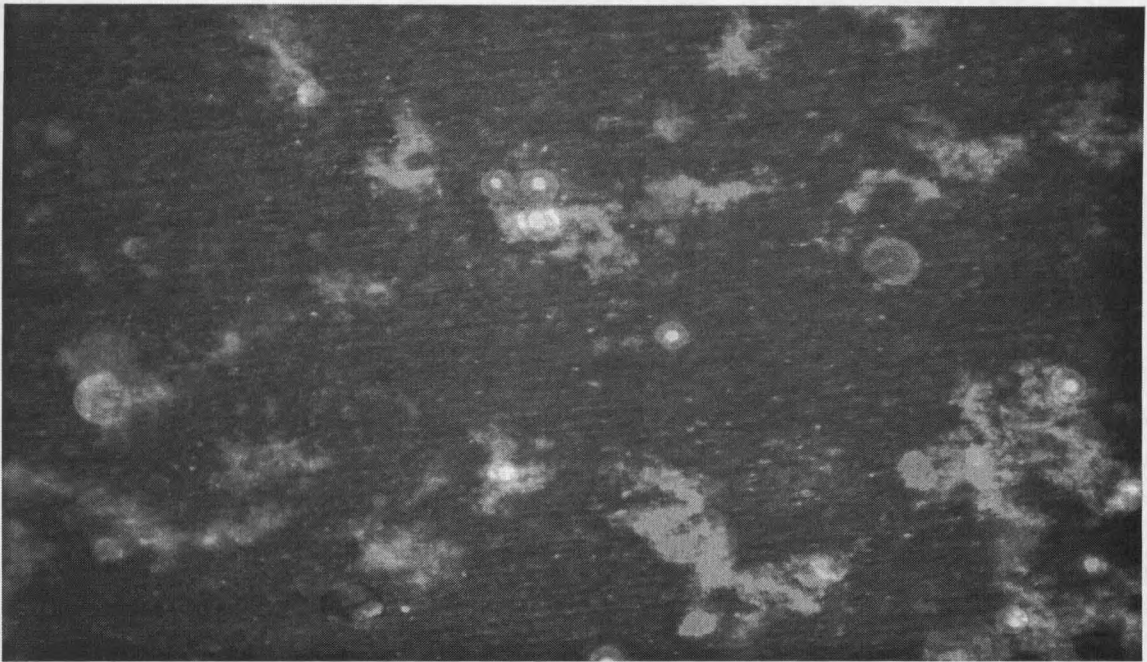


Image 41c: (20X) Rhodamine B-view of *Dunaliella tertiolecta* & Associated Organisms on Aluminum

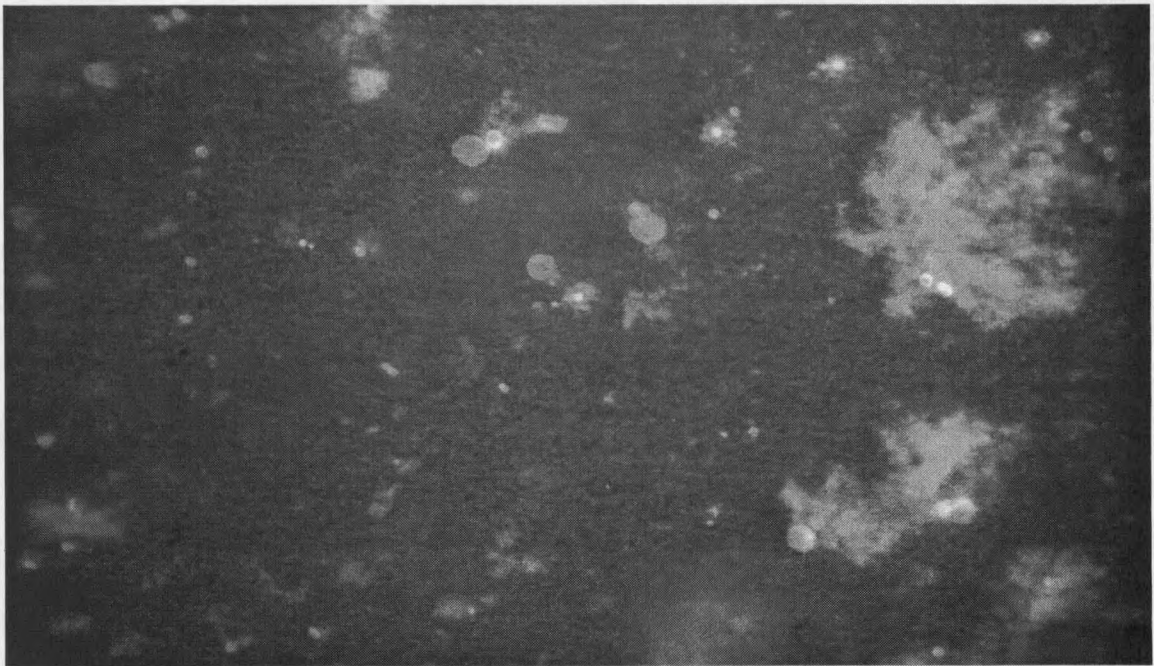


Image 42: (20X) Color Combined-view of DAPI, Auto-fluorescence and Rhodamine B of *Dunaliella tertiolecta* & Associated Organisms on Aluminum

