Effect of substratum topography on algal turf colonization and productivity under different
nutrient conditions

by

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ABSTRACT

Algae can act as a promising source for biofuel production, pollution recovery from natural waters and nutrient recovery from wastewaters. Typical algae cultivation involves algae in the suspended form, and separation methods including flocculation, filtration, and centrifugation contribute to high cultivation costs. Benthic algae, which grow attached to a growth substratum, is a good alternative to suspended algae for cultivation, as algal biomass can be harvested using mechanical scraping and vacuuming. This approach, called algae turf scrubbers (ATS), have been used for benthic algae cultivation at a large scale in outdoor algae cultivation for pollutant recovery from natural waters and wastewaters. There is little control, however, over the environmental conditions (temperature, light intensity, nutrients and pH) in outdoor ATS systems, and design of the reactor components, such as the growth substratum topography characteristics, can be key to determining the quantity and quality of the biomass produced. The characteristics of the substratum topography can be altered to control the colonization of algae, maximize algal biomass densities, and determine species selectivity to affect the quality and quantity of biomass. The objective of this research is to test the effect of substratum surface topography, using additive manufacturing (AM) technology to prototype, on the biomass density (biomass per unit area) and species selectivity under varying nutrient concentrations (low, medium and high).

Substratum test samples were designed using hemispheres of 500μm, 1000μm and 2000μm radius with AM technology and a plain surface was kept as control. Replicates of each of surface

topography were made using clay. Four Algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris)* were seeded into a laminar flow lane reactor, and cultivated under different nutrient treatments (low, medium, and high). Repeated harvests of algal biomass were analyzed for biomass density, ash content, and species abundance, and correlations between these parameters, surface topography, and nutrient treatment were investigated.

Results demonstrated that nutrient concentration has a primary effect on algal biomass density. The highest nutrient concentration had 186% more biomass density than the lowest concentration (control) and 136% more than the medium concentration. Substratum topography had a secondary effect on the biomass density, and different surface topographies had different biomass densities under each nutrient concentration. The surface topography with 2000 μm radius hemispheres has the highest average biomass density (1.06 ± 0.53 mg/cm^2) followed by the surface with 500 μm radius hemispheres (0.92 ± 0.41 mg/cm^2) for seven day harvest period. Biomass from the medium nutrient concentration had the highest ash content ($17.16\% \pm 0.71\%$), whereas the highest nutrient concentration had lowest ash content percent ($14.11\% \pm 0.32\%$).

Nutrient concentration also has a primary effect on the abundance of algal species in the system. At the lowest concentration, *Microspora floccose* was in abundance ($40.00\% \pm 1\%$), and at medium nutrient concentration *Microspora floccose* ($45.68\% \pm 0.76\%$) and *Mougeotia Scalaris* ($43.50\% \pm 0.84\%$) were in abundance. *Oedogonium crassum* ($34.14\% \pm 1.25\%$) and *Sirogonium scalaris* ($39.14\% \pm 1.19\%$) were most abundant at the highest nutrient concentrations. Substratum characteristics affect the species abundance only at the lowest nutrient concentrations, where *Microspora floccose* was the only species out of the four affected by substratum characteristics, where it was observed to be more abundant on 500 μm radius hemispheres and 2000 μm radius

hemispheres. These results demonstrate the efficacy of using substratum design to control biomass

characteristics and quantity in attached growth algae cultivation systems

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Chapter 1 Introduction

1.1 Economic importance of Algae

Algae cultivation has been investigated as a promising source of nutrient recovery from wastewaters and surface waters (Hoffman, 1998; Oswald, 2003; Roeselers et al., 2008), pollution recovery from natural waters (Adey et al., 1993), biofuels and other renewable energy production (Craggs et al., 1996; Chisti, 2007; Adey et al., 2011), and CO_2 bio fixation (Benemann, 2003; Brune et al., 2009). Nutrient recovery from wastewater and surface waters with algae biomass cultivation can be cheaper and, as being solar driven through photosynthesis, is a potentially more sustainable way as compared to other physical and chemical processes (Tchobanoglous and Burton, 1991; Graham et al., 2009).

1.2 Problems with Wastewater treatment -Algae Biofuel production scenario

Despite many years of investigation and development, economically-viable systems that couple wastewater treatment with algal biomass production for biofuels have not been attained, for a variety of reasons including mixed algal cultures, uncontrolled culture conditions, and high harvesting costs (Sheehan et al., 1998; Cui et al., 2014; Park et al., 2011). The industry has focused primarily on the development of microalgae for biofuels production, the cultivation for which has used mostly raceway ponds, if the systems are open, or tubular photo bioreactors, if the systems are closed (Mata et al., 2010; Davis et al., 2011; Leite et al., 2013). Either technology typically requires large capital expenditures for bioreactor design, making algae production scenarios non-economical (Sheehan et al., 1998; Molina Garima et al., 2008;

Gross et al., 2016). In addition, these type of reactors present major challenges for biomass recovery, as the algal biomass obtained from these processes is in the suspended form with solids concentration typically less than 1%, resulting in high operational costs for biomass harvesting and separation. Biomass can be separated by filtration, flocculation, sedimentation, centrifugation, or with decantation, but most of these methods are costly (Sheehan et al., 2008; Roeselers et al., 2008; Cao et al., 2009; Cui et al., 2013; Gross et al., 2016). In open pond systems biomass harvesting alone contributes 21% of the capital costs of algae cultivation systems (Davis et al., 2011).

In contrast, the use of benthic algal biofilm systems for wastewater treatment and biomass production, while still less characterized, may have more operational advantages over suspended algal systems (Hoffmann, 1998). Algal biofilm cultivation on solid carriers can be more economical, as the biomass can be easily harvested to a higher solids content by mechanical methods including scraping and vacuuming (Cao et al., 2009; Adey et al., 2011; Christenson and Sims, 2012; Cui et al., 2013; Gross et al., 2016).

Algal turfs are short mats of attached benthic algal filaments (Adey et al., 1993) that often have high production rates (Mulbry and Wilkie, 2001). The use of algal turf systems for biomass production has been limited, however, because of the typically lower quality of the non-specific biomass that is generated, resulting from the process recruitment of wild indigenous algal community that is cultivated in polyculture conditions (Adey et al., 1993; Adey & Loveland, 1998). To remedy this, design of the reactor materials, such as the substratum, can offer an approach for controlling the population of species that colonize and dominate the benthic biofilm community (Cardinale et al., 2002; Murdock and Dodds, 2007; Whitehead and Verran, 2009). Substratum properties, such as surface roughness and

topography, can enhance the colonization process of the community, potentially affecting the surface binding forces of the cell, enhancing the cell attachment for the biofilm colonization (Burkholder and Wetzel, 1989; Murdock and Dodds, 2007; Whitehead and Verran, 2009) and stimulating nutrient availability to the cells through transport processes (Bright and Fletcher, 1983; Murdock and Dodds, 2007). Crevices or valleys on the surface of a rough substratum may reduce the local water velocities, which helps the colonizing algal spores to settle and attach, and physical disruption of the flow by substratum roughness can lead to settlement and attachment of turf algae on the substratum (Adey et al., 1993).

1.3   Research justification

Previous research suggests that algal turf cultivation can be a reasonable alternative to suspended algal cultivation because of the reduction of harvesting costs of algal biomass (Gross and Wen, 2015; Gross et al., 2015; Gross et al., 2016), and that this performance can be enhanced through optimization of the reactor design (Gross et al., 2015). A direct approach affecting reactor design is through understanding the role of substratum roughness on algal growth and biomass characteristics. It can be hypothesized that a change in the substratum roughness properties can affect the species recruitment and nutrient availability to the algal turf species by changing the velocity boundary layer at the surface, such that some species can become dominant over the others through competitive exclusion in the colonization process mediated by surface roughness. Little work has been done, however, to understand the effects of controlled substratum properties on the recruitment, colonization and growth characteristics of different algal turf species that typically dominate these systems In this study, we will use additive manufacturing technology to design the substratum with controlled surface topography to test the recruitment, colonization and biomass characteristics of selected

filamentous algal species under different nutrient concentrations. We can also hypothesize that there will be an optimal range of surface feature sizes for any particular algal turf species at which its biomass density will be highest. We will test the hypothesis that a change in nutrient concentration will increase the competition among species for colonization by affecting their relative growth rates, thus affecting the characteristics of algal biomass on different substratum characteristics environments.

1.4 Goals and Objectives of research

The goal of this research is to investigate the effect of surface topography of a growth substratum on the community and biomass characteristics of a benthic filamentous algal community in polyculture. To attain this goal, the specific objectives of this study are as follows:

1. To determine the difference in algal biomass characteristics (especially biomass density (biomass per unit area) and ash content) under varying surface topography conditions and nutrient concentrations compared to baseline environmental conditions of light intensity, pH and flow velocity.

2. To determine the effects of surface topography and nutrient concentration on recruitment of select filamentous algal species under baseline environment conditions of light intensity, pH, and flow velocity.

Chapter 2: Review of Literature

2.1 Algae

Algae are photosynthetic organisms found in various types of habitats in all parts of the world (Daneshwar et al., 2006; Wang et al., 2008). These are prokaryotic or eukaryotic organisms that can grow in a wide range of conditions and can be unicellular or multicellular (Li et al., 2008). Cyanobacteria (*Cyanophyceae*) are prokaryotic organisms, while green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) are eukaryotic organisms (Mata et al., 2010). According to size, there are two types of algae: macroalgae and microalgae. Macroalgae can range from centimeters to meters in size and are often seen in flowing waters, whereas microalgae size is in the range of micrometers and are found in suspension in water bodies. Microalgae is a broad term that includes the prokaryotic cyanobacteria and eukaryotic microalgae living in a wide range of environmental conditions (Masojídek et al., 2008). It is estimated that more than 50,000 species of microalgae exist, although only a limited number, of around 30,000, have been studied (Richmond, 2004). Algae biomass has become popular for the production of renewable energy due to their photosynthetic ability, fast growth rate, and lipid production efficiency (Feng et al., 2011). Microalgae are the basis of food chains in the aquatic environments, as they are CO_2 consumers and primary producers, converting solar energy into biomass very efficiently compared to other primary producers (Masojidek et al., 2008).

2.2   Composition of Algal Biomass

Algal biomass contains three main components including carbohydrates, proteins, and lipids/natural oil (Johnson et al., 2009). In addition to these main components, algal biomass also includes different vitamins, pigments, chlorophyll, and enzyme contents (Masojídek et al, 2008). The chemical composition of algal biomass differs from strain to strain and depends on a number of environmental factors including temperature, nutrients, light, pH, CO_2 supply and mineral content of the medium (Becker, 2004). Composition of algal biomass makes it suitable to use in different applications, e.g., algal biomass that is high in oil and lipid content can be used for the production of biofuel products (Adey et al., 2013). Due to the presence of vitamins and enzymes, algal biomass can be used as food supplements. Chlorophyll content in algae makes it suitable to use in pharmaceutical industries for antibiotic and antioxidants production (Harun et al., 2010).

Oil content of algal species varies from 20-60% of their dry weight but the terrestrial crops have oil content 5% of the dry weight of crop (Chisti, 2007; Gouveia and Oliveira, 2009). According to Chisti (2007), between 1% and 3% of the total U.S. cropping area would sufficiently produce algal biomass that can satisfy 50% of the transport fuel needs. He compared the oil yields of some terrestrial crops used for biofuel production with microalgae (Table 2.1). The oil content of the various micro algal species ranges from 4% to 63% (Table 2.2).

Table 2.1: Comparison of microalgae with terrestrial crops in terms of oil yield (adapted from Chisti, 2007)

Crop	Oil Yield (L/ha)
Corn	172
Soybean	446
Canola	1190
Jatropha	1892
Coconut	2689
Oil palm	5950
Microalgae	136900

Table 2.2: Oil Content (% dry weight) for various algal species (adapted from Chisti 2007; Gouveia and Oliveira, 2009; Kumar et al., 2011).

Microalgae	Oil Content (percent dry weight)	Reference
Botryococcus braunii	25-80	Chisti, 2007
Chlorella	14- 40	Chisti, 2007
Spirogyra	14.82	Kumar et al., 2011
Tolypothrix	12.78	Kumar et al., 2011
Cladophora	11.76	Kumar et al., 2011
Rhizoclonium	11.64	Kumar et al., 2011
Chlorella emersonii	63	Gouveia and Oliveira, 2009
Neochloris oleabundans	29	Gouveia and Oliveira, 2009
Scenedesmus obliquus	17.7	Gouveia and Oliveira, 2009
Spirulina maxima	4-9	Chisti, 2007

2.3   Use of algae in different fields

2.3.1   Algal Biofuels

Having high lipid and low ash content, algae can be used for biofuel generation (Mulbry et al., 2008). They can be converted into biogas and oil-based biofuels by thermochemical conversion methods including gasification, pyrolysis, hydrogenation, and liquefaction of the algal biomass (Miao and Wu, 2004). The potential productivity of oil from microalgae can be significantly greater than oilseed crops such as soybean (Sheehan et al., 1998), as algae can grow much faster than other terrestrial crops like soybean or corn, which require a complete season to grow (Chisti, 2007). Microalgae require sunlight, CO_2 and some nutrients for growth, and the growth rates can be modified by the addition of other nutrients (Renaud et al., 1999), some of which can potentially be obtained from wastewater sources.

2.3.2   Algae in water treatment and nutrient recovery

Water pollution is a major global problem caused by growing populations and nutrient enrichment. In the past century, human activities involving fossil fuel combustion and agricultural fertilizers almost doubled the nitrogen and phosphorus concentrations in natural ecosystems (Canfield et al., 2010). Excessive nutrient flow into aquatic ecosystems leads to eutrophication of surface waters such as lakes, ponds, and rivers, and is the major cause of the degraded water quality worldwide (Carpenter et al., 1998), which induces problems such as fish kills, pH shifts, low dissolved oxygen concentrations, and conditions leading to an increase in water borne human diseases. The main driver of eutrophic conditions is excess nitrogen and phosphorus, which stimulates nuisance algae production downstream of discharges and results in consequent ecosystem damage (Correll, 1998).

As an autotrophic microorganism with a rapid growth response to nutrient availability, algae have been investigated for pollutant recovery from natural waters (Adey et al., 1993; Hoffman et al., 1998; Adey et al., 2013) and nutrient recovery from wastewaters (Craggs et al., 1996; Mulbry and Wilkie, 2001; Mulbry et al., 2010). Algae are capable of taking up these nutrients from wastewaters for self-nourishment, and treating wastewater with algae production can be less expensive. Being solar driven through photosynthesis, algae cultivation is a potentially more sustainable way as compared to other physical and chemical processes for wastewater treatment (Tchobanoglous and Burton, 1991). Algae can also be used to remove many toxic heavy metals from wastewater. A number of algal turf species have been used in the removal of heavy metals from wastewaters (Table 2.3).

Table 2.3: Heavy metal removal from wastewaters by algal turfs biomass

Algal turf species	Metals	References
Spirogyra sp.	Cr	Gupta et. al.,2001
Ulothrix zonata	Cu	Nuhoglu et al., 2002
Cladophora crispate	Cr	Nourbakhsh et al.,1994

2.3.3 CO_2 bio fixation

As photosynthetic organisms, algae require CO_2 for their metabolism (Wang et al., 2008) and can therefore be used for the reduction of CO_2 emissions from power plants (Briggs, 2004). Carbon in the exhaust gases from various industrial and atmospheric processes can be fixed by setting up algae cultivation plants near the industrial area, and CO_2 produced from the

power plant could be utilized as a carbon source for algal growth. This process recycles waste CO_2 from power plants into clean burning biodiesel and helps in the reduction of global warming impact on the atmosphere (Danielo, 2005; Suresh and Ravi Shankar, 2004).

2.4   Algae Cultivation Systems

Large-scale cultivation systems have been designed to supply the biological requirements of algae with the physical and operational characteristics of the engineered system (Terry and Richmond, 1985). The most important factor affecting mass algae cultivation is light, which as sunlight is available freely. The amount of light energy received by each algal cell depends on several factors including photon flux density, cell density, thickness of culture layer, and rate of mixing (Masojídek et al., 2008). A second important factor affecting algae growth is temperature. Many algal species can tolerate wide ranges of temperature (Masojídek et al., 2008). For optimal algal growth of most of the algal species, the temperature range should be between 20˚C to 30˚C (Chisti, 2007). The growth medium used for algal cultivation or wastewater must contain essential nutrients such as nitrogen, phosphorus, iron, and sometimes silicon for efficient algal growth (Grobbelaar and Bornman, 2004). Other factors that should be monitored for algae cultivation system are pH and oxygen concentration (Masojidek et al., 2008). With these considerations for the design of reactors, algae can be cultivated in open ponds or in closed systems called photo bioreactors. Benthic or attached algae can also be cultivated in flow way systems such as ATS. Each of these cultivations systems has its advantages and disadvantages.

2.4.1   Open Ponds or Raceway ponds

Raceway ponds for algae cultivation have been used since at least 1950 (Terry and Richmod, 1985; Chisti, 2007). These systems are made up of a closed loop recirculation raceway channel and are often built in earth with concrete and lined with plastic. Mixing and circulation is done by paddlewheel. Flow is guided around bends by baffles placed in the flow channel. On completion of the circulation loop, cell-laden broth is harvested behind the paddlewheel (Figure 2.1). The paddlewheel operates all the time to prevent sedimentation. These ponds are easy to build and operate, and are usually less expensive than photo bioreactors. They are known to have problems in cooling, however, as most of the cooling is done by evaporation, which leads to significant water loss. Because of these evaporation losses, the use of carbon dioxide in raceways is also much less efficient than in photo bioreactors (Chisti, 2007). Maintaining the optimum culture conditions in the raceway ponds is difficult, and cultures can be easily contaminated resulting in poor productivity. Harvesting costs are also high, as the medium in these ponds is dilute, which increases the filtration and processing costs and makes the system expensive to use (Ugwu et al., 2008; Pittman et al; 2011). The main limitations of open ponds are evaporation losses, diffusion loss of CO_2 to the atmosphere, large area requirements, and contamination of the algal culture with unwanted species (Ugwu et al., 2008).
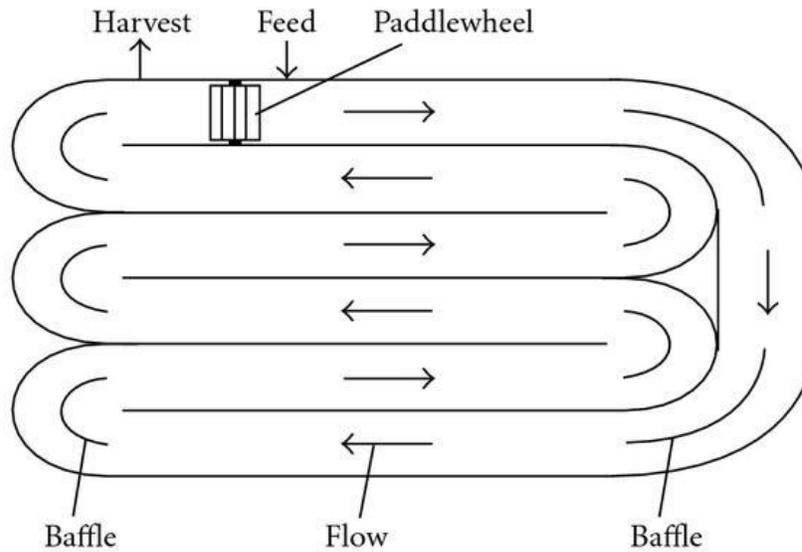
Figure 2.1: Arial view of raceway pond (Chisti, 2007).

2.4.2 Photo Bioreactors

Photo bioreactors can be used in the cultivation of single algal species for prolonged durations, unlike open raceway ponds that are subject to potential species contamination (Molina et al., 2000; Pulz, 2001; Chisti, 2007). Because of their highly controlled conditions, photo bioreactors can be used for the production of a large quantity of biomass as compared to raceway ponds. Photo bioreactors can be flat plate, tubular type, or vertical column bioreactors according to their shape and use (Ugwu et al., 2008). A tubular photo bioreactor consists of an array of straight transparent tubes of plastic or glass that is placed in the sunlight source (Chisti, 2007). The diameter of the tubes is kept less than 0.1 m so that light can easily penetrate into the tubes. Micro algal broth is circulated continuously from a reservoir to the solar collector and back to the reservoir (Chisti, 2007). The tubes are always oriented North–South. The ground beneath the solar collector is often painted white, or covered with white sheets of plastic

(Figure 2.2). Biomass sedimentation in tubes is prevented by maintaining highly turbulent flow (Chisti, 2007).
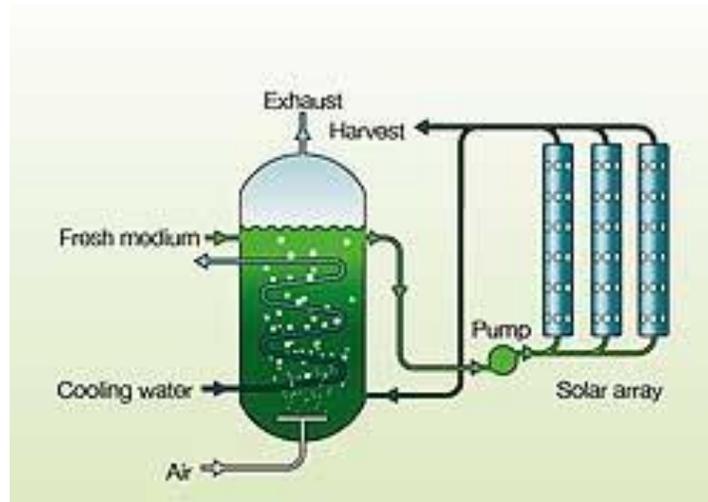
Figure 2.2: Schematic of a closed photo bioreactor system.

(Source: http://www.massey.ac.nz)

The biomass concentration produced in photo bioreactors can be nearly 30 times than that obtained in raceways (Chisti, 2007). Harvesting costs of the algal biomass in a photo bioreactor are typically less than those of raceway ponds, and biomass can be easily separated by filtration or centrifugation process. Because of the controlled conditions in the reactor, the quality of biomass separated in photo bioreactors is good as compared to the biomass collected from open ponds (Garima et al., 2003). Limitations exist on the use of photo bioreactors, however, as photo bioreactors are not cost effective when they are scaled up in the mass cultivations. They require high cost support material, and temperature variation and wall growth of algae can cause stress to the algae culture (Ugwu et al., 2008).

2.4.3 Algal Turf Scrubbers

The Algal Turf Scrubber (ATS) is a technology for the cultivation of benthic or attached filamentous algal biomass that has the advantage of reduced harvesting costs compared to suspended microalgae. In microalgae culture, the algae remain in suspended form in open ponds or photo bioreactors, and harvesting methods like centrifugation, flocculation, and filtration need to be used, leading to high harvesting and processing costs. In ATS technology, the algae are attached to the substratum and can be easily harvested by mechanical scraping or vacuum harvesting methods (Adey et al., 2013). This technology has been investigated for the treatment of polluted water through uptake of various dissolved inorganic compounds such as nitrates, phosphates, and metals from the water (Adey and Loveland, 1998), and has also been studied for nutrient removal from dairy manure (Mulbry and Wilkie, 2001), aquaculture (Adey & Loveland, 1998), sewage (Craggs et al.,1996), and agricultural runoff (Adey et al., 2011). The ATS was developed in the early 1980's at the Smithsonian Institution as a biomimicry of coral reef primary productivity, and was initially used as a tool to manage water quality in an extensive series of living microcosm and mesocosm models of wild coral reef ecosystems (Adey and Loveland, 2007). Typically, algal turfs are grown on polyethylene mesh in laboratory-scale ATS units and on nylon netting in pilot and field-scale units. A tipping bucket or other such mechanism is used to create a frequent wave surge that prevents boundary layer formation, increases nutrient and metabolite exchange, and prevents the light shielding of internal portions of the algal turf (Adey and Loveland, 1998; Mulbry and Wilkie, 2001). Performance of the algae turf scrubber can be manipulated by changing the flow rates and water depth in the flow way, and light can also be provided by artificial lightening sources for indoor applications (Figure 2.3). Algae turf scrubbers can be easily scaled up to

large systems according to the need, and full-scale systems at the hectare scale are in operation in some parts of the United States (Figure 2.4). One of these systems was designed for removing phosphorus from agricultural drainage water in southern Florida (Hydromentia Inc., 2005). When the nutrient supply is moderately high and solar energy is moderately abundant, productivities from common ATS systems have ranged from 25 to 45 g m^{-2} d^{-1} (Adey et al., 2011).
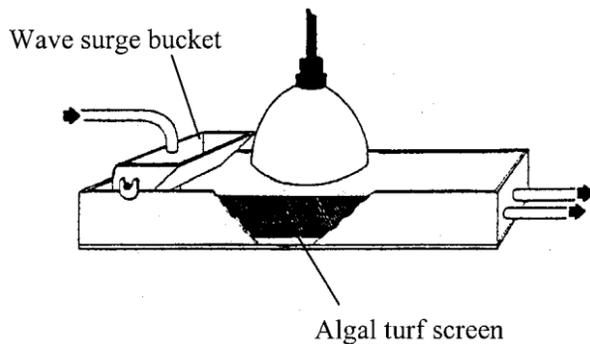
Figure 2.4: Lab Scale ATS System (Adey and Loveland, 1998).

Figure 2.3 : ATS on the Great Wicomico River off the Central Chesapeake Bay (Adey et al, 2013).

In different studies, ATS systems are used in non-point pollution recovery, manure effluent treatment, and wastewaters treatment. Adey et al. (1993) used ATS technology to remove phosphorus from natural waters in the Florida region and found that the organic content produced in the experiment was extraordinarily high, with levels of phosphorus in the biomass varying from 0.38% to 0.42%. Biomass productivity and nutrient recovery from the ATS from wastewater is often quite high, and production costs of algae are lower in ATS than in photo bioreactors (Adey et al., 2011). Mulbry and Wilkie (2001) used ATS technology to treat dairy manure with freshwater algae cultivation. They used liquid digested dairy manure to supply an

ATS with nutrients at a rate of 0.6– 0.96 g total nitrogen day^{-1}, and observed an approximate dried algal yield of 5 g m^{-2} day^{-1}. The dried algae contained 1.5% to 2% phosphorus and 5% to 7% nitrogen. Mulbry et al. (2008) conducted similar experiments on raw dairy manure and swine manure effluents with varying loading rates of total nitrogen, finding similar productivities. As such, ATS technology has the potential for sustainable tertiary treatment of sewage for removal of nutrients and other contaminants (Craggs et al., 1996).

2.5   Factors affecting algal colonization and characteristics

2.5.1   Temperature

Temperature plays a major role in the growth and chemical composition of micro algal species (Oliveira et al., 1999; Renaud et al., 2002; Adey et al., 2013). The ratio of saturated to unsaturated fatty acids has been shown to decrease with decreasing temperature in some micro algal species (Oliveira et al., 1999). The optimum temperature range for maximum growth rates varies from species to species. Oliveira et al. (1999) tested the effect of temperature on the growth characteristics of two species of *Spirulina*, i.e., *S. maxima* and *S. platensis*. It was observed that cell production was at a maximum at temperatures of 30˚C to 35˚C for *S. maxima* and 25˚C to 30˚C for *S. platensis*. Also, an increase in temperature decreased the protein content of both species and stimulated carbohydrate production. Both the species had a wide temperature tolerance range from 20˚C to 40˚C, and an increase in temperature increased the growth rate, but at temperatures below 17˚C, the growth rate of algae decreased. Renaud et al. (2002) observed the effect of temperature change on four Australian algal species (*Chaetoceros* sp., *Cryptomonas* sp., *Rhodomonas* sp. and *Prymnesiophyte* NT19). It was observed that all the species had low protein content at temperatures above 27˚C and no consistent trend in carbohydrate content with temperature. The optimum temperature for

growth was 25˚C to 27˚C for *Rhodomonas* sp. and 27˚C to 30˚C for *Prymnesiophyte* NT19, *Cryptomonas* sp., *Chaetoceros* sp. and *Isochrysis s*p. *Chaetoceros* sp. grew well at high temperatures of 33˚C to 35˚C. There were no chlorophyll changes in all the five species with temperature change. Converti et al. (2009) observed the effect of temperature on growth rate and lipid accumulation in *Nannochloropsis oculata* and *Chlorella vulgaris*. *Chlorella vulgaris* growth rate was highest at 30˚C, and a decrease of 17% occurred when the temperature was increased to 35˚C. Also, lipid content increased with a decrease in temperature to 25˚C, whereas the biomass productivity remained the same. For *Nannochloropsis oculata*, the optimum temperature for high growth rate was 20˚C, and there was a decrease in the growth rate when the temperature was increased to 25˚C, and an increase in lipid content when the temperature was reduced to 15˚C. It can be concluded that temperature of the surrounding environment has a great impact on the biomass productivity, lipid content, fatty acids content and carbohydrate content of algal species, where every algal species has a range of temperature tolerable to its cells.

2.5.2   Light

In the case of photoautotrophic algae, both the intensity and wavelength of photons contribute to the major energy source for algal cells, affecting algal productivity (Adey et al., 2013). Sunlight is the main energy source for phototrophic algal cells. The availability of light is crucial for algal cultures and availability of light affects the growth rates by increasing photosynthetic activity until reaching a threshold point, but further increases in light intensity will no longer increase photosynthesis rate. Higher intensities can damage light receptors in the chloroplasts of the cells (Lee, 1999). The main requirement of algal cultivation systems is uniformity of light to get high cell densities (Grobbelaar, 1994).

Newly developed light emitting diodes (LEDs) with characteristics of narrow band wavelength and low power consumption can be considered the optimal light sources for cultivating algae at the laboratory scale and studying the effect of light wavelength on algal cells (Wang et al., 2007; Michel and Eisentraeger, 2004). In 2007, Wang et al. observed the effect of light intensity and wavelength on micro algal colonization. They selected white (380–760 nm), red (620–645 nm), yellow (587–595 nm), green (515–540 nm) and blue (460–475 nm) LEDs to test their effect on the growth rate of the blue-green alga *Spirulina platensis*. It was observed that biomass density was lower with blue LEDs because absorption bands of chlorophyll were not present in wavelengths more than 460 nm. Red LED had highest biomass density because the red color was absorbed through green pigment of chlorophyll and the blue color has least biomass because absorption bands of chlorophyll were not present in wavelengths more than 460 nm. Optimum light intensity is an important factor for the algae cultivation in the lab, and light intensity requirements differ for different algal species according to their cell absorbance.

2.5.3 pH

pH is another physiochemical factor that affects the cell growth and formation of omega-3 fatty acids in the cultivation of microalgae. Jiang and Chan (2000) studied the effect of pH on the growth characteristics of *Crypthecodinium cohnii* and observed that it can grow well in a wide pH range of 5.5-9, but the highest amounts of dry cell weight, fatty acid saturation and glucose formation occurred when the pH was 7.2, and no growth occurred at pH values of 4 and 10.

2.5.4 Nutrients

The primary nutrients required for algae production are nitrogen, phosphorus and carbon. The source of carbon is typically CO_2 for autotrophic algae and organic carbon for heterotrophic algae. Nitrogen and phosphorus can be taken up by microalgae mostly in the form of nitrates and phosphates, respectively, which are available in abundance in wastewaters and natural waters (Rawat et al., 2011). Other trace elements required for the production of algae are silica, calcium, magnesium, potassium, iron, manganese, sulfur, zinc, copper, and cobalt (Knud-Hansen et al., 1998). Redfield (1958) observed that planktonic biomass contains C, N and P in an average atomic ratio of 106:16:1, which is similar to the ratio of C, N and P in marine waters. However, according to Rhee (1978), different algal species require different proportions of nitrogen and phosphorus for their optimum growth, and their growth rate is limited by the nutrient in shortest supply. Species specific optimum nutrient ratios may be the basis of exclusion or co-existence of competing species (Rhee 1974, 1978). For example, the optimum growth for *Scenedesmus* sp. occurs when the N: P ratio is 30 (Rhee, 1978). Xin et al. (2008) studied the effect of nutrient concentration on growth rate of *Scenedesmus* sp., observing that for high removal efficiencies for both nitrogen and phosphorus, the N: P ratio should be controlled in the range of 5:1–8:1 (Xin et al., 2010). According to Converti et al. (2009), a 75% reduction in the nitrate concentration of the growth medium increased the lipid content of *Nannochloropsis oculata* from 7.90% to 15.31% and of *Chlorella vulgaris* from 5.90 to 16.41%, with no change in the biomass productivity. It can be concluded that nutrients levels should be maintained in the production of microalgae for different fields, e.g., if the algae is to be used in biofuel production, the biomass lipid content should be high as compared to the algal species to be used in other fields like cosmetics, medicine, etc.