Dunaliella tertiolecta on Steel-

Geographic association of these organisms, assumed to be bacteria, with algae was also present when a steel coupon was used. Bacterial cells covered the entire surface on the aluminum coupon; the bacterial cells were mostly seen surrounding the attached algae on the steel coupon. Images 43 and 44 are both color combined photographs taken at 20X magnification. These images show that the bacteria were almost exclusively seen colonizing areas of the coupon in close proximity to the *Dunaliella tertiolecta*. Images 45 and 46 illustrate the same phenomenon at a greater magnification (60X). Some bacterial colonies totally enclosed individual algal cells, while others were seen very near, but not surrounding, the algae. Future investigations, using this phenomenon, could possibly shed some light on the physiological state of the algal cells. For example, the cells that were completely surrounded by bacteria could be unhealthy. The limiting nutrients will have to diffuse through the bacterial cluster to reach the algae. These bacterial could also be in the process of degrading the cellular structure of *Dunaliella tertiolecta* (Ike et al., 1997). On the other hand, the algal cells that are close proximity but not surrounded by the bacteria could indeed be involved in some form of symbiotic relationship.

Image 47 shows an uncharacteristic feature that was not observed at other locations of the steel coupon. In this image, the cells colored in blue do not show a great degree of association with the algal cell. The blue cells themselves seem to be larger and more filamentous in appearance (unlike cells seen in Images 49 and 50). These filamentous cells could be another species of bacteria or even fungi.

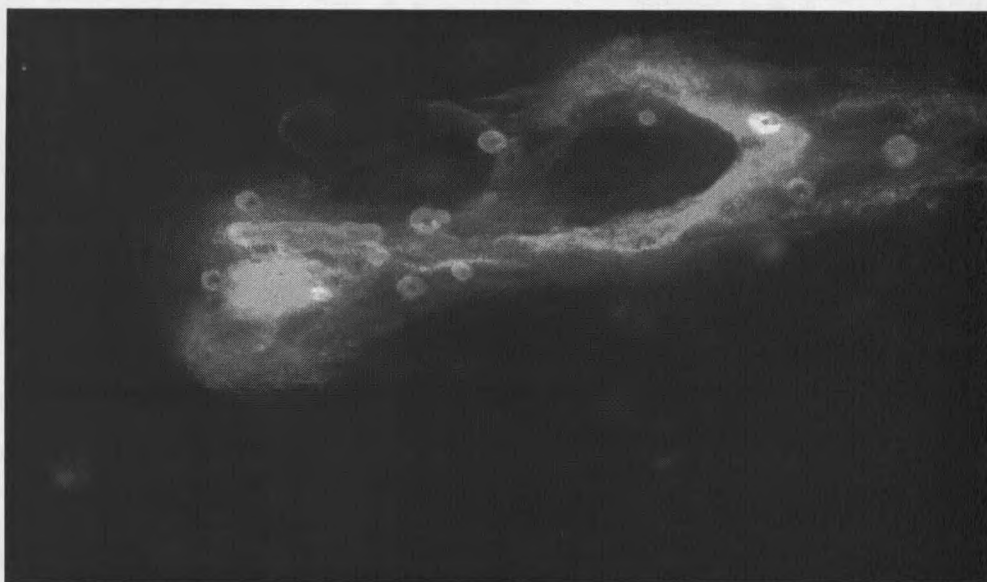


Image 43: (20X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Steel

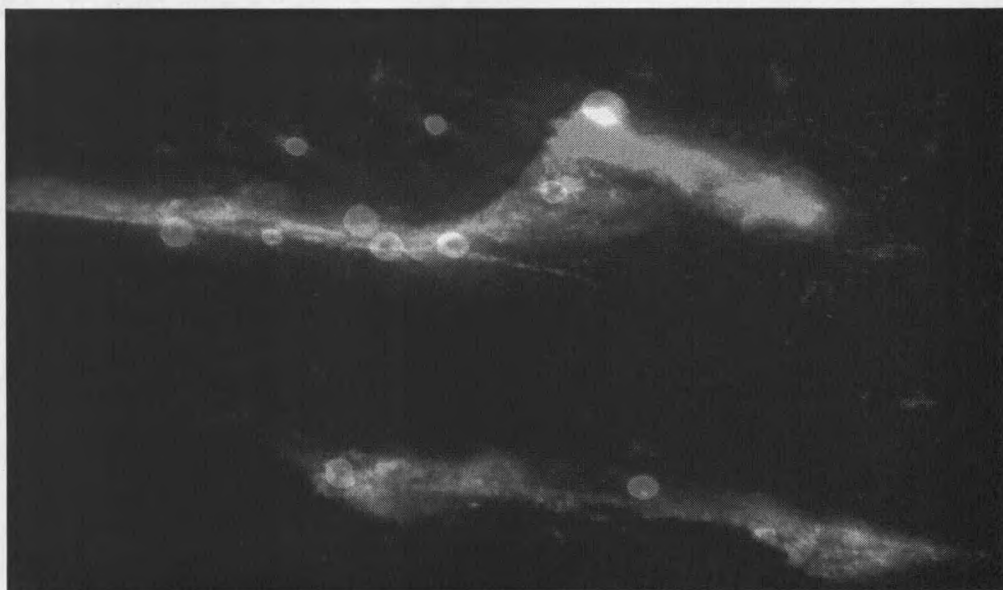


Image 44: (20X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Steel



Image 45: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Steel

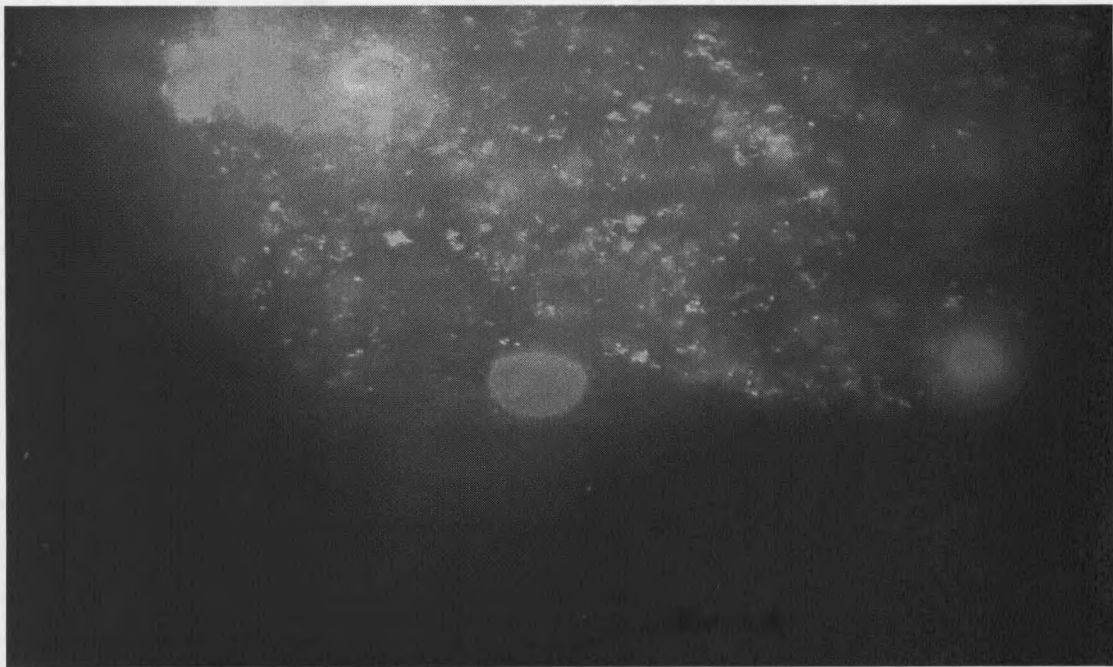


Image 46: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Steel

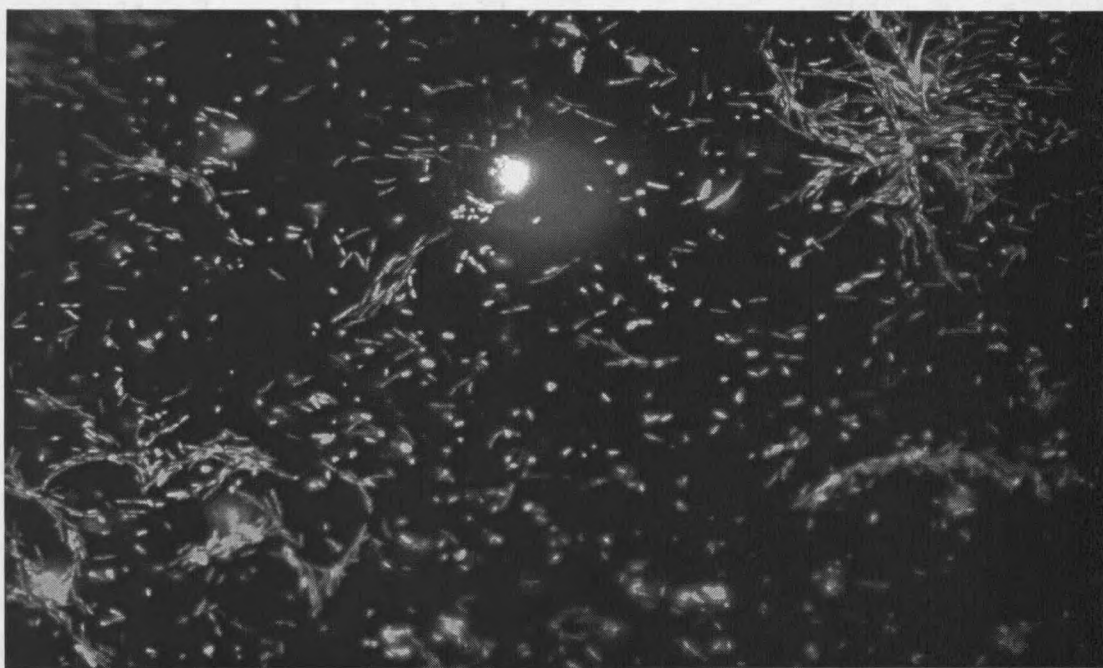


Image 47: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Steel

Dunaliella tertiolecta on Teflon-

The number of both associated organisms and algal cells presents on the Teflon surface was much less than the numbers seen on the aluminum and steel coupons. Image 48 shows a 60X picture at a location that had the greatest density of bacterial cells. Images 49 and 50 show the presence of both *Dunaliella tertiolecta* and bacteria. The bacteria surrounding the algae are in much smaller numbers than seen on the other coupons. Like the algae, the bacteria might also find it difficult to attach to the Teflon surface. The relatively low amounts of bacteria could also be due to the small population of attached algal cells. There seems to be a direct correlation between the presence of the algae and the presence of the unknown bacteria. Perhaps the polymeric substances