2.5.5   Substratum characteristics

Substratum characteristics are known to be the key factor in the determination of the extent of cell adhesion to surfaces (Crawford et al., 2012). Algal abundance and species composition are controlled by substratum topographical features and time available for substratum colonization (Burkholder, 1996). Harlin and Lindberg (1977) conducted an experiment to see the effect of surface relief on the algal turf development and population structure in a natural wave marine environment, integrating variations caused by modifying factors like inclination, distance from the shore and depth. They divided acrylic discs into four quadrants. Three quadrants were cemented by three grades of discrete monolayers of hard particles differing only in the diameter (0.1 to 0.5 mm, 0.5 to 1.0 mm and 1.0 to 2.0 mm) and the fourth quadrant was left smooth. Surfaces were painted with dissolved plastic to ensure the chemical uniformity of all the particles. Twenty five similar discs were placed in spring, fall and winter (1972-1974) in Narragansett Bay in Rhode Island. It was discerned that there was no difference in the initial settlement of algal species according to the surface below the dissolved plastic. On the largest two grades, the population of algal species *Chondrus crispus* and *Ulva lactuca* were 79.5 and 85.2%, respectively. On the smallest grade, the population was significantly lower as 20.1% and 30.8%, respectively, and the smooth quadrant had even less colonization. Abundant species observed during this study were *Chondrus crispus*, *Ulva lactuca, Corallina officinalis* and *Polysiphonia harveyi*. This study described the influence of substratum characteristics on the recruitment of different algal species according to the cell dimensions of marine macroalgal species. If cells are a comparable size to the features, they get enough space to settle and will resist flow motion, resulting in colonization. Substratum roughness is also known to increase the colonization and biomass density of algal species.

Norton and Fetter (1981) conducted an experiment on the brown weed *Sargassum muticulum* to analyze its settlement in stationary and flowing waters and to investigate the effect of substratum characteristics on its settlement. They concluded that in still water, cells of the seaweed remain in contact with the surface wherever they land. In flowing waters they accumulate in depressions rather than on the 'peaks' of the micro topography. The number of propagules that settle out from a given inoculum decreases with increasing water velocity over the range tested, 22-55 cm s^{-1}. They concluded that smooth substrata were the least favorable for the algal spores to settle; more settlement occurred on substrata with increasing surface relief up to an optimum roughness, where the depth of the depressions averaged 800 μm, and settlement was least at higher rugosity. A heterogeneous topography on the substratum will include depressions where flow velocity is reduced, allowing spores to settle down and colonize (Stevenson 1983, Stevenson 1997).

Cao et al. (2009) conducted an experiment in which green microalgae *Scenedesmus dimorphus* was grown on two stainless steel sheets, of which one was smooth (the control) and the other sheet was laser textured with dimple sizes of about 6-8 μm in diameter and 2-3 μm in depth and 40 μm apart. Results from this study also demonstrated that algae preferred textured stainless steel surfaces as compared to the smooth ones. Microscopic images indicated that algal cells filled into the dimples and clustered around them. Again they tested two dimple sizes of about 250 μm and 1 mm in diameter and observed that more cells of *Scenedesmus dimorphus* were found to attach to surfaces with dimples of 250 μm diameter than with 1 mm diameter. This research suggests that specific algal cells could select certain sizes of dimples for attachment.

In 2013, Cui et al. observed the effect of micro textured substratum on microalgae cell attachment. They investigated the attachment of two microalgae species, *Scenedesmus dimorphus* and *Nannochloropsis oculata*, on two textured polymers, polycarbonate and nylon. Three texture patterns (ridge, groove, and pillar) of varying width and depth were designed on the surfaces of both polymers. In this study it was observed that, independent of surface chemistry, surface texture plays an important role in algal attachment. Feature spacing affected the selection of algae attached to the surface when the spacing between two features was of the same size as the algae species. Any spacing smaller or larger than the cell size will reduce the adhesion strength. The grooved surface had a better attachment for both algal species, and there was no significant difference between pillar and ridge for *N. oculata* in both materials. Compared with the smooth control surfaces, *N. oculata* showed reduced attachment on polymers with ridges and pillars. *S. dimorphus* showed attachment on polycarbonate with pillars while there was reduced attachment on nylon with the same pattern. Polymer with ridges seemed to have no influence on the attachment of *S. dimorphus*. Considering the properties of the microalgae cells, *S. dimorphus* is a freshwater unicellular alga with length of 10-16 μm and width of 3-5 μm, whereas the marine species *N. oculata* is known to be spherically shaped and 2-5 μm in diameter. The ridge spacing was close to the cell size of *N. oculata*, but the depth was only 1 μm so the attachment was still point contact where cells might bridge between or align on the features, whereas *S. dimorphus* was larger than the feature size, so bridging became necessary for settlement. In case of two grooves with the same width and different depth (larger than the cell size), the deeper groove could achieve more cell colonization due to the increased surface area available for cell-substratum contact. This study demonstrates that substratum characteristics have a greater role in the selection of the algae species that colonize.

Granhag et al. (2004) tested the settlement and adhesion of green alga *Ulva linza* on defined substratum topographies. They tested a range of substratum topography (R_z: 25-100µm) by creating patterns with ridges and depressions. They concluded that fewer spores were removed from surface roughness of 25 µm by a water jet as compared to a smooth surface and a 100 µm roughness surface, i.e., roughness has a strong effect on the strength of attachment of different algal species to its substratum. Wirtanen et al. (1995) also reported a positive correlation between surface roughness and adhesion of benthic species to the surface.

 Hassan et al. (2012) investigated the effects of surface roughness and shear on the attachment of *Oscilltoria* algal species filaments onto stainless steel coupons in a spinning cylindrical environment. The surfaces in this study were manufactured with traditional abrasive processes (sanding and hand tools). Six coupons with average roughness (Ra) increasing from 0.801 µm to 1.309 µm were utilized. It was found that the amount of algae strands deposited in the coupons increased with the average roughness. From the above studies it is evident that physical and chemical compositions of the substratum greatly impact the selection of species in mass cultivation, and there can be a range of substratum feature size that is optimum for any given species.

 Substratum also influences the availability of inorganic phosphorus, nitrogen, and carbon for associated algae, thus substratum can alter the ability of periphyton to deal with different resource availability the aquatic environments (Vadeboncoeur and Lodge, 2000). Adey et al. (2013) investigated nutrient-substratum interactions in a study conducted in the Great Wicomico River in the Chesapeake Bay. They installed two ATS units in the river to compare two dimensional and three dimensional substrata for algal biomass productivity and nutrient removal rates from the river water. They concluded that yearly mean biomass

productivity with two-dimensional substrata was 15.4 g m^{-2} d^{-1}, and this increased to 39.6 g m^{-2} d^{-1} with a three- dimensional (3-D) screen. Nutrient removal rates increased by 3.5 times with the use of three-dimensional substratum as compared to two-dimensional substratum.

Control of the ATS algal community's composition is important to influence the quality of the biomass for post-harvest applications, for example, using the biomass as a feedstock for biofuels production (Adey et al., 2013). In ATS systems, there is little control over the species selection and dominance when the algae is grown with natural waters or wastewaters in open environments, as the conditions like temperature, light intensity, and nutrient availability are not directly controlled. To combine wastewater pollution recovery with biofuel production, however, it is preferred that species dominance should be controlled, as the species with high oil content can be converted to biofuels economically (Adey et al., 2013). Substratum heterogeneity will determine the flow characteristics on the substratum surface and thus will affect the biofilm colonization, growth, and metabolism. There are few detailed studies in the literature related to the behavior and dominance of species over a range of controlled substratum characteristics, and to the role of substratum topographies and features in recruitment of different algal species under controlled conditions. Vadeboncoeur and Lodge (2000) suggested that substratum alters the dominance of periphyton communities according to nutrient availability, but there is no detailed study on the effect of substratum on algal turf colonization in varying nutrient concentrations environments.

2.6   Use of 3D (Three Dimensional) or additive manufacturing (AM) printing in the biological field

3D printing or additive manufacturing (AM) is a newly available technology in which three dimensional surface characteristics can be fabricated at the micron level from plastic and

powder through a variety of processes. Due to the ability to design complex structures, 3D printing can also be used in non-manufacturing processes related to biological phenomena. AM techniques have been investigated for the fabrication of organized tissue constructs to repair or replace damaged or diseased human tissues and organs (Melchels et al., 2012). Other studies have demonstrated the use of AM technology in tissue engineering, in which 3D scaffolds were developed that guide cells to form functional tissue and match bone elastic properties with desired porosity (Hutmacher et al., 2004).

Connel et al. (2013) reported a micro-3D printing strategy for creating "designer" ecosystems tailored to investigate the interaction and integration of multiple bacterial populations within any 3D arrangement. In this study they tested the behavior of two pathogens (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) under different spatial structures.

As shown in Figure 2.5, microstructures using 3D printing have been established around the desired cell to enhance its colonization. 3D printing technology has also been used in the manufacturing of antimicrobial medical devices. In the medical industry, surfaces are generally treated externally with antimicrobial substances. Sandler et al. (2014) conducted a study in which they used nitrofurantoin (NF) and polylactic acid (PLA) as a biodegradable polymer. Two samples were tested. In one sample both PLA and NF were printed using 3D printing and in the second one printing is done by PLA only and NF was externally applied. It was observed that the surfaces printed with 3D printing from these substances has 85% more inhibition to biofilm formation.
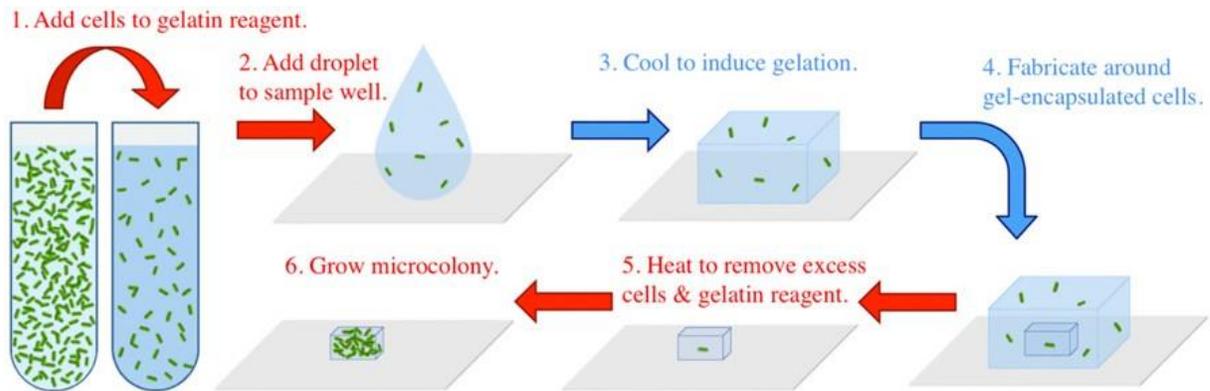
Figure 2.5: Gelatin-based micro-3D printing in the presence of bacteria (Connel et al, 2013).

2.6.1 Using 3D printing or AM technology to replicate surface roughness

3D printing is a well-known technology that has replaced many conventional manufacturing techniques. 3D printing is a very versatile field, and different types of materials can be used for printing structures, such as plastics, ceramics and metal particles (Dimitrow et al., 2006). AM technology can be used for the design of features with micron level replication of surface roughness, such that micro-topography can be controlled to affect the flow characteristics and cell behavior at the boundary condition. Preliminary level work has been done in this field to test whether chemical composition of polymer used in 3D printing is favorable for algae biofilm colonization and to determine the effect of surface roughness of the substratum on algal biofilm colonization (Kardel et al., 2015). The chemistry at the surface of a colonized substratum can strongly affect the type and characteristics of the attached algal biofilm. To test this, smooth tiles of size same as ceramic tiles were designed using Solidworks (Dassault Systems SolidWorks Corp., Waltham, MA) and fabricated with an Objet 30 printer. A mesh having smooth printed photopolymer plastic tiles and ceramic tiles were put in natural streams to investigate if algal species will colonize plastic surfaces as compared to ceramic

tiles. Tile mesh replicates were put in the 6 different streams for a period of 21 days. It was observed that the printed tiles had more colonization than ceramic tiles (Figure 2.6). All the biomass was harvested by vacuum harvesting, and samples were observed under microscope for species identification. Five different filamentous genera, including *Cladophora*, *Microspora, Mougeotia, Oedogonium* and *Sirogonium* were observed on the tiles.
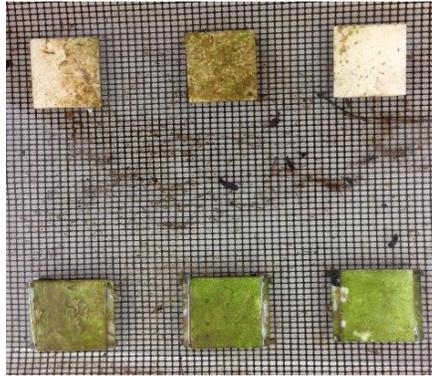
Figure 2.6: Comparison of ceramic tiles and printed plastic tiles in natural streams (Kardel et al., 2015).

In addition, two experimental plates with roughness variation were designed for examination of substratum effects on algal colonization. A rectangular plate (90 mm x 100 mm) with four parallel channels and 5 mm collimating walls was designed with hemispherical surface features of increasing scale. The first channel was smooth while the remaining three channels had a pattern of hemispheres of diameters 500, 1000, and 2000 μm, respectively. A second plate was circular in shape (diameter of 100 mm) having 4 quadrants with each quadrant containing the same pattern and scale of features as in the rectangular plate. Both plates were put in a laboratory-scale algal turf scrubber for a period of 45 days. All 4 sections of the tiles were harvested separately to analyze the difference in biomass characteristics (Figure 2.7).

Figure 2.7: Circular and rectangular tiles before and after biofilm colonization in an ATS.

Preliminary results from dried biomass harvested from different portions of the tiles are shown in Figure 2.8. The circular tile appears to have generally more biofilm colonization as compared to the rectangular tile. This might be explained by the effect of the lane boundaries present on the rectangular tile, which could restrict free water flow from all directions. In the case of the circular tile, however, there were no such restrictions, and turbulent flow would move from all sides on the surface, possibly to the benefit of biofilm colonization. In addition, the surface characteristics seem to affect the algal biomass density, where the surfaces with larger hemisphere diameters had higher biomass per unit area.
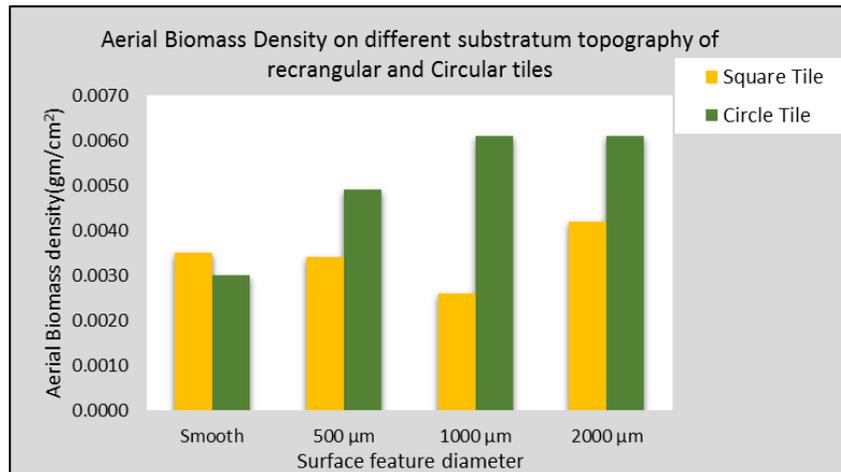
Figure 2.8: Biomass density on different surfaces of both the tiles.

2.7 Current limitations in algae industry

Wastewater treatment and recycling must be incorporated with algal biofuel production to be economical (USDOE, 2010). Two main limitations about this process were listed in the Department of Energy's Aquatic Species Program, i.e., efficient removal of algal biomass from the growth medium (suspended algae) and contamination of unwanted species in the culture (Sheehan et al., 1998). A major problem related to biofuel production from algae is the high cultivation costs of algae, stemming mainly from harvesting. Removal of suspended algae from water is a major cost, as the various methods used for the separation like filtration, sedimentation, flocculation or centrifugation are very expensive (Cao et al., 2009; Cui et al., 2010). When cultivating at large scales in open environments with wastewaters, there is little control over factors including temperature, pH, light and dominance of species. Biomass and growth characteristics of algae are mainly dependent on the type of culture species and the reactor design. In the literature, there is little knowledge regarding the effect of substratum characteristics on algal species dominance or biomass productivity in mass cultivation. In addition, there is no detailed study about how the precise design of controlled surface micro topography of the substratum would allow for specific zones for biofilm colonization and continued growth, which might affect performance of the system at the larger scale. By designing the substratum characteristics, we can test the hypothesis that substratum features and topography will affect the recruitment of algal species according to their cell sizes, boundary layer characteristics, and flow characteristics, and whether this can increase the quality of benthic algal biomass regardless of the environmental conditions.