produced by the attached bacteria are necessary for *Dunaliella tertiolecta* to adhere to certain surfaces.



Image 48: (60X) Color Combined-view of DAPI and Auto-fluorescence of *Associated Organisms* on Teflon



Image 49: (60X) Color Combined-view of DAPI and Auto-fluorescence of *Associated Organisms* on Teflon

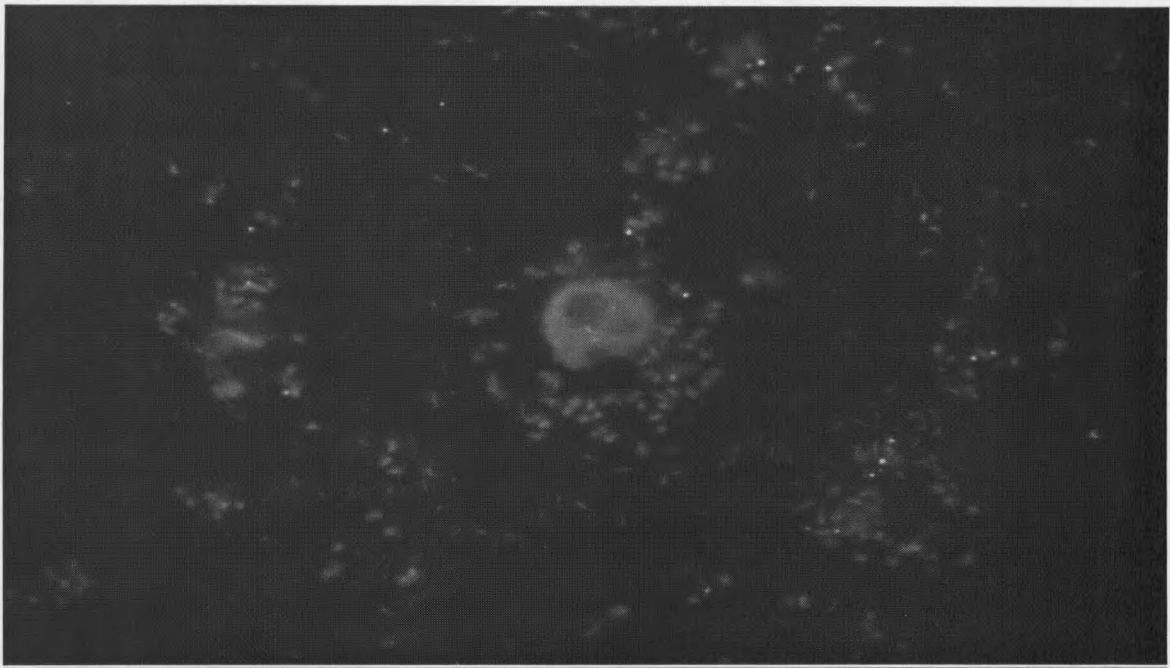


Image 50: (60X) Color Combined-view of DAPI and Auto-florescence of *Dunaliella tertiolecta* & Associated Organisms on Teflon

Botryococcus braunii on Aluminum-

Colonies of *Botryococcus braunii* and associated organisms on the surface of the aluminum coupon are shown in Images 51 through 55. The DAPI stained flow-cell experiments with *Botryococcus braunii* produced similar results to the experiments with *Dunaliella tertiolecta* that were discussed in the previous section. Bacteria were seen in close association with both algae. According to the statements from the University of Texas Algal Culture Center, almost all frozen culture systems will contain associated bacteria. Some of the bacteria which have been found in these cultures were certain species of *Pseudomonas*, *Flavobacterium*, and *Algaligenes* (Chirac et al., 1985). Image 51a depicts an auto-florescing colony of *Botryococcus braunii* (10X). Image 51b is the

combination of Image 51a and a DAPI stained image. Bacterial accumulations are seen covering the entire surface of the aluminum coupon.

The auto-florescent images of *Botryococcus braunii* demonstrate the apparent presence of 'algae-empty crevices' between the highly dense cellular regions on the colonies. Image 51b shows that these 'empty crevices' are actually filled with DAPI stained bacteria. Images 53a, b and 55a, b clearly depict this feature. It is also possible that these seemingly empty areas contain the extra-cellular products from algae, including algal hydrocarbon botryococcene, polysaccharides (hydrocarbons and polysaccharides will not appear in auto-florescent images). The presence of, or easy access, to the hydrocarbons might attract the bacteria to these specific regions. The hydrocarbons are usually concentrated within the outer walls of the *Botryococcus braunii* cells. The bacteria that are known to be present in these algal cultures, notably certain species of *Pseudomonas* and *Flavobacterium*, are capable of growing on hydrocarbons as their sole organic carbon source (Chirac et al., 1985). In addition, the presence of various microorganisms could influence the total amount of hydrocarbons produced by the *Botryococcus braunii*. Bacterially produced CO₂ was previously shown to be sufficient to fulfill algal requirements (Chirac et al., 1985).

According to the literature, in the absence of associated microorganisms, *Botryococcus braunii* lost aspects of its colonial habitat (Murray et al., 1977). Since associated organisms affect the cell-cell adhesion within the algal colony, they could also be involved in the adhesion of the algae to the substratum. Identification of the specific bacterial species that could aid algal attachment would possibly enhance our ability to grow *Botryococcus braunii* biofilms.



Image 51a: (10X) Auto-florescent – View of *Botryococcus braunii* on Aluminum

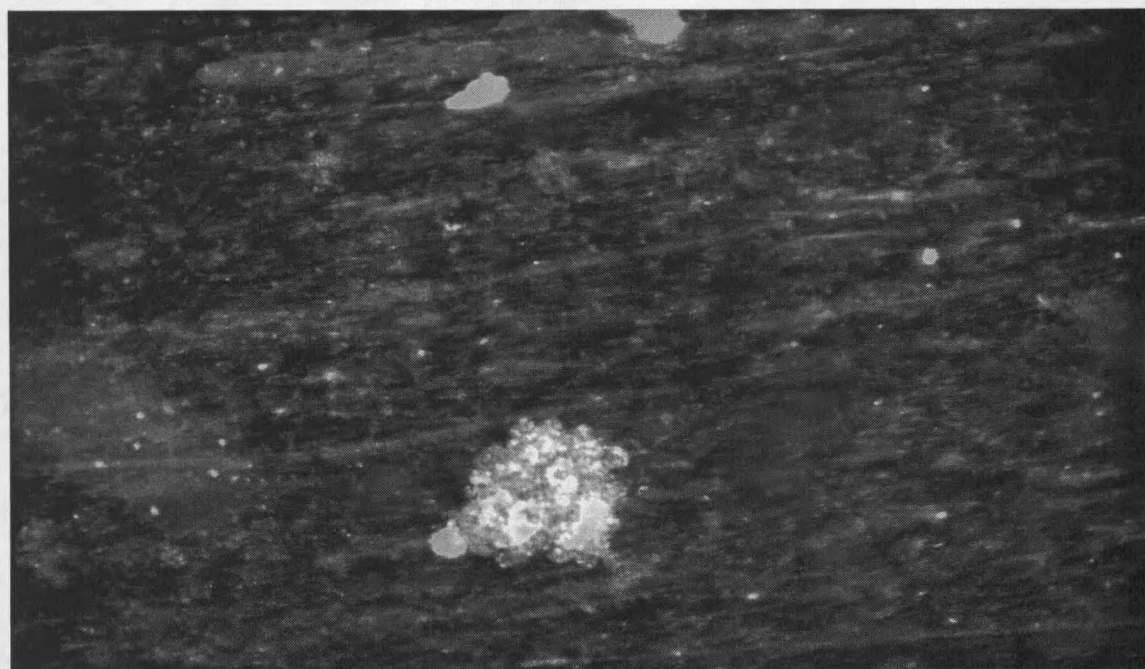


Image 51b: (10X) *Botryococcus braunii* & Associated Organisms on Aluminum Stained with DAPI