Physical characteristics of the substratum can affect the growth dynamics of algae by limiting the micro- or macro-nutrients availability through transport processes. Substratum

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also influences the availability of inorganic phosphorus, nitrogen, and carbon for associated algae (Vadeboncoeur and Lodge, 2000). Therefore, substratum designs can be tested under different nutrient conditions for algal species dominance and biomass productivity to investigate whether substratum alters the algae growth under different resources availability.

Chapter 3: Material and Methods

3.1   Introduction to the Study

This chapter describes the methods used to test the effect of substratum characteristics on four selected filamentous algal species recruitment and algal biomass characteristics under different nutrient concentrations. The experiment was conducted in a re-circulating flow-lane photo- incubator specially designed for benthic algal biofilm cultivation experiments (Rains and Blersch, 2015). The flow lane incubator consists of five geometrically identical flow lanes with the base of each covered with smooth unglazed ceramic tiles and fed from a common reservoir. Four out of five lanes of the flow lane incubator were used in this study. Controlled substratum characteristics were designed by the use of 3D printing technology. Square shaped tiles (0.102 m x 0.102 m or 4 in. x 4 in.) having four different substratum characteristics were designed and fabricated in an Objet 30 machine (Stratasys Ltd., Eden Prairie, MN), which uses poly-jet technology to deposit a layer (28 µ m thick) of UV - light – cured acrylic polymer. One tile was kept smooth, and surface topographies on the other three tiles were designed using closely-packed hemispheres of radius 500 µm, 1000 µm, and 2000 µm. These tiles were used as a template mold to fabricate unglazed ceramic clay replicates tiles. Five clay replicates of each of the four substratum topographies were made for each treatment. Clay tiles were placed in the flow lane incubator in a pseudorandom pattern by replacing a number of in situ ceramic tiles. The reservoir was filled with distilled water and algae cultivation medium. The algae cultivation medium used in the study was Proline F/2 algae food (Pentaire Aquatic

Ecosystems). The flow lane incubator was seeded using four different filamentous algal species named as *Oedogonium crassum, Sirogonium sticticum, Microspora floccose,* and *Mougeotia scalaris* from different lab and natural sources. Environmental conditions including temperature, pH, and conductivity were monitored on a daily basis and kept as constant as possible. Flow rate, flow uniformity and flow velocity were kept uniform and under control for each flow lane. The effect of substratum characteristics on algal species recruitment and algal biomass characteristics were tested under three different nutrient concentrations.

3.2   Experimental Set Up

Experiments were conducted in a re-circulating flow lane photo incubator located in the Green Infrastructure Lab, Hubbard Center for Advanced Science, Innovation and Commerce (CASIC), Auburn University, Auburn, AL, USA (Figure 3.1; Figure 3.2). Rains and Blersch (2015) designed this incubator to be used in experimentation on the effects of substratum characteristics on selection, attachment and growth of filamentous benthic algae in a flow environment. The incubator consists of five geometrically identical flow lanes (10 cm wide and 100 cm long) fed with a recirculating flow from a common reservoir. The flow lanes are covered with detachable 5.02 cm (2 in.) square sized unglazed ceramic tiles. Each flow lane consists of 38 similar ceramic tiles in a 2x19 array. An adjustable 0.03-0.32 L s^{-1} (0.5-5 gallon min^{-1}) flow meter (Hydronix AFM-055 flow meter, Chino Hills, CA, USA) is installed on each lane for flow rate control. Removable collimators are fixed at the inlet of each flow lane to collimate the flow from the flowmeter, and detachable weirs are placed at the downstream end of each lane to set the flow depth. Uniform light to the incubator is provided by T5 fluorescent plant grow lamps (Envirogro Hydrofarm, Petaluma CA, USA). Five lamps are placed perpendicularly across the flow lanes.

Figure 3.1: Flow lane incubator and frame assembly.

A reservoir having a 76 L (20 gal) capacity rests at the bottom of the flow lane incubator on the aluminum frame, allowing gravity flow return from the outlet manifold of the incubator, and water recirculates to the flow lanes through the flowmeters using a submersible Pondmaster 4500 L h^{-1} (1200 gal h^{-1}) magnetic drive pump (Danner Manufacturing, Islandia, NY, USA). Four out of five flow lanes of the incubator were used in this experiment to ensure uniformity in the light intensity across the lanes, as it was reported that the exterior lanes had a lower light intensity compared to the center lanes (Rains and Blersch, 2015).
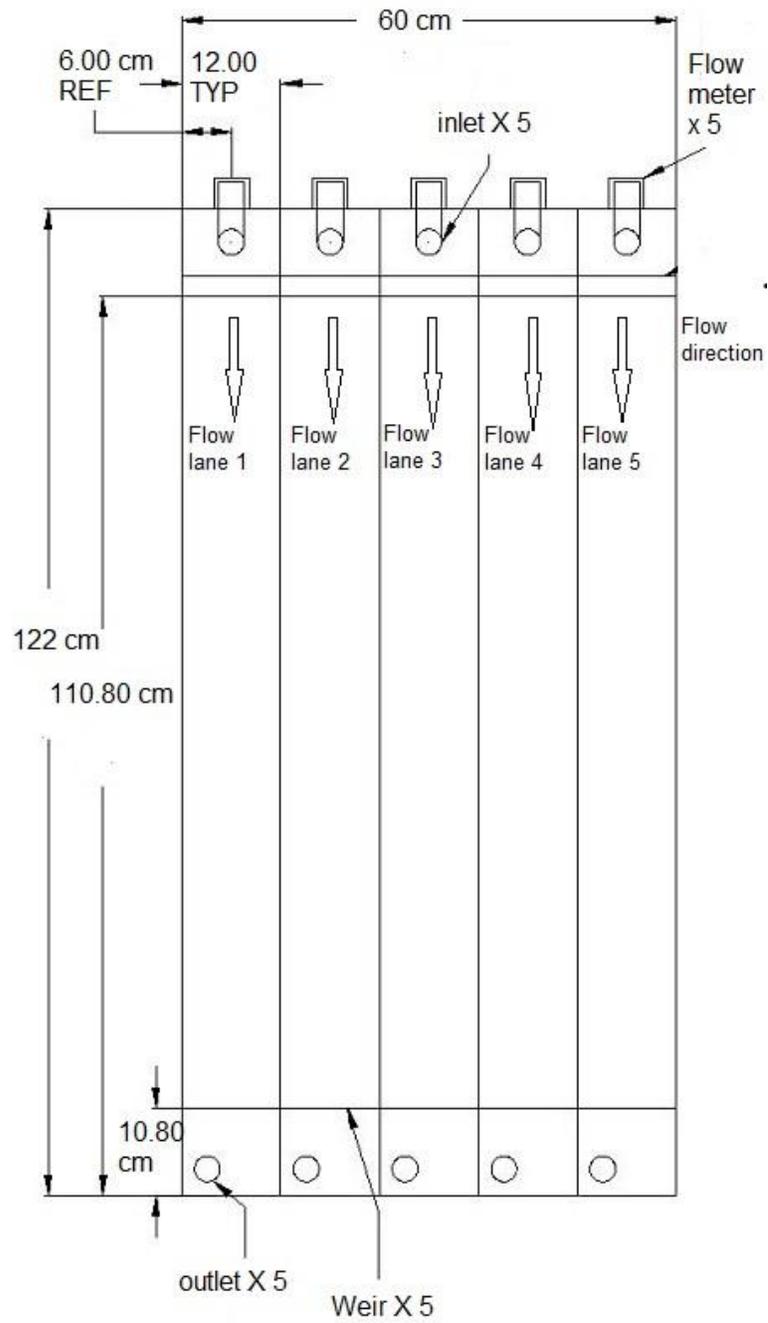
Figure 3.2: Schematic diagram of flow lane incubator with dimensions.

3.2.1 Preliminary experimentation on Flow lane incubator

To test the effect of substratum characteristics in the flow lane incubator, it was required that operating parameters such as flow rate, flow velocity, and light intensity should be uniform throughout the experiment under controlled environmental conditions. Preliminary experimentation was done to test these properties.

3.2.1.1 Flow rate and Flow Velocity

Flow rate for each lane was set at 0.03 L s^{-1} (0.5 gal min^{-1}) using the adjustable flow meters. Flow velocity and uniformity in each flow lane was observed by video analysis of food coloring dye injected at equal intervals in each lane. Video analysis was done by mounting a camera (Sony Webbie HD MP4 and 5MP all-in-one camera) to a bracket above the incubator to allow the complete view of all flow lanes. Movement of dye down the length of each lane provided the velocity estimation and flow dispersion among lanes. Mean velocity in all four channels was 0.042 ± 0.003 ms^{-1} (Figure 3.3). The significant difference in average velocity among different lanes has been tested using one way Analysis of Variance (ANOVA). Velocity values among all four lanes were not significantly different ($p=0.24$). Reynolds number for the flow lane incubator varied from 100-1000. It indicates that flow in the incubator was laminar.
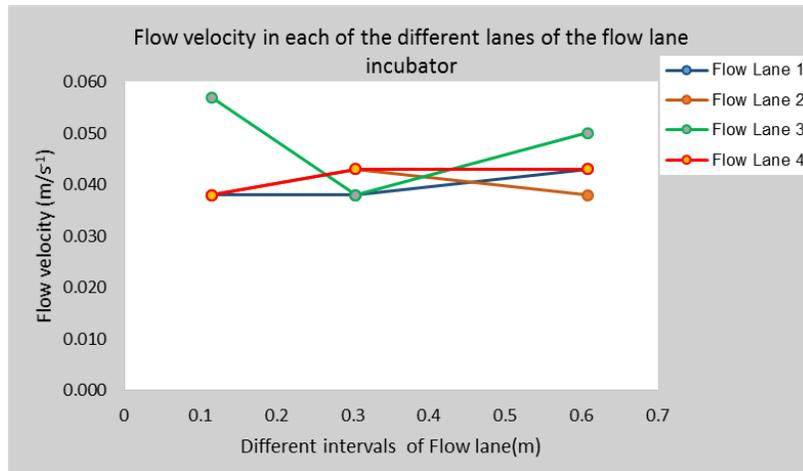
Figure 3.3: Flow velocity at different intervals of each flow lane when set to 0.03 L s^{-1}.

3.2.1.2 Light Characteristics

The incubator lighting characteristics were measured by a photon flux sensor (Apogee MQ-200; Logan, UT). The photon flux sensor was used to measure the amount of photosynthetically active radiation (PAR) across each individual tile. A heat map of the PAR data collected by photon flux sensor was generated using Matlab 8.5 (Mathworks, Inc.) (Figure 3.4). Flux variability across the four lanes of the flow lane photo incubator was tested using Analysis of variance (ANOVA) and there was no significant difference in flux across the selected four lanes (P value=0.25)
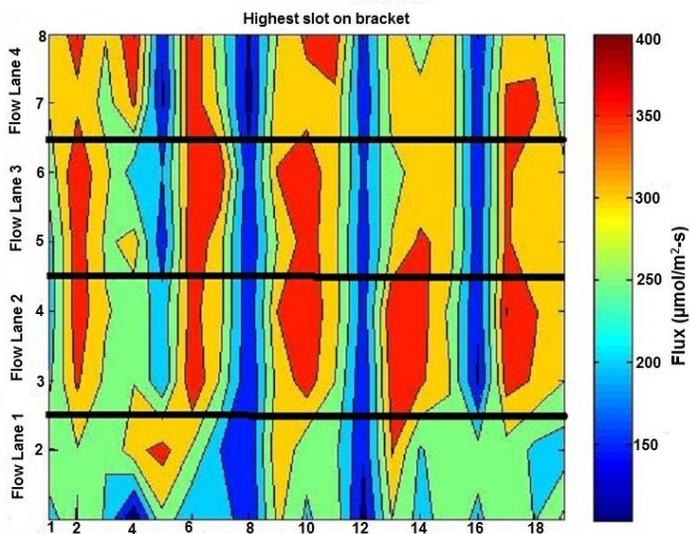
Figure 3.4: Schematic diagram of heat map generation of flux values at each tile of four flow lanes.

3.2.1.3 Biomass Characteristics

The incubator was run at the nutrient media concentration recommended by the manufacturer (0.5 ml L^{-1} or 0.02 oz. gal^{-1}) and baseline environmental conditions (temperature= 24°C $\pm$2°C, pH = 8 $\pm$1.5 and conductivity= 0.20 $\pm$ 0.03 mS cm^{-1}) to test the total harvested biomass in each of four flow lanes. Three subsequent harvests were done on every fourth day to test the total harvested biomass variability among the selected four lanes. Total dry biomass and percentage ash content were measured and calculated for each flow lane. Statistical analysis was done using one-way ANOVA to test for any significant differences in the total dry biomass content and percentage ash content among the lanes. There were no significant difference among different flow lanes (p value of 0.61 and 0.67, respectively) for biomass density and ash content.

3.3 Three Dimensional printing of tiles their replication using clay

Four square sized (0.102 m x 0.102 m or 4 in. x 4 in.) acrylic polymer tiles were designed in Solidworks® and fabricated with a Stratasys® Objet30 3D printer with a 28 μm layer thickness. One control tile was kept smooth (Sa =1.19 μm) and other three tiles had adjacent hemispheres of radius 500 μm, 1000 μm, and 2000 μm respectively. All of the four tiles were replicated using clay in 3D Arts Building, College of Liberal arts, Auburn University, Auburn, AL, US by Dr. Gary Wagoner (Figure 3.5).
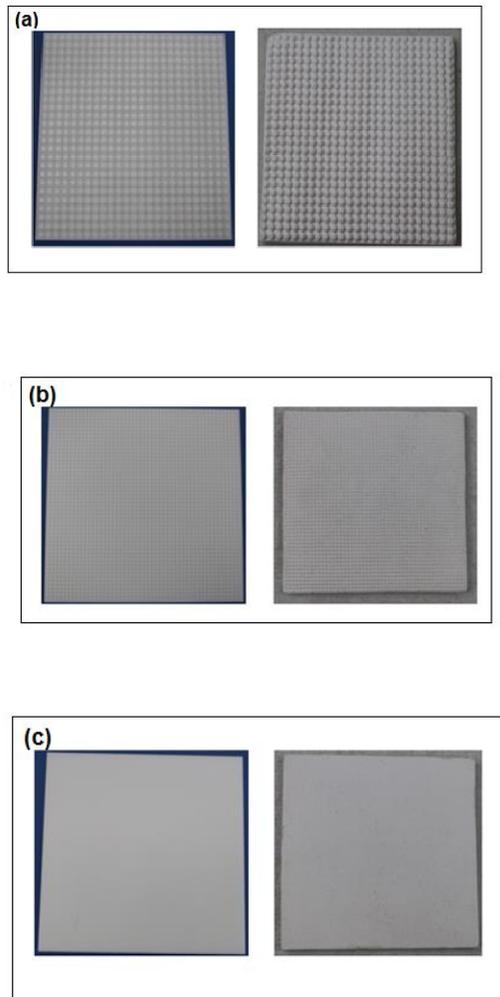
Figure 3.5: 3D printed plastic tile (left) and its replicated clay tile with substratum having radius of hemispheres (a) 2000 μm, (b) 1000 μm and (c) 500 μm.

Smooth tiles have same nominal and actual surface area. Tiles having hemispheres features of different dimensions on substratum have different nominal and actual surface area (Table 3.1).

Table 3.1: Nominal and actual surface area of all the four tiles having different substratum characteristics.

Surface topography on tile	Nominal surface area (cm^2)	Actual surface area (cm^2)
Smooth tile	103	103
Hemispheres of radius 500μm	103	184
Hemispheres of radius 1000μm	103	183
Hemispheres of radius 2000μm	103	183

3.3.1 Set up of the tiles in the incubator

Five replicates of each type of surface topography including five control smooth tiles were selected in each of three treatments and placed in the selected four flow lanes of flow lane photo-incubator in a pseudorandom pattern (Figure 3.6). The initially placed ceramic tiles were removed from the specific locations and replaced by clay tiles (Figure 3.7). The location of the clay tiles was kept similar in both the longitudinal and transverse directions in each of the flow lane. Twenty tiles per treatment, and 60 tiles overall, having four different surface topographies were used in the complete experiment.
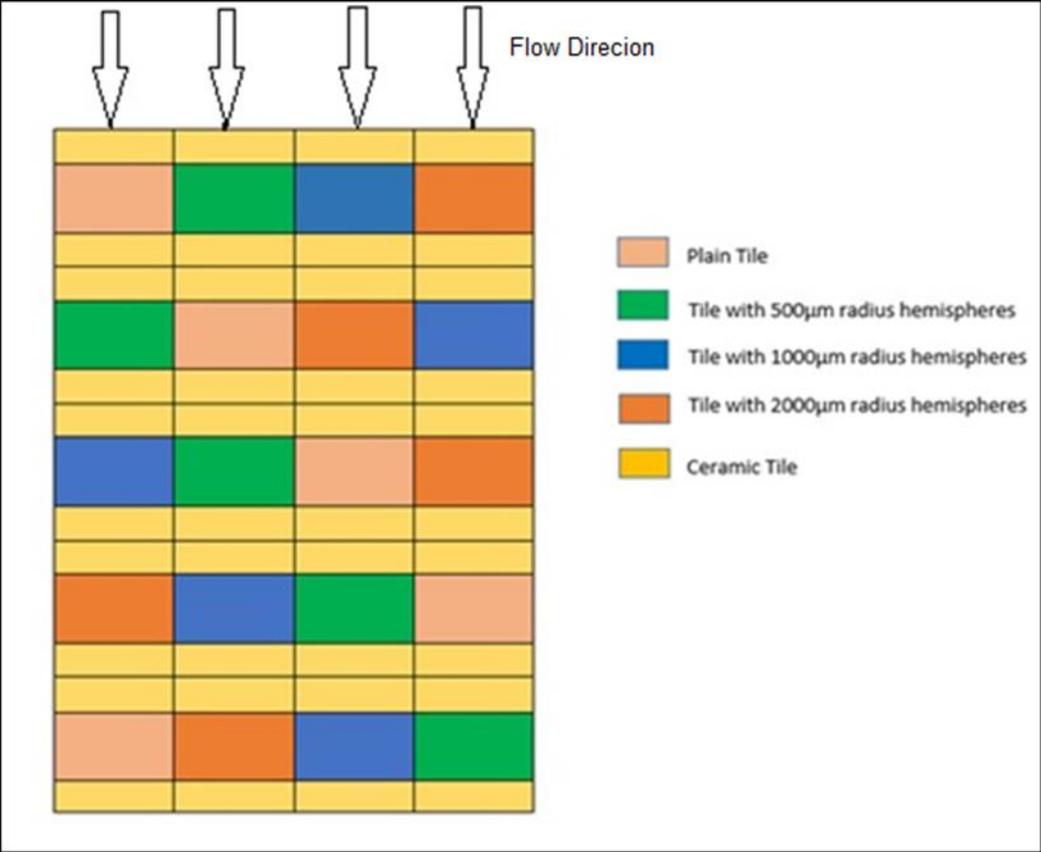
Figure 3.6: Pattern of different clay tiles in the flow lane photo incubator.

Figure 3.7: Placement of tiles on the flow lane photo incubator.

3.4   Algae collection and seedling the incubator

Four different filamentous algal species named as *Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris* were collected from different natural and lab sources for seeding the incubator (Figure 3.8). Filaments of each algal species were removed from the sample with the help of tweezers and poured into a 10 ml glass cylindrical container. All the four algal species were mixed in the container. Three different samples of algae were taken from the mix and stored in formalin vials for microscopic analysis before seeding the incubator with the remainder. The algal species sample was added to the reservoir with distilled water and F/2 medium for 15 days incubation period. A Motic optical microscope (Motic Corp., Richmond, BC) was used at 400 X to take 10 random images from three

subsamples of each sample, and the number of times each species appeared in the micrograph was counted and analyzed for differences using one way ANOVA. It was observed that there was no significant difference (p value= 0.62) in the occurrence of four species in all the three samples.
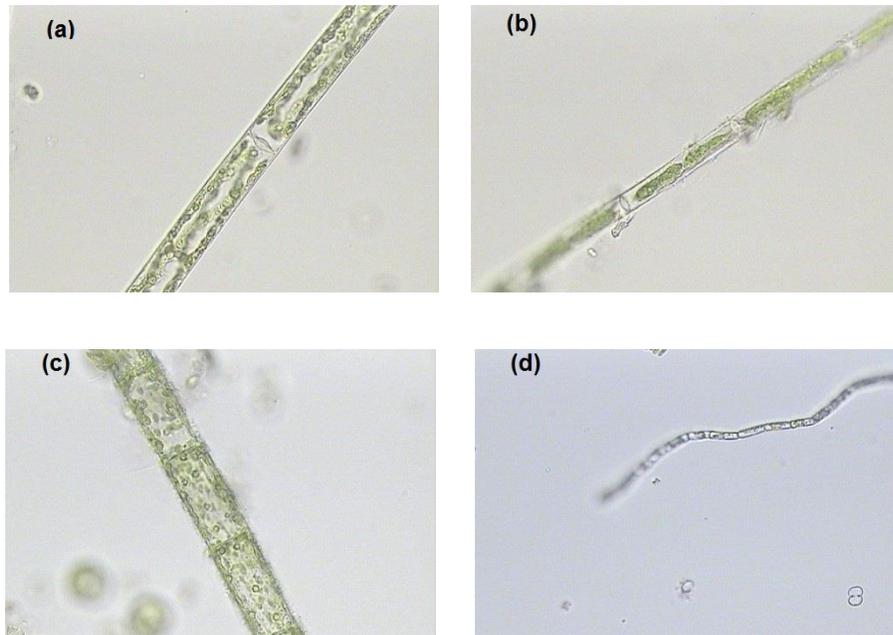
Figure 3.8: Algal species used for seeding the incubator (a) *Sirogonium sticticum*, **(b)** *Mougeotia scalaris*, **(c)** *Oedogonium crassum* **and (d)** *Microspora floccose*.

3.5   Nutrient concentrations selected for the experiment

Microalgae require various nutrients, minerals and vitamins in specified ratios for growth. Proline F/2 algae food (Pentaire Aquatic Ecosystems) based on the Guillard (1975) F/2 formation recipe (Table 3.2) was used for providing required nutrients for algae cultivation.

Table 3.2: Recipe of F/2 algae food (Guillard and Ryther 1962, Guillard 1975)

Chemical Component	Mass (gmol^{-1})	Final concentration (M)	Final concentration (gL^{-1})
NaNO$_3$	84.98	8.82×10^{-4}	0.075
NaH$_2$PO$_4$·H$_2$O	137.97	3.62×10^{-5}	0.005
FeCl$_3$·6H$_2$O	270.30	1.17×10^{-5}	0.0032
MnCl$_2$·4H$_2$O	197.01	9.10×10^{-7}	1.79×10^{-4}
ZnSO$_4$·7H$_2$O	186.00	7.65×10^{-8}	2.19×10^{-5}
CoCl$_2$·6H$_2$O	237.00	4.20×10^{-8}	9.95×10^{-6}
CuSO$_4$·5H$_2$O	249.00	3.93×10^{-8}	9.79×10^{-6}
Na$_2$MoO$_4$·2H$_2$O	237.88	2.60×10^{-8}	6.18×10^{-6}
Thiamine · HCl (vitamin B1)	333.27	2.96×10^{-7}	1.00×10^{-4}
Biotin (vitamin H)	242.45	2.05×10^{-9}	5.00×10^{-7}
Cyanocobalamin (vitamin B12)	1355.4	3.69×10^{-10}	5.00×10^{-7}
Na$_2$SiO$_3$·9H$_2$O	284.04	1.06×10^{-4}	0.030
Na$_2$EDTA·2H$_2$O	374.24	1.17×10^{-5}	0.0044

The experiment was divided into three treatments according to the concentration of F/2 medium provided for algae cultivation. The first treatment, established as the baseline medium concentration, was at the concentration recommended by the medium manufacturer using the recipe of 0.53 ml L^{-1} (0.02 oz. gal^{-1}) required for the algae to grow. The second and third treatments were conducted at the nutrient concentrations of two and four times, respectively, of the concentration used in the first treatment (Table 3.3).

Table 3.3: Concentration of N and P used in all three treatments

Treatment	Concentration of F/2	Concentration of N $(mg\ L^{-1})$	Concentration of P $(mg\ L^{-1})$
1	1X	12.34	1.122
2	2X	24.68	2.244
3	4X	49.38	4.88

3.6   Initial start of the incubator and its daily operations

The incubator reservoir was filled with 57 liters (15 gallons) of distilled water at the start of experiment. The seeding algae sample was also put in the reservoir. The incubator pump was started, and 7.5 ml each of Proline F/2 algae food A and B was added to the reservoir. The volume of water lost from the reservoir by evaporation and spilling was estimated and replaced with distilled water after every 24 hours. According to the loss, a proportional amount of F/2 medium was also added on a daily basis. Light intensity was provided for 24 hours a day during the whole period of the experiment. Water quality parameters including temperature, conductivity and pH were monitored on a daily basis during the experiment using a Hannah HI 98130 meter (Hanna Instruments). The flow rate of the flowmeters was also measured every other day to maintain the uniformity in flow characteristics in all the four flow lanes.

3.7   Biomass harvesting, storage and restarting the incubator

Each treatment consisted of four harvests, and the first harvest was done after 15 days of incubation period to allow for sufficient algal colonization. The subsequent three weekly harvests were performed and considered for analysis. The incubator pump was turned off at the time of harvest, and each weir was removed to drain all the water from flow lanes to the reservoir. One clay tile at a time was removed from the incubator for harvesting. The tile was

photographed at that time and then placed back to its location in the incubator. Algal samples were removed from each tile for the microscopic algae species identification with the help of tweezers from 10 selected locations throughout the tile (Figure 3.9). These samples were stored in a formalin vial (VWR prefilled 10% formalin vials, Radnor, PA, US) (Figure 3.10) and refrigerated at 4°C for later microscopic analysis.
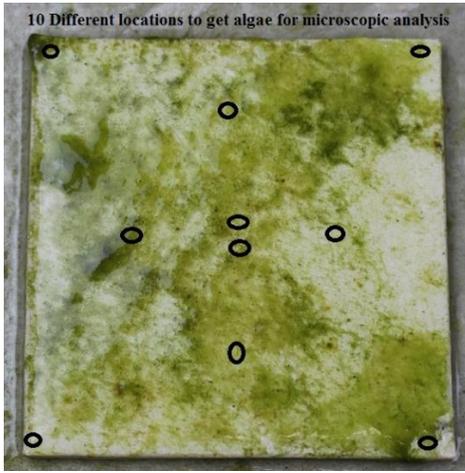
Figure 3.10: Different location to get algae for microscopic work.

Figure 3.9: Storage of algal biomass in VWR formalin vial.

The remainder of the biomass from the tile was removed by vacuum harvesting using a vacuum flask apparatus (Figure 3.11). The tile was rinsed with distilled water and again vacuumed. The whole process was repeated three times to recover all visible biomass from the tile. Following this harvest, the biomass slurry was poured into 125 ml plastic sample bottles. The vacuum flask was rinsed with distilled water to get all the residual algal biomass into the plastic storage bottle. All the algal samples were stored in the refrigerator (Thermoscientific, model MH45PA-GAEE-TS, Asheville, NC, USA) before they were analyzed for total biomass,

ash content and species identification. After harvesting the clay tiles, all the ceramic tiles were also harvested jointly from all the flow lanes by using vacuum harvesting.

Figure 3.11: Vacuum harvesting apparatus (left) and vacuuming process (right).
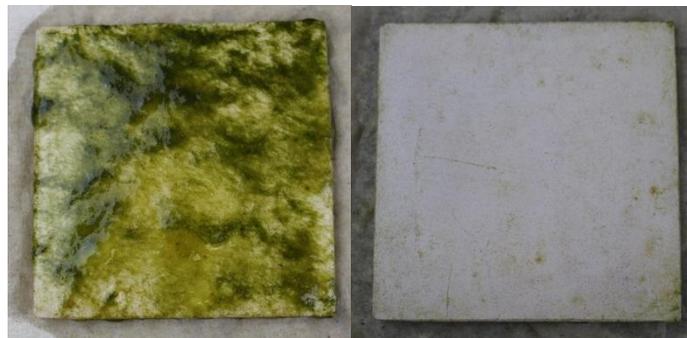
Figure 3.12: Smooth tile before and after harvesting.

A water sample of the reservoir water was taken at the time of harvesting for later water chemistry analysis. Up to 100 ml water was taken from the reservoir and stored in the refrigerator in 125 ml plastic bottles for later analysis of total nitrogen (TN), nitrate (NO_3-N), and phosphate (PO_4-P).

3.7.1   Restarting the incubator after each harvest

After each harvesting was completed 19 L (5 gal.) of water was removed from the reservoir to remove excess salt build up and replaced with fresh distilled water. A proportional

amount of F/2 medium was also added to the reservoir. Then the incubator pump was started, and the incubator was fed daily until the next harvest was done.

3.7.2 Restarting the incubator after each treatment

Light intensity (PAR) at each individual tile was measured using photon flux meter after every treatment to ensure there was no significant difference in the light in radiation across all the flow lanes and individual tiles. A new set of clay tiles were used in each new treatment, and the whole process was repeated, except for the amount of F/2 medium added daily to the reservoir, which was 0.53 ml L^{-1} (0.02 oz. gal^{-1}) and 1.06 ml L^{-1} (0.04 oz. gal^{-1}) for treatment two and treatment three, respectively.
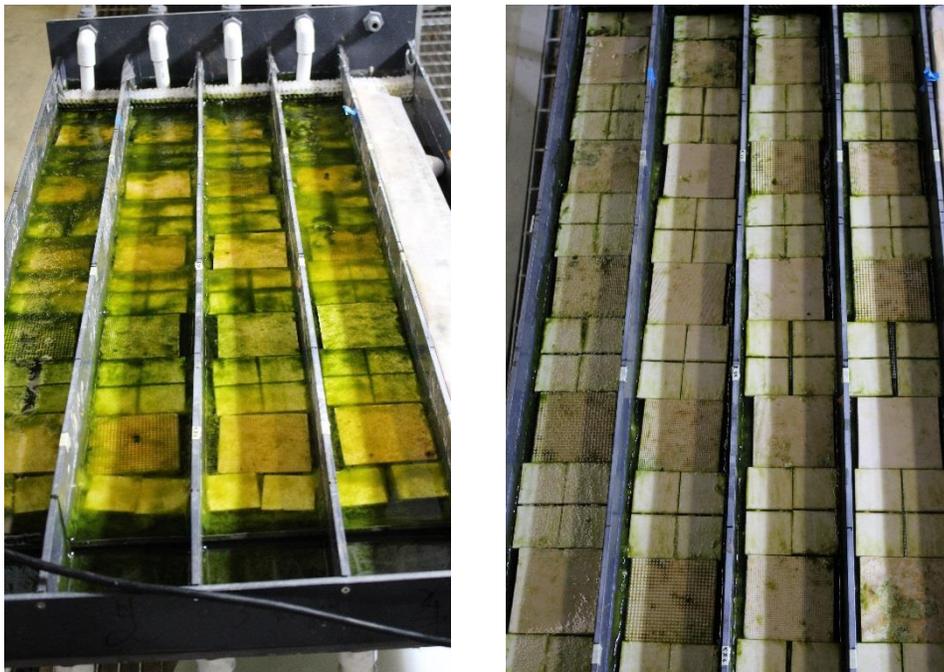
Figure 3.13: Flow lane photo incubator before (left) and after (right) harvesting.

3.8 Biomass Analysis

3.8.1 Biomass density (Biomass per unit area)

Total dry biomass density was calculated using a conventional oven according to standard methods (APHA, 2012). The aluminum weighing pans were pre-dried in the conventional oven (VWR International model 1370FM, Sheldon Mfg., Cornelius, OR, USA) for a period of four hours and then placed in the desiccator. The pans were weighed with an analytical weighing balance (ALC 80.4 Scales Galore, Brooklyn, NY, USA). The biomass sample from each tile was thoroughly mixed with a VWR Analog Vortex Mixer (Marshall Scientific, Hampton, NH, USA) for a period of 120 sec to make the samples uniform (Figure 3.14). The entire volume of the liquid was measured with a graduated cylinder and three subsamples each of 10 ml were drawn with the help of a 10ml graduated cylinder from each sample and poured into three different pans (Figure 3.15). All the pans having liquid biomass samples were put in the conventional oven for a period of 24 hours at 105˚C. Samples were removed from the oven (Figure 3.16) and allowed to cool to room temperature in the desiccator (Figure 3.17). Then the weighing pans having oven-dried biomass were weighed using an analytical weighing balance.