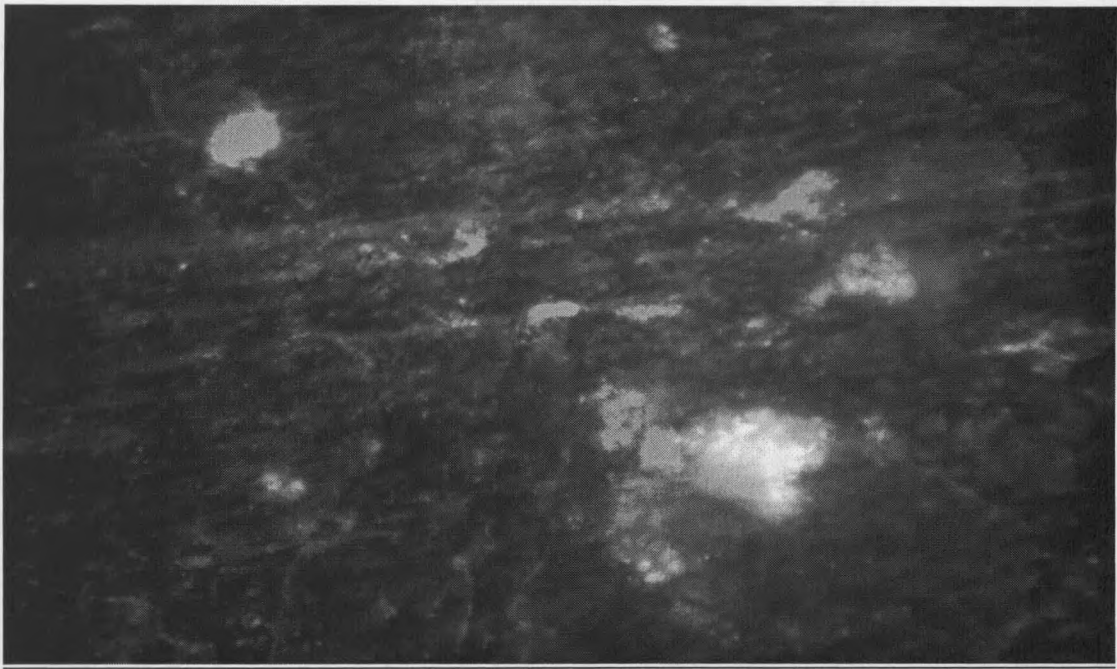


Image 52: (10X) *Botryococcus braunii* & Associated Organisms on Aluminum Stained with DAPI



Image 53a: (10X) Auto-florescent – View of *Botryococcus braunii* on Aluminum

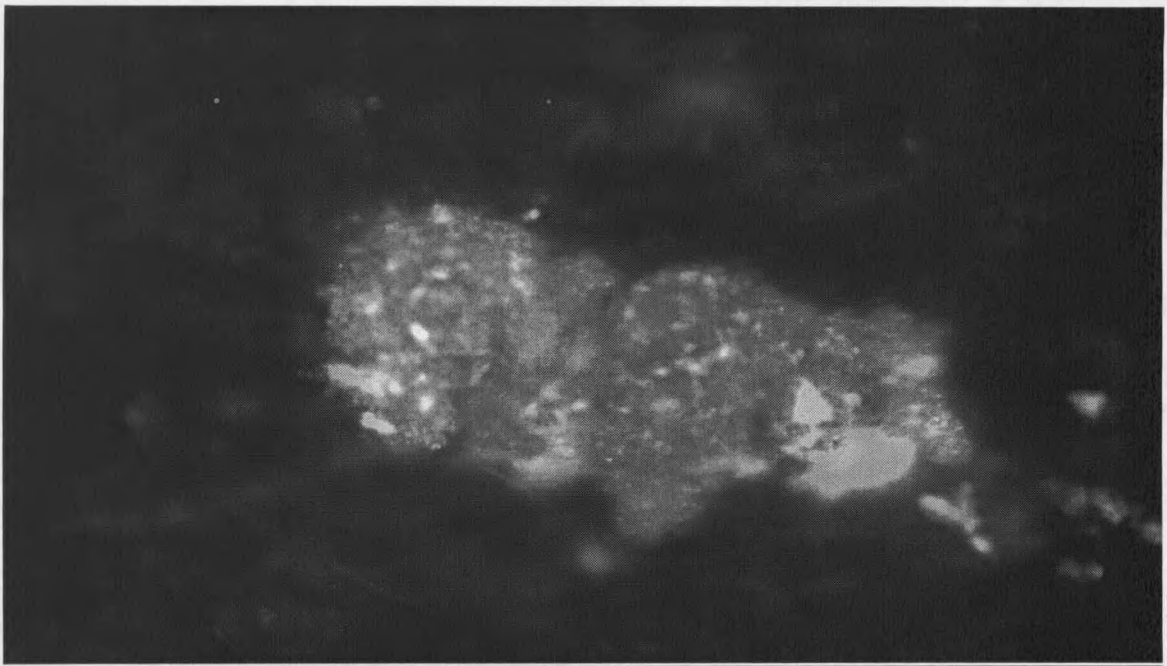


Image 53b: (10X) *Botryococcus braunii* & Associated Organisms on Aluminum Stained with DAPI