Figure 3.14: Mixing of the biomass samples by VWR Analog Vortex Mixer.

Figure 3.15: Algal samples before putting in the conventional oven at 105°C.

Figure 3.16: Samples after removing from the conventional oven.

Figure 3.17: Dried algal samples in the desiccator.

Following this, the total biomass for each subsample was calculated (equation 3.1)

$$Biomass(g) = W_{pan+biomass} - W_{pan} \qquad (3.1)$$

Where

$W_{pan + biomass}$ = Weight of Pan and biomass after oven drying (g)

W_{pan} = Weight of empty Pan (g)

The average of all the three subsamples were taken, and the total harvested biomass from the whole tile sample was calculated (equation 3.2)

$$Total\ Biomass\ (g) = \frac{W_{average} \times V_{total}}{V_{subsample}} \qquad (3.2)$$

Where

$W_{average}$ = Average biomass from three 10 ml subsamples (g)

Vtotal = Total Volume of sample from each tile (ml).

$V_{subsample}$ = Volume of each subsample (ml).

The biomass density of algal biomass on each tile was calculated by using equation 3.3

$$Biomass\ Density(mg/cm^2) = \frac{(Biomass \times 1000)}{Area} \qquad (3.3)$$

Where

Biomass = Total Biomass from the sample tile (g)

Area = Actual surface area of sample tile (cm^2)

3.8.2 Percentage Ash Content

Dry samples were used in the percentage ash content determination. After weighing the samples for oven dry biomass, the algal samples were placed in the muffle furnace having a

ramping program for ash content (Thermoscientific, model F6020C, Dubuque Iowa, USA). Samples were removed from the muffle furnace (Figure 3.18) and placed directly into the desiccator to cool until the weighing pans came to room temperature (NREL, 2005). Aluminum pans were weighed in an analytical balance (ALC 80.4 Scales Galore, Brooklyn, NY, USA) and weight was recorded.

Figure 3.18: Algae samples after removing from the muffle furnace.

Percentage ash content was calculated as follows (equation 3.4):

$$Percent\ ash\ content\ = \frac{(Weight_{Pan+ash} - Weight_{Pan}) \times 100}{ODW_{sample}} \qquad (3.4)$$

Where

Weight $_{Pan+ash}$= Final mass of the pan with ash (g)

Weight $_{Pan}$= Mass of empty pan (g)

ODW $_{sample}$= Oven dry biomass of the sample (g)

3.9   Water Chemistry Analysis

Water chemistry analysis for nitrates and phosphates (NO_3-N and PO_4-P) of the water samples collected at the start of experiment and at time of every harvest was carried out using direct reading photometer (Model YSI 9500, Yellow Springs, OH) (Figure 3.20) for lower nutrient concentrations. For higher concentrations of NO_3-N (greater than 20 mgL^{-1}) water quality test strips (Hach Company, Loveland, CO) were used (Figure 3.19). Higher concentrations of PO_4-P (greater than 4 mg/L) were tested using phosphorus Insta-test water quality test strips (Motte, Chestertown, MD) (Figure 3.20). Conductivity, pH, and temperature readings of water were taken on daily basis (Table 3.3).

Figure 3.19: Nitrate and Phosphate test strips.

Figure 3.20: YSI 9500 Photometer.

Table 3.4: Water chemistry analysis in all the three treatments

Treatment	NO$_3$-N (mgL^{-1})	PO$_4$-P (mg/L^{-1})	pH	Conductivity (mScm^{-1})	Temperature (˚C)
1	38.5±0.24	0.38±0.04	7.65±0.31	0.24±0.02	24.65±0.64
2	43.25±5.54	0.64±0.15	7.22±0.51	1.58±0.44	24.41±0.57
3	45.50±7.79	0.91±0.07	8.50±0.44	1.37±0.76	24.05±0.61

3.10 Microscopic Identification

Species identification was done using digital light microscopy (400 X) using a Motic optical microscope (Motic Corp., Richmond, BC) (Figure 3.21). The sample preserved in the formalin vial was mixed well by shaking by hand, and one drop (0.0625 ml) was transferred to the glass slide by using a dropper. The sample was covered with the microscopic glass cover slip, and micrographs were taken at selected 15 different locations of the glass cover (Figure 3.22) and saved in JPEG format using the Motic software for further image analysis (Figure 3.23). Three subsamples were taken from each tile sample, and 15 micrographs were taken for each subsample in a similar way. In this way, forty five micrographs from each tile sample were taken.

Each micrograph was observed for each of the four algal species named as *Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*. The number of times each of the four species was present in each micrograph was recorded, and the total number for each species was counted for all the fifteen micrographs of each subsample. Algal samples from each tile during all the three treatments were observed in a similar way. A total

of 8100 micrographs were analyzed for the algal species identification to test the effect of substratum topography and nutrient concentration on recruitment and dominance of each algal species.

Figure 3.21: Microscope Set Up.
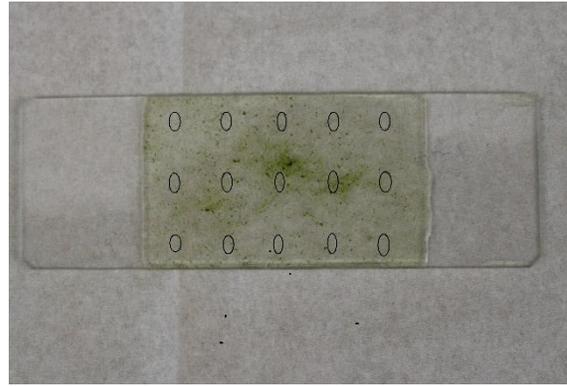
Figure 3.22: 15 different locations where micrographs were taken.
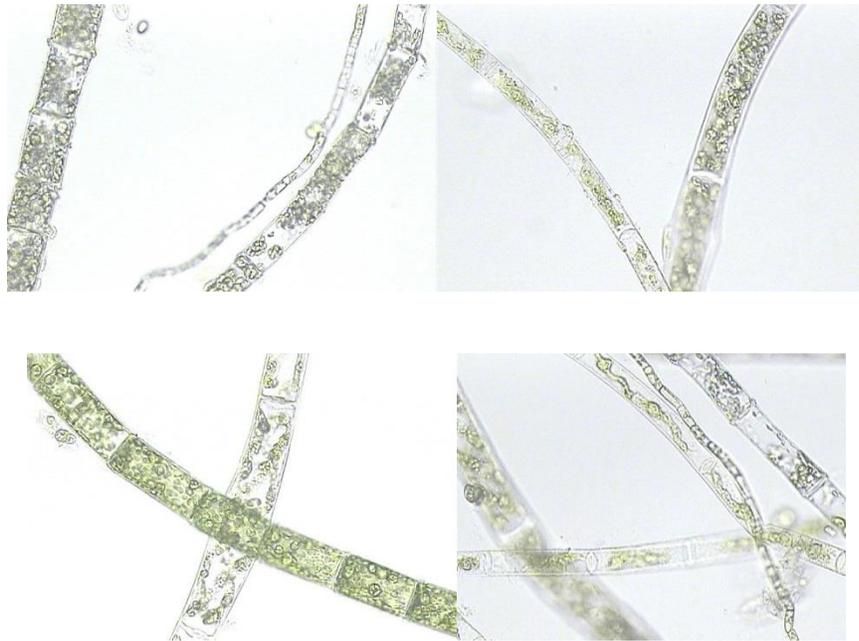
Figure 3.23: Different micrographs obtained by Motic optical microscope.

3.11 Simpsons Diversity Index

Simpson's diversity index was calculated as a measure of the diversity of species on different substratum topographies in each of the three treatments (Simpson, 1949).

Simpson's diversity index was calculated (equation 3.5):

$$D = \frac{\sum n(n-1)}{N(N-1)} \qquad\qquad (3.5)$$

Where

D = Simpsons Index

1-D = Simpson's Diversity Index

n = the total number of organisms of a particular species

N = the total number of organisms of all species

The value of Simpsons diversity index ranges from 0 (no diversity) to 1 (maximum diversity). The greater the value of Simpson's diversity index, the greater is the diversity of algal species found on that particular substratum topography.

3.12 Statistical analysis

Biomass density, percent ash content, and count and relative percent occurrence of each algal species on different substratum topographies recorded for each harvest and treatment were organized using an Excel spreadsheet. Minitab® 17.2.1 (2015) software was used to determine significant differences (at significance level of 95%) between the means of different substratum topographies across different harvests in each treatment and among different treatments. Tests of normality including QQ plots were used to test the normality of data.

Nested analysis of variance (ANOVA) was performed to determine significant differences among the means of biomass density and percent ash content for different substratum topographies in each treatment and among different treatments. Significant differences between different pairs of biomass density and ash content in terms of substratum topography and treatment were compared using the Tukey Kramer procedure.

The count of each tested algal species in different treatments and substratum topography was found to be non-normal and was analyzed using non-parametric procedure, i.e., the Kruskal Wallis Rank Sum test. When significant differences were indicated at a significance level of 95%, multiple comparison tests were performed to determine the significant difference between specific pairs of substratum topography and treatments. A Mann Whitney non-parametric multiple comparison tests were also performed in Minitab software.

Chapter 4: Results and Discussion

4.1 Introduction

This chapter includes the analysis of biomass growth characteristics and species selectivity of four selected filamentous algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) under four different surface topography conditions in three different treatments. Each of three treatments had different nutrient concentrations (low, medium and high), and all other environmental conditions (temperature, light intensity, flow rate and flow velocity) were kept uniform throughout the experiment. Three different dimensions of hemispherical features were selected on square clay tiles (0.102 m x 0.102 m) to test the effect of surface topography on algal biofilm colonization. One surface was kept smooth (control) and three surfaces have repeated patterns of hemispheres having radius of 500 μm, 1000 μm and 2000 μm.

Each treatment consisted of four harvests, and the first harvest was done after 15 days of incubation period to allow the algal biofilm to establish on the clay tiles. Next, three weekly harvests were done and analyzed for biomass density, percent ash content and algal species recruitment. Graphical and statistical analyses of biomass density, ash content, species count and relative abundance of species were tested under different surface topographies as a function of nutrient concentration.

4.2   Experimental results: Objective 1

4.2.1   Biomass Density (Biomass per unit area)

Figure 4.1 illustrates the main effects of nutrient concentration and surface topography on the algal biomass density. It was observed that biomass density increased with increase in nutrient concentration. Treatment 3 had the highest and Treatment 1 had the lowest mean biomass density regardless of the surface topography. The average total biomass density for a harvesting frequency of 7-days was 0.55 mg cm^{-2} for Treatment 1 (lower nutrient concentration), 0.67 mg cm^{-2} for Treatment 2 (medium nutrient concentration), and 1.59 mg cm^{-2} for Treatment 3 (higher nutrient concentration). There was an increase of 21.03% in the biomass density from Treatment 1 to Treatment 2, and then there was an abrupt increase of 136% from Treatment 2 to Treatment 3. These results are similar to Rodolfi et al. (2009), where a 20% increase in biomass productivity was observed when there is a change from nutrient deprived media to nutrient sufficient media. Similar results were reported by Burton et al. (1991), Lohman et al. (1991), and Xin et al. (2010), where the concentration of nitrogen and phosphorus present in water had a direct influence on algal growth kinetics, and their enrichment increases the algal biomass productivity. A number of studies have similar outcomes, where nutrient supply (N and P) sets the primary productivity (algal and sea grass productivity) in aquatic ecosystems when light and temperature are adequate (Howarth, 1988; Hecky and Kilham, 1988).

For seven day harvest frequency, a total average biomass density of 0.90 mg cm^{-2} was observed on the plain surface (control) in all three treatments. Surface topography sections having feature radius of 500 μm,1000 μm and 2000 μm have average biomass densities of 0.92 mg cm^{-2}, 0.87 mg cm^{-2} and 1.06 mg cm^{-2} respectively. The surface having 2000 μm radius

features has the highest biomass density (21% more as compared to control plain surface) and the 1000 µm surface has lowest biomass density (5% less as compared to the control) when tested as a mean in all the three treatments. The surface having 500 µm radius feature size has 2% more biomass density as compared to the control. Cao et al. (2009) obtained similar results, in which surfaces with dimples of 250 µm and 1 mm diameter each had more dense algal attachment than smooth surfaces, suggesting that algal cells would be selected by certain sizes of dimples for attachment. Also, higher attachment is seen on rough surfaces when compared to smooth surfaces in the case of both titanium and stainless steel coupons (Sekar et al, 2004). It is expected that different species will have different attachment preferences as determined by surface topography. According to Adey et al. (2013), 3-D growth substrates allowed greater packing and retention of filaments and diatom cells in the flowing environment of moderate turbulence and mixing, causing greater algal densities. However, the algal density also depends on the diameter of the selected algal species and surface feature dimensions. Surface topographies with scales that match with the diameter of the cell attaching to the surface have more colonization (Cui et al., 2013). Similar results were observed by Callow et al. (2002), who measured a five-fold increase in spore settlement of the alga *Enteromorpha* on 5 µm deep valleys as opposed to valleys of 1.5 µm deep.
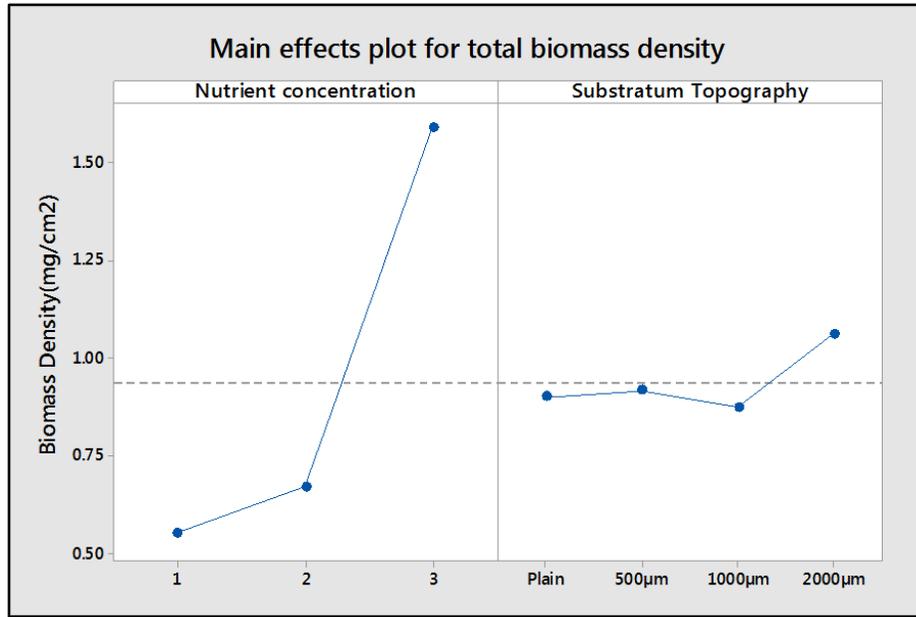
Figure 4.1: Main effects plot of mean biomass density as a function of nutrient concentration and surface topography.

4.2.1.1 Effect of surface topography on algal biomass density in each of the three treatments

Figure 4.2 and Table 4.1 presents the mean biomass density with standard deviation from seven day harvest frequency for all the four substrata in each of the three treatments. It can be observed that nutrient concentration has the primary effect on mean biomass density, and the effect of surface topography is varied under different treatments.

During Treatment 1, the 500 μm surface has highest biomass density (0.61 ± 0.09 mg cm^{-2}) followed by the plain surface (0.59 ± 0.12 mg cm^{-2}), and the surface having the 1000 μm feature size (0.47 ± 0.07 mg cm^{-2}) has lowest biomass density.

In Treatment 2, the surface with 2000 μm radius features had the highest biomass density (0.84±0.22 mg cm^{-2}) followed by the 500 μm surface (0.72 ± 0.19 mg cm^{-2}), and the 1000 μm surface had lowest biomass density (0.49 ± 0.08 mg cm^{-2}).

During Treatment 3, the surface with the 2000 μm radius features had highest biomass density (1.79±0.84 mg cm^{-2}), followed by the 1000 μm surface (1.66 ±0.42 mg cm^{-2}), and the surface with 500 μm radius features had the lowest biomass density (1.42 ±0.50 mg cm^{-2}). A majority of the algal biomass in Treatment 3 was composed of unidentified diatoms and unicellular algal species. Similar results were reported by Bothwell (1985) in which enrichment of phosphorus results in dominance by diatoms. Differences in the biomass density might be caused by species selectivity under different treatments, as each of the selected algal species responded differently to each tested surface topographies.

A nested ANOVA indicated significant differences between treatments (*p* value=0.000) and surface topographies (*p* value= 0.017). A Tukey-Kramer test was used to test the pairwise differences in mean biomass density between different treatments and surface topographies in each of the three treatments. Treatment 3 was significantly greater than both Treatment 1 and Treatment 2. There was no significant difference between Treatment 1 and Treatment 2.

In Treatment 1, one way ANOVA indicated that there was significant differences in biomass density between different surface topographies (*p* value= 0.00). A Tukey Kramer procedure for pairwise comparison indicated that biomass density on plain surface and 500 μm radius feature surface were significantly different than that of 1000 μm radius feature size.

In Treatment 2, one way ANOVA indicated that there was also significant differences in biomass density between different surface topographies (*p* value= 0.00). A Tukey Kramer

procedure for pairwise comparison indicated that total biomass density on plain surface was significantly different than that of surface 2000 μm radius feature size, and surfaces with feature radius of 500 μm and 2000 μm were significantly different from 1000 μm feature radius.

There was no significant difference in biomass density among different surface topographies at Treatment 3. In this Treatment, a diatom community as compared to selected algal species contributed most of the biomass, and there was an abrupt increase in biomass that covered the entire surface area of the incubator reducing the substratum effect.
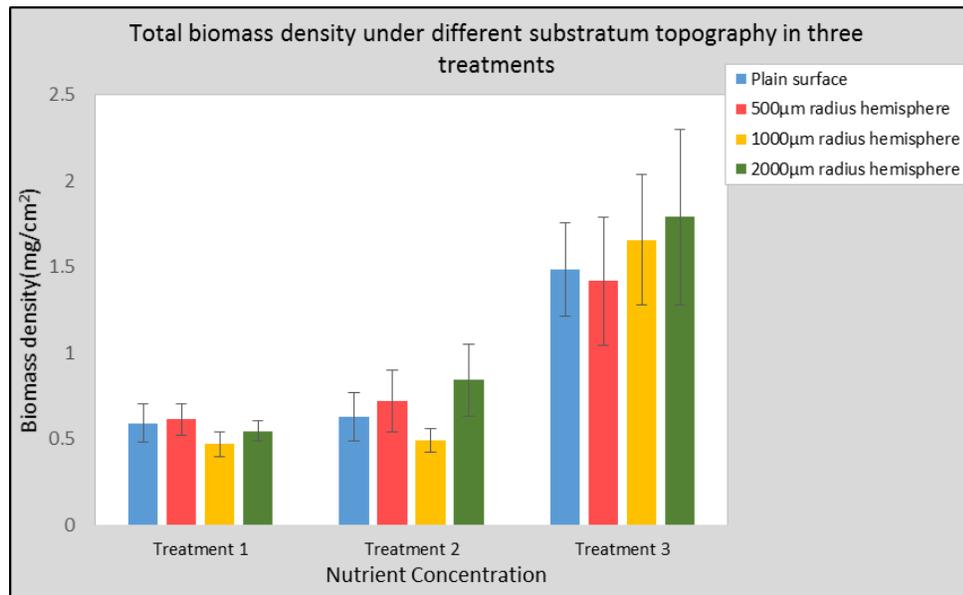
Figure 4.2: Bar plot for total biomass density under different surface topography in all the three treatments

Table 4.1: Biomass density results under different surface topography in all three treatments.

Concentration	Plain surface (mg/cm^2) (Mean±S.D.)	500µm feature radius (mg/cm^2) (Mean±S.D.)	1000µm feature radius (mg/cm^2) (Mean±S.D.)	2000µm feature radius (mg/cm^2) (Mean±S.D.)
1	0.59±0.12[a]	0.61±0.09[b]	0.47±0.07[a c]	0.55±0.06[a d]
2	0.63±0.15[a]	0.72±0.19[a b]	0.49±0.08c [d]	0.84±0.22[a d]
3	1.49±0.39[a]	1.42±0.50[a]	1.66±0.40[a]	1.79±0.84[a]

Note: Treatments with same letters in specific rows are not significantly different at α=0.05

4.2.2 Percent ash content

Figure 4.3 illustrates the main effects of nutrient concentration and surface topography on the percent ash content of algal biomass. It shows that percent ash content changes as a function of nutrient concentration. Treatment 2 had the highest percent ash content (17.16%±0.71%), and Treatment 3 had the lowest percent ash content (14.11%±0.32%) irrespective of surface topography. Algae contain three kinds of organic substances: protein, carbohydrates and natural lipids. Nutrient variations can alter the lipid accumulation and triglyceride content in algal species (Xin et al., 2010; Rodofie et al., 2009) which might be a reason for variation in percent ash content under different treatments. The different nutrient concentration treatments also have different species population abundances, and their chemical composition may alter the ash content at different nutrient concentrations.

As for the effect of surface topography, the plain surface had highest percent ash content, which generally decreases with increasing surface topography feature size. The surface with 1000 µm radius hemispheres had the lowest percent ash content (14.63% ± 1.49%). The

average percent ash content in the algal biomass in all the samples in the experiment was 15.21% ± 0.73%. Surface topography does not significantly affect the percent ash content in the algal biomass. However, change in nutrient concentration alters the species dominance in the algal sample, and at higher concentrations there was shift in species dominance, with an increase in diatoms and other unidentified unicellular algal species in harvested biomass that might alter the percent ash content.
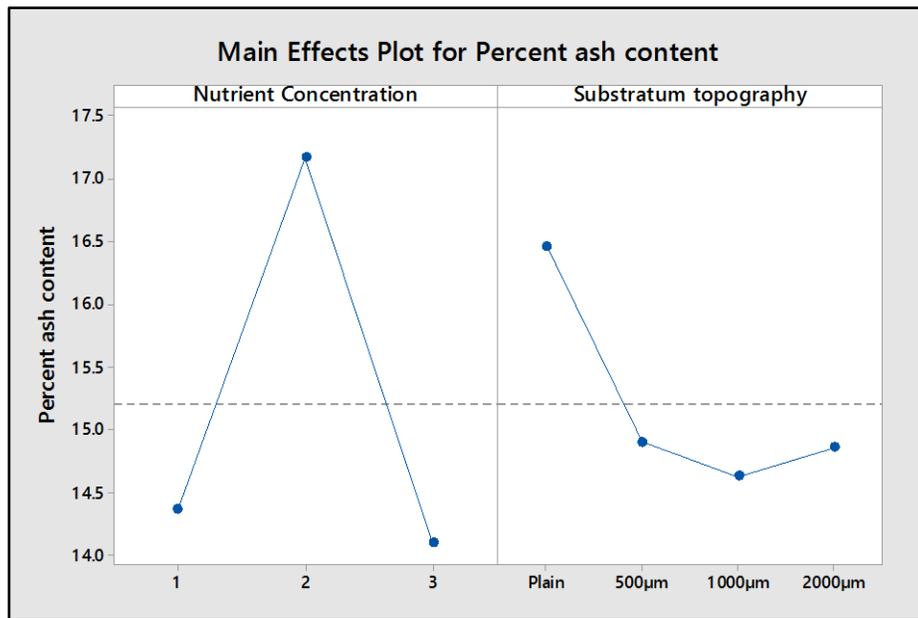
Figure 4.3: Main effects plot of mean percent ash content as a function of concentration and surface topography.

Figure 4.4 shows the mean of percent ash content under different surface topographies in all the three treatments. It can be observed that both nutrient concentration and surface topography had no significant effect on percent ash content.

Across all three treatments, whereas the plain surface had the highest ash content, surfaces with different surface topography did not significantly affect the ash content percent. Each of the topography surfaces with features of radius 500 μm, 1000 μm and 2000 μm had

almost same percent ash content within the treatment, and generally lower as compared to the plain surface.
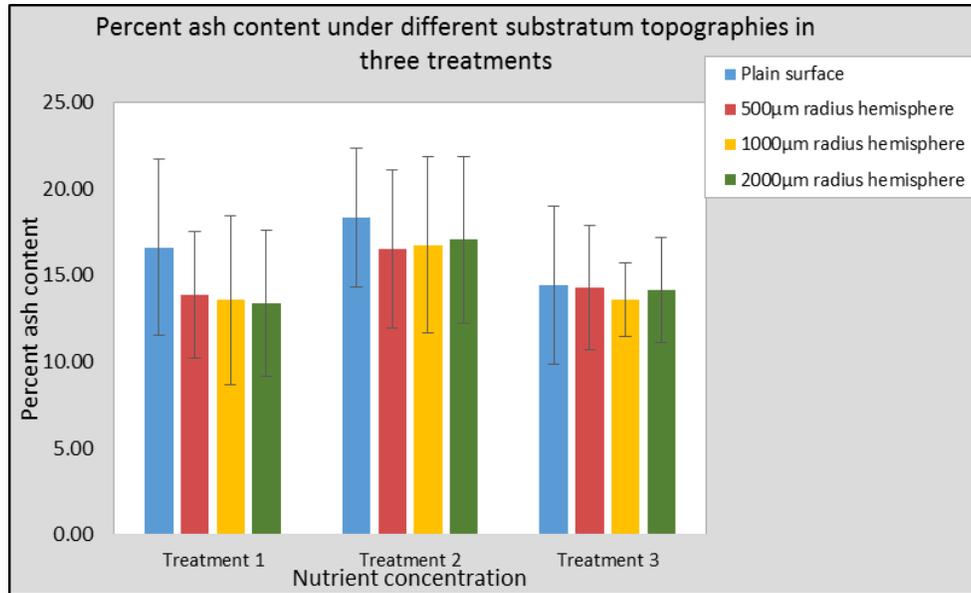
Figure 4.4: Bar plot for percent ash content under different surface topographies in all the three treatments

4.3   Experimental Results: Objective 2

The second objective of this work was related to the species selectivity under different surface topography in each of the three treatments. Each of the tile samples was analyzed using digital microscopy (400X), and each of the 8100 micrographs at that magnification was observed for number of times each of algal species appeared in the micrograph. The species count data for all the four algal species violated the ANOVA assumptions for normality, so non-parametric statistical tests were employed. The Kruskal-Wallis test (α=0.05) was performed on the medians and Mann-Whitney test (α=0.05) was used for pairwise comparison.

4.3.1 Microspora floccose

Figure 4.5 illustrates the mean of species count for *Microspora floccose* on all the selected surface topographies during three treatments. The mean species count increased from Treatment 1 to Treatment 2 and then decreased from Treatment 2 to Treatment 3. It was observed that the occurrence of *Microspore floccose* in Treatment 3, i.e., at higher nutrient concentrations, was very low as compared to the other two treatments. A Kruskal-Wallis test suggests that nutrient concentration had significant effect on the abundance of *Microspora floccose* species (p value = 0.00). The species count in Treatment 2 was significantly greater than in Treatment 3 (p value = 0.00).

Surface topography also had a significant effect on the abundance of *Microspora floccose* species (p value = 0.049). During Treatment 1, the occurrence of *Microspora* was significantly greater on the 500 µm radius feature surface compared to the plain surface and 1000 µm surface (p value = 0.00 for both). Also, the occurrence of *Microspora floccose* on the 2000 µm surface was significantly greater than on the 1000 µm radius feature surface and plain surface (p value = 0.00 and 0.003, respectively). At Treatment 1, the surface with 2000 µm radius features had the highest species count for *Microspora floccose,* followed by the 500 µm surface.

During Treatment 2, the occurrence of *Microspora floccose* was significantly greater on the surface with 500 µm radius features compared to the 1000 µm and 2000 µm surfaces (p value = 0.01 and 0.02, respectively). At Treatment 2, the surface with 500 µm radius features size had the highest species count for *Microspora floccose,* and the 2000 µm surface has the lowest species count, in contrast Treatment 1.

During Treatment 3, the occurrence of *Microspora floccose* was significantly greater on the surface with 500 µm radius features compared to the plain surface and the surface with 2000 µm radius feature (*p* value = 0.007 and 0.034, respectively).
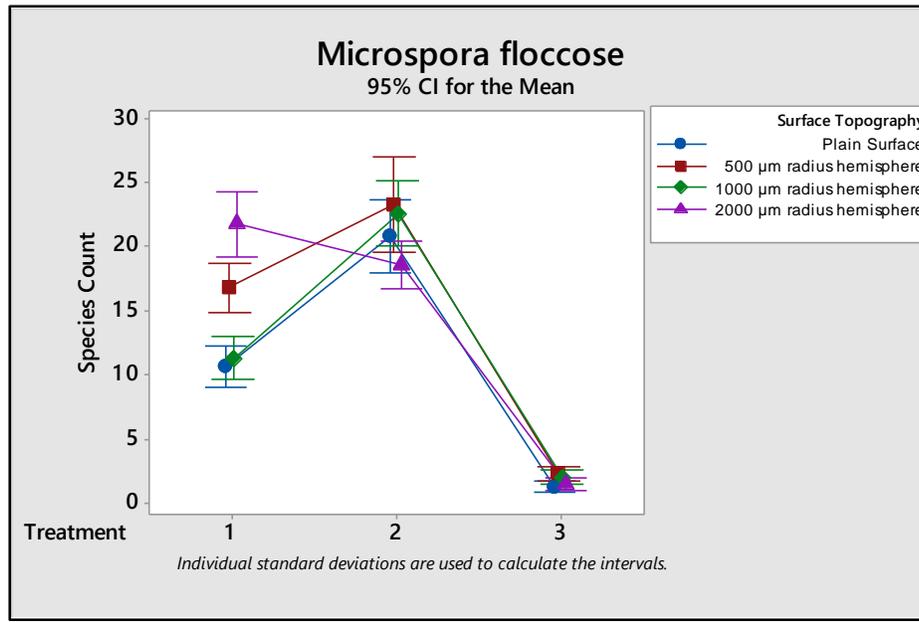
Figure 4.5: Species count versus treatment of *Microspora floccose* on different surface topographies.

4.3.2   Mougeotia scalaris

Figure 4.6 illustrates the mean of species count for *Mougeotia scalaris* on selected surface topographies during three treatments. The mean species count for *Mougeotia scalaris* increased from Treatment 1 to Treatment 2 and then decreased from Treatment 2 to Treatment 3. It was observed that the occurrence of *Mougeotia scalaris* in Treatment 3, i.e., at higher nutrient concentrations, was significantly lower compared to the other two treatments. The highest population for *Mougeotia scalaris* was observed at Treatment 2 (medium nutrient concentrations).

A Kruskal Wallis test suggests that nutrient concentration has a significant effect on the abundance of *Microspora floccose* species (*p* value = 0.00). The species count in Treatment 2 was significantly greater than in Treatment 1 and Treatment 3 (*p* value = 0.00).

The surface topography had no significant effect on the abundance of *Mougeotia scalaris* species (*p* value = 0.83). A Mann-Whitney test was used for pairwise comparison between different surface topographies in each of the three treatments. During Treatment 1, the species count on the surface with 500 µm radius features was significantly less than the plain surface (*p* value = 0.012) and 1000 µm surface (*p* value = 0.018). The plain surface had the highest species count for *Mougeotia scalaris,* and the 500 µm surface had lowest species count

During Treatment 2, the surface topography had no significant effect on the abundance of *Mougeotia scalaris* species. The surface with 500 µm radius features had the highest species count, and the surface with 1000 µm radius features had the lowest species count, which was in contrast to Treatment 1.