Image 54a: (60X) Auto-florescent – View of *Botryococcus braunii* on Aluminum

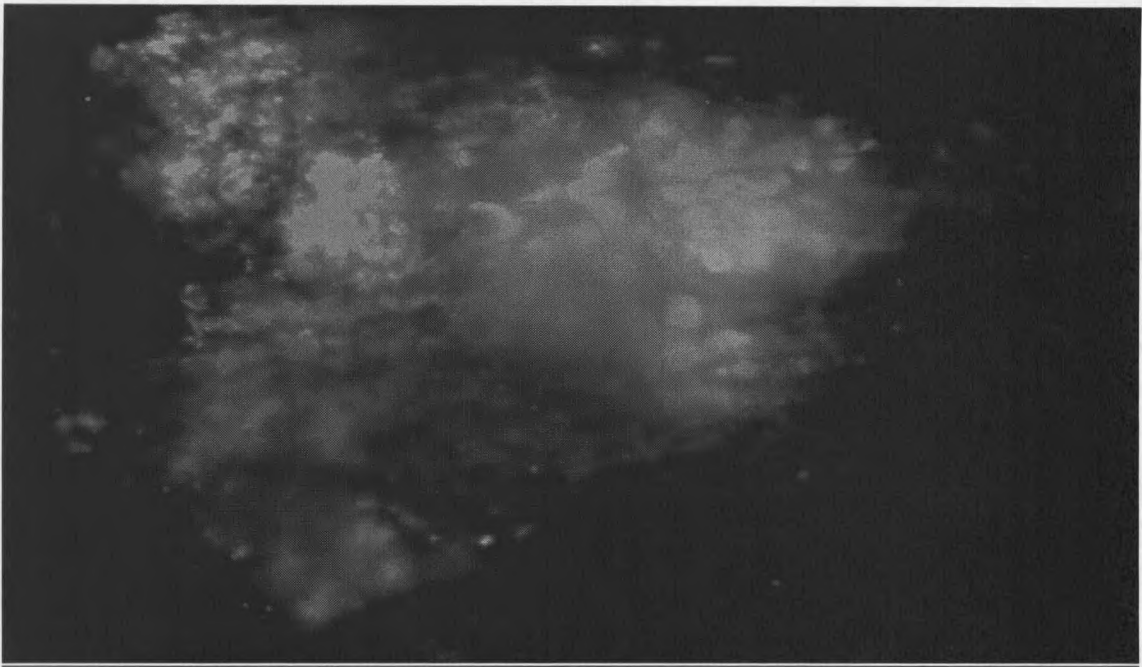


Image 54b: (60X) *Botryococcus braunii* & Associated Organisms on Aluminum Stained with DAPI

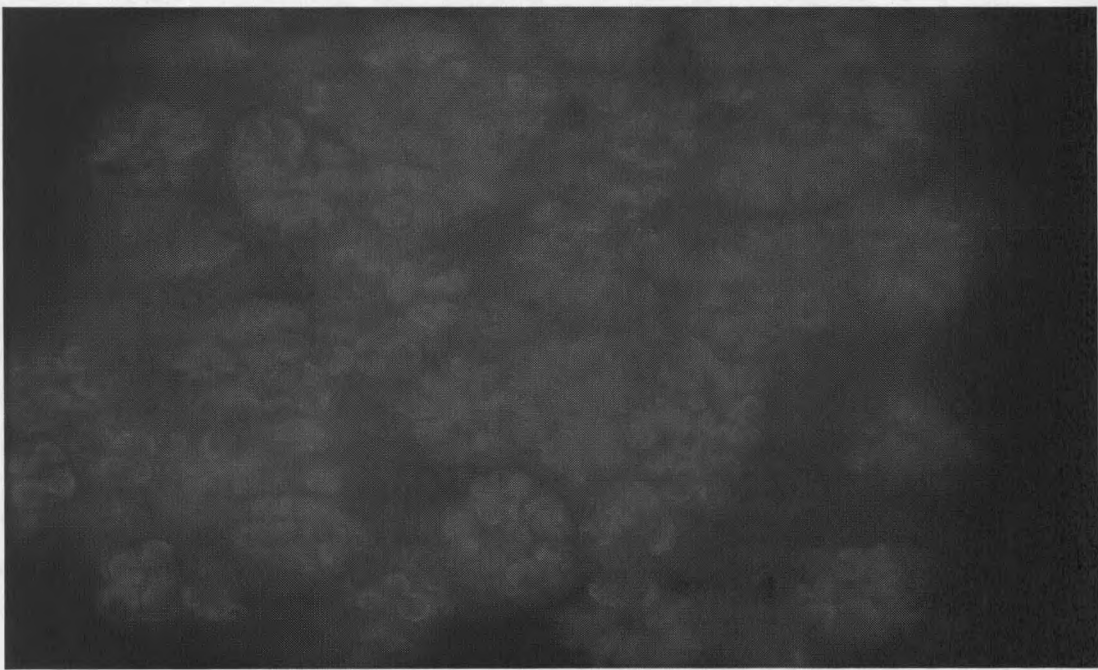


Image 55a: (60X) Auto-florescent – view of *Botryococcus braunii* on Aluminum

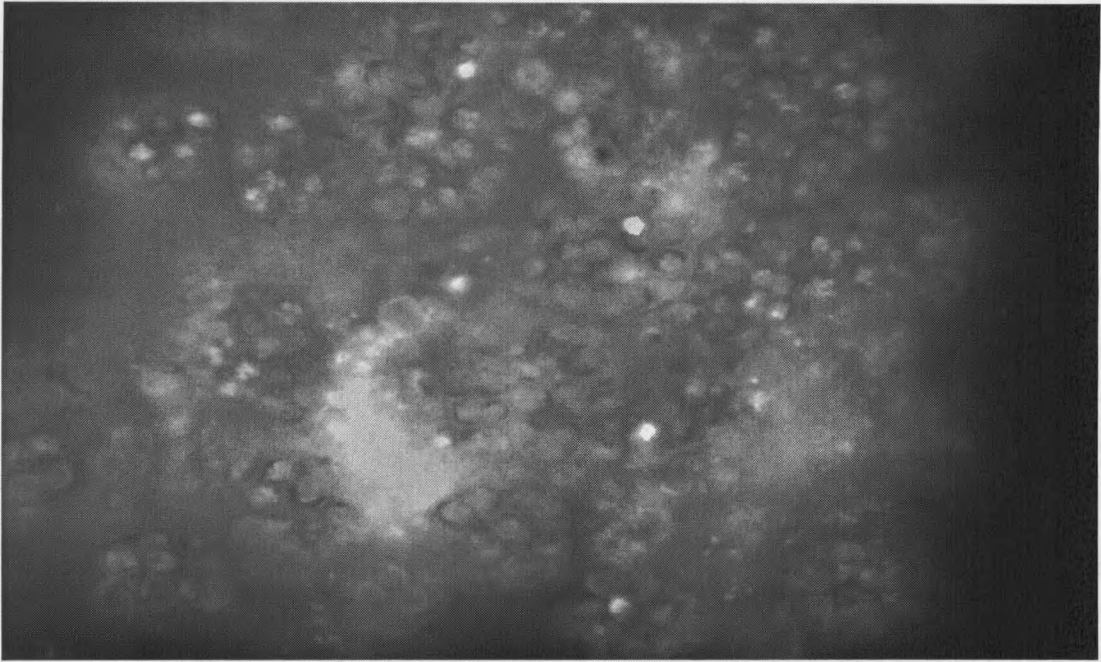


Image 55b: (60X) *Botryococcus braunii* & Associated Organisms on Aluminum Stained with DAPI

CONCLUSIONS AND RECOMMENDATIONS

A set of techniques and methods were developed to analyze attachment, detachment and morphology of two micro-algal species *Botryococcus braunii* and *Dunaliella tertiolecta* and associated cells, presumably bacteria. Flow experiments were conducted on glass, aluminum, steel and Teflon surfaces. The results, especially in the case of *Dunaliella tertiolecta*, were similar to attachment and detachment results obtained by Mueller, Sears, Characklis and Jones (Mueller, 1990). The *in situ* observations have revealed some possible factors that could influence attachment and detachment of these two algal species to the specified surfaces. The following conclusions have been derived from this work:

1. *Dunaliella tertiolecta* did exhibit a tendency to adhere to glass, forming biofilm-like formations in both flow and static systems. There was no conclusive evidence that attachment of *Botryococcus braunii* to glass did or did not occur. However, the lack of cells indicates attachment probably did not occur.
2. Under flow conditions, *Dunaliella tertiolecta* had a greater tendency to attach to aluminum and steel than Teflon. *Botryococcus braunii* had a greater attachment tendency for aluminum than Teflon. The surface properties (i.e. surface roughness and surface hydrophobicity) possibly influence attachment of these two algal species.
3. On the onset of flow, *Dunaliella tertiolecta* displayed varying degrees of detachment. Cells of *Dunaliella tertiolecta* detached from Teflon more rapidly

than aluminum. A general pattern was seen in the detachment of *Dunaliella tertiolecta* from the steel and aluminum surfaces; many cells appeared to detach quickly, but other cells were eroded away much more slowly, reminiscent of first-order detachment.

4. With the onset of flow, *Botryococcus braunii* adhered to aluminum more strongly than Teflon. A general trend was seen in detachment of *Botryococcus braunii* from aluminum. Initial dispersion of peripheral cells (or small groups of cells) from the parent colony occurred, followed by a sloughing event where the remaining colony was washed down stream.

5. *Dunaliella tertiolecta* were capable of forming biofilm structures. Large individual colonies of *Botryococcus braunii* appeared to adhere to certain substrata, but evidence of true biofilm formation was not observed.

6. Associated microorganisms (presumably bacteria) present in the algal cultures adhered to steel and aluminum more strongly than Teflon. Such association was identified as a possible factor affecting surface attachment of algae.

7. Evidence suggesting a possible synergistic relationship between associated organisms (bacteria) and algae (*Botryococcus braunii* and *Dunaliella tertiolecta*) was observed.

Future research work may be necessary to fully establish some of the suggestions in this thesis.

1. Additional research is required to identify the associated organisms that were seen attached to the aluminum and steel coupons. This can be done using a

stain that would preferentially target prokaryotic cells. For more specific detail such as speciation, genetic probes could be employed.

2. The extra-cellular substances seen adhering to the coupon should be analyzed for chemical composition. This would probably indicate the source (algal or other) of the extra-cellular material. Known bacterial species with extra-cellular polysaccharide proclivity could be added to the algal system to identify the effects of EPS on algal attachment and surface morphology.
3. Attachment/detachment studies should be done using pure micro-algal cultures (without associated organisms) to see if there is reduction in adherence, implying a correlation between the presence of the associated organisms and surface adherence tendency of the algae. Then associated organisms could be introduced into the system to examine if a change in surface adherence occurs.
4. Methods need to be utilized to investigate the relationship between the health of the algae and surface adherence. The physiological states of the attached algal cells should be studied, starting with some type of live/dead staining. Assuming a true synergistic relationship, by monitoring the physiological states of these attached algal cells we could possibly identify the type of relationship (synergistic, opportunistic or parasitic) that exists between the algal and associated organisms.
5. The methods used in this study could be used to identify other variables that could influence algal attachment such as light intensity location in the flow

cell, nutrients content, viscosity, temperature, type of flow and the presence of other surface conditioning agents.

6. Additional studies could be conducted to quantify the dependence on light and growth of the multi-species biofilm (algal and associated organisms).

Substrate and light penetration could be investigated using microprobes.

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