During Treatment 3, the species count on the 500 µm surface was significantly greater than that of the 2000 µm surface (*p* value = 0.006). The surface with 1000 µm feature size had a species count significantly greater than the 2000 µm surface (*p* value = 0.000). The plain surface had a species count significantly greater than the surface with 2000 µm radius features (*p* value = 0.000). The plain surface had the highest species count for *Mougeotia scalaris,* and the surface with 500 µm features had the lowest species count, which is similar to Treatment 1 and opposite to Treatment 2.
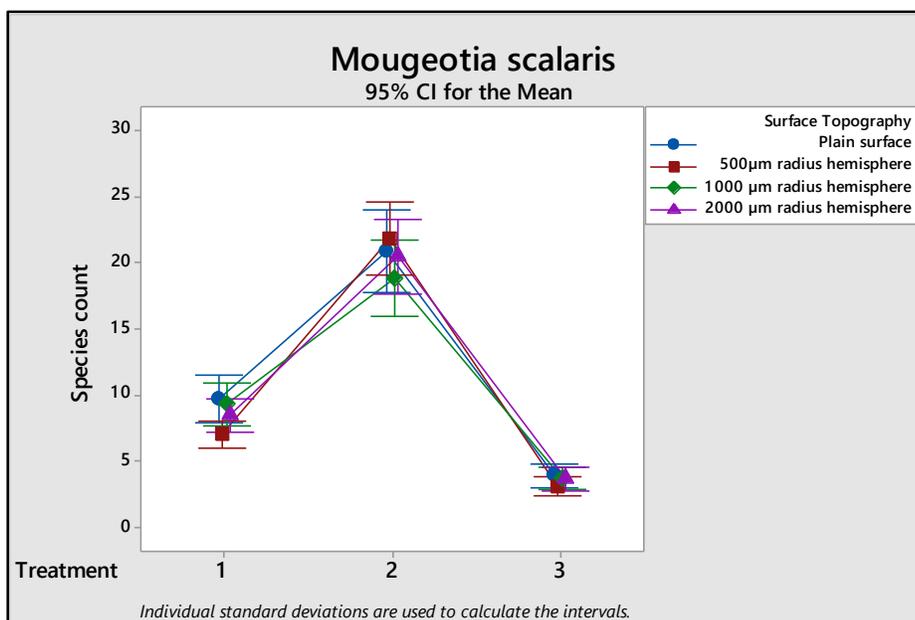
Figure 4.6: Species count versus treatment of *Mougeotia scalaris* on different surface topographies

4.3.3 Oedogonium crassum

Figure 4.7 illustrates the mean of species count for *Oedogonium crassum* on all the surface topography conditions during three treatments. The mean species count for *Oedogonium crassum* decreased from Treatment 1 to Treatment 2 and then increased from Treatment 2 to Treatment 3. It was observed that the occurrence of *Oedogonium crassum* in Treatment 3, i.e., at higher nutrient concentrations, was highest as compared to the other two treatments. However, the population of *Oedogonium crassum* in the experiment was lowest as compared to other competitive species, i.e., *Microspora floccose*, *Mougeotia scalaris* and *Sirogonium sticticum,* in the selected environmental conditions.

A Kruskal Wallis test suggests that nutrient concentration has significant effect on the abundance of *Oedogonium crassum* species (p value = 0.00). The species count in Treatment 3 was significantly greater than in Treatment 1 and Treatment 2 (p value = 0.000).

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Surface topography had no significant effect on the abundance of *Oedogonium crassum* species (*p* value = 0.38). Also, with pairwise comparison by a Mann-Whitney test, there was no significant effect ($\alpha=0.05$) of surface topography on the abundance of this species during all the three treatments.
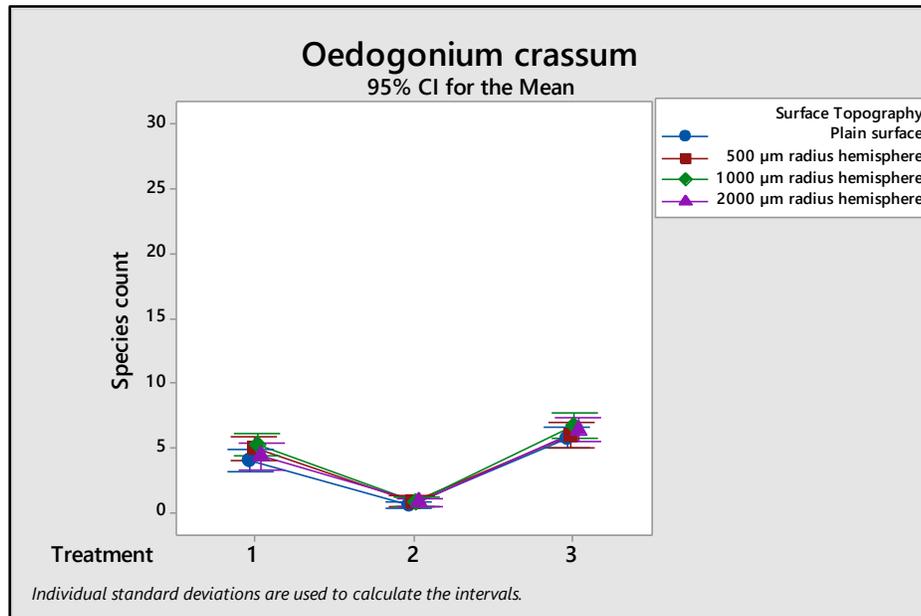
Figure 4.7: Species count versus treatment of *Oedogonium crassum* on different surface topographies.

4.3.4 Sirogonium sticticum

Figure 4.8 illustrates the mean of species count for *Sirogonium sticticum* on all the surface topographies in each of the three treatments. The mean species count for *Sirogonium* decreased from Treatment 1 to Treatment 2 and then increased from Treatment 2 to Treatment 3. It is observed that the occurrence of *Sirogonium sticticum* in Treatment 1, i.e., at lower nutrient concentrations, was highest as compared to the other two treatments. The population of *Sirogonium sticticum* in the experiment was also low, however, as compared to other competitive algal species in the experiment, e.g., *Microspora floccose* and *Mougeotia scalaris*.

A Kruskal Wallis test was used to test the significance of the effect of nutrient concentration on the abundance of *Sirogonium sticticum* species count (*p* value = 0.00). The species count in Treatment 1 was significantly greater than in Treatment 2 and Treatment 3 at α=0.05 *(p* value = 0.0022 and 0.0000 respectively).

Surface topography had no significant effect on the abundance of *Sirogonium sticticum* species in any of the three treatments *(p* value = 0.93). A Mann Whitney test for pairwise comparison showed that surface topography had no significant effect (α = 0.05) on the abundance of this species during Treatment 1 and Treatment 3. During Treatment 2, however, the surface with 500 µm radius features showed significantly greater abundance of *Sirogonium* than 1000 µm surface *(p* value = 0.049).
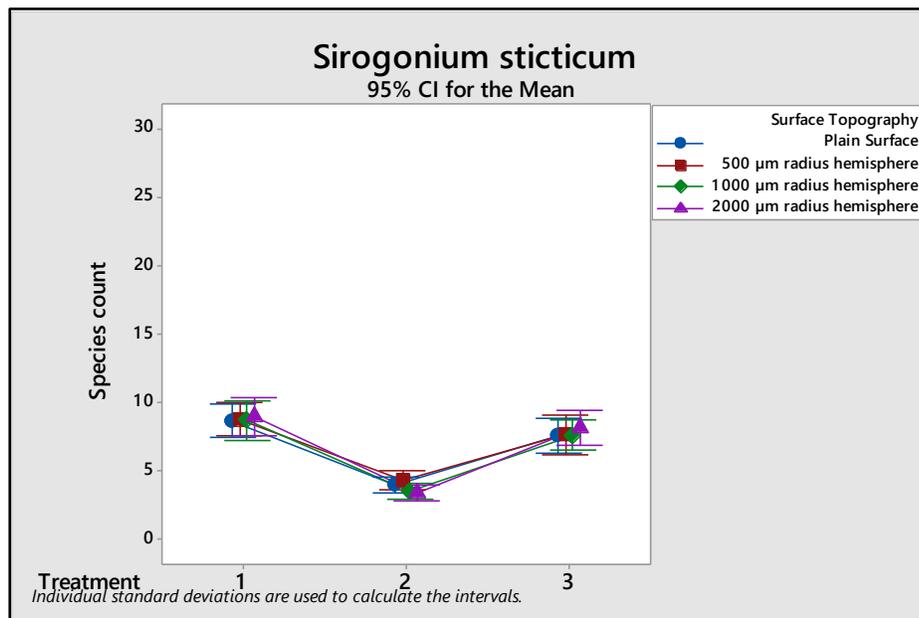
Figure 4.8: Species count versus treatment of *Sirogonium sticticum* on different surface topographies.

4.4 Relative abundance of algal species at different surface topography in all three treatments

Relative abundance was calculated to see how the different species compete with each other for existence on different surface topographies in each of the three treatments. The relative percent occurrence of each algal species on each surface topography was calculated.

4.4.1 Treatment 1

Figure 4.9 illustrates the relative abundance of four selected algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) at lower nutrient concentration under controlled environmental conditions. It can be observed that for all four species percent occurrence varied in algal biomass despite the same initial seeding. Most of the biomass at Treatment 1 was composed of *Microspora floccose* (40.00% ± 1.00%) followed by *Sirogonium sticticum* (24.16% ± 0.78%) and *Mougeotia scalaris* (23.28% ± 0.82%). *Oedogonium crassum* (12.30% ± 0.49%) was the least abundant species observed at lower nutrient concentration. The nutrient requirements for different algal species may be different, and it will affect the biomass production differently (Munn et al., 2010). Based on this interpretation*, Microspora floccose* is more competitive in freshwaters with lower nutrient concentrations compared to other component species in the community. It can be observed that surface topography (*p* value= 0.004) affected the recruitment of algal species at Treatment 1. Most of the population of *Microspora floccose* species occurred at 500 μm and 2000 μm substratum topography, possibly because of their smaller cell diameters (14-18 um) and unbranched cylindrical filament shape (Fritsch, 1935).

When tested using Kruskal Wallis test at significance level of 5%, it was observed that different surfaces topographies recruit different populations of *Microspora floccose (p* value =

0.000) species, *Oedogonium crassum (p* value = 0.005), and *Mougeotia scalaris* species *(p* value 0.004). Algal populations were not significantly different for only *Sirogonium sticticum* on different surfaces topographies *(p* value= 0.312).
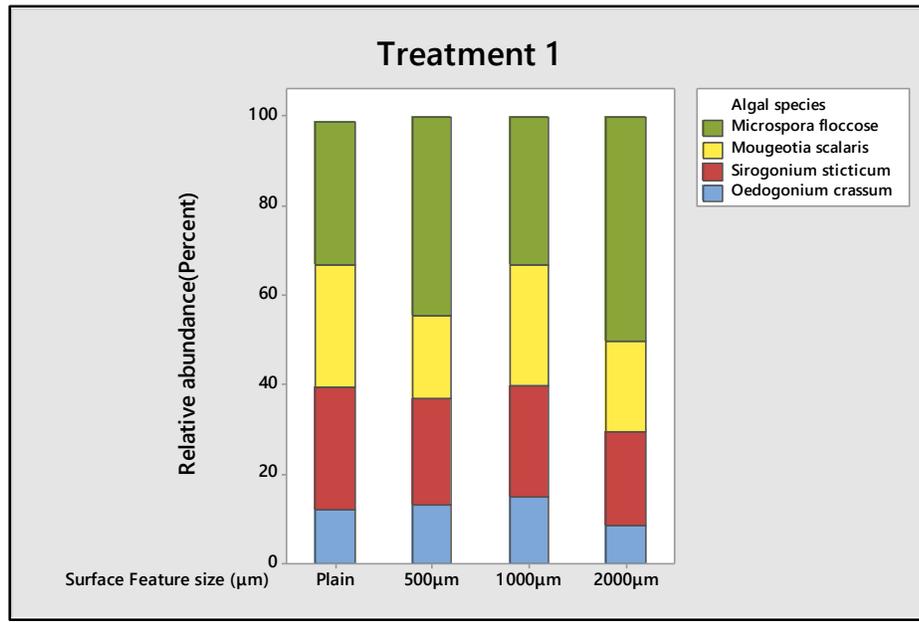
Figure 4.9: Relative abundance of four selected algal species at different surface topographies during Treatment 1.

4.4.2 Treatment 2

Figure 4.10 illustrates the relative abundance of four selected algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris)* at medium nutrient concentration under controlled environmental conditions. It can be observed that for all four species relative abundance varied in algal biomass at Treatment 2 as compared to Treatment 1. Most of the biomass was composed of *Microspora floccose* (45.52%± 0.76%) *and Mougeotia scalaris* (42.06%±0.84%) followed by *Sirogonium sticticum* (8.84%± 0.35%). The percent occurrence of *Oedogonium crassum* (2.16%± 0.24%) was the lowest at medium nutrient concentration. The rate of nutrients loaded to a system can exert a strong influence on

the algal species that will thrive in the system (Kilham and Hecky, 1988). Abundance of *Microspora floccose and Mougeotia scalaris* at medium nutrient concentrations may be a result interspecific competition between existing species for resources (Tillman, 1977). *Mougeotia scalaris* was also found in abundance at medium nutrient concentrations (Deyab and El-Katony, 2015).

Surface topography did not significantly affect the recruitment of any of the selected algal species. The relative abundance was not significantly different for different surface topography for the selected species in the experiment, i.e., *Microspora floccose* species *(p* value = 0.265), *Oedogonium crissum (p* value = 0.93), *Sirogonium sticticum (p* value = 0.673) and *Mougeotia scalaris (p* value = 0.581) when tested using the Kruskal Wallis test at α=0.05.
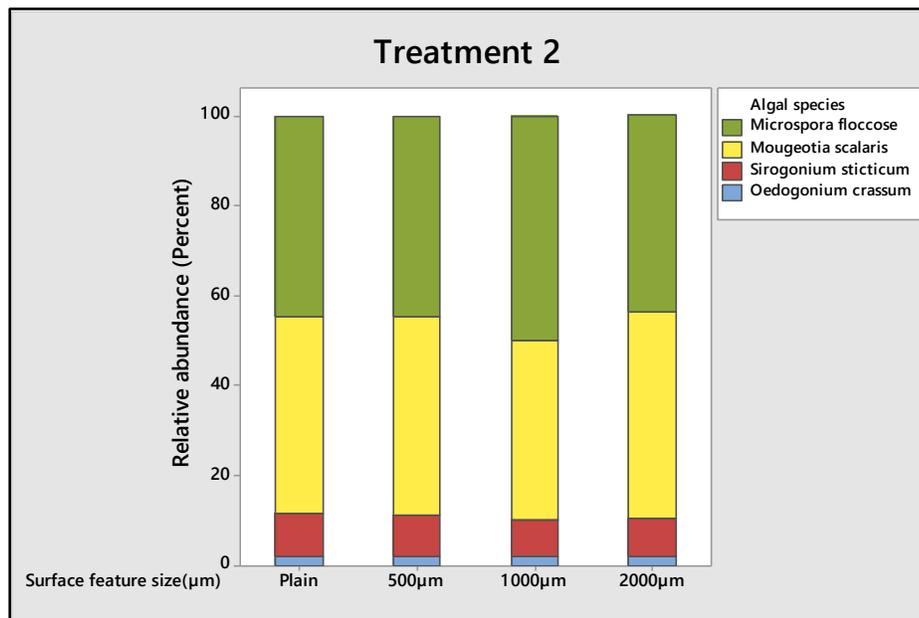
Figure 4.10: Relative abundance of four selected algal species at different surface topographies during Treatment 2.

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4.4.3 Treatment 3

Figure 4.11 illustrates the relative abundance of four selected algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) at high nutrient concentration under controlled environmental conditions. It can be observed that for all four species percent occurrence in overall algal biomass varies in Treatment 3 compared to Treatment 2. Most of the biomass was composed of *Oedogonium crassum* (34.67% ± 1.25%) *and Sirogonium sticticum* (38.92%±1.19%) followed by *Mougeotia scalaris* (18.82% ± 0.86%). *Microspora floccose* (9.38% ± 0.76%) was the least abundant species observed at higher nutrient concentration, which was totally opposite to what was observed in the low and medium nutrient concentrations. Survival of *Oedogonium crassum and Sirogonium sticticum* at high nutrient ratios might be due to their high nutrient uptake rates as compared to other competitive species. At higher nutrient concentrations, it was clear from the micrographs that unidentified diatom communities were abundant. More salt build up in the higher concentrations (especially Si) might be a reason for the diatom community abundance over algal species.

Surface topography did not significantly affect the recruitment of any of the tested algal species. The relative abundance is not significantly different for different surface topography for the selected species in the experiment, i.e., *Microspora floccose (p* value = 0.082) species, *Oedogonium crassum (p* value = 0.995), *Sirogonium sticticum (p* value = 0.920), and *Mougeotia scalaris* species *(p* value = 0.763) when tested using Kruskal Wallis test.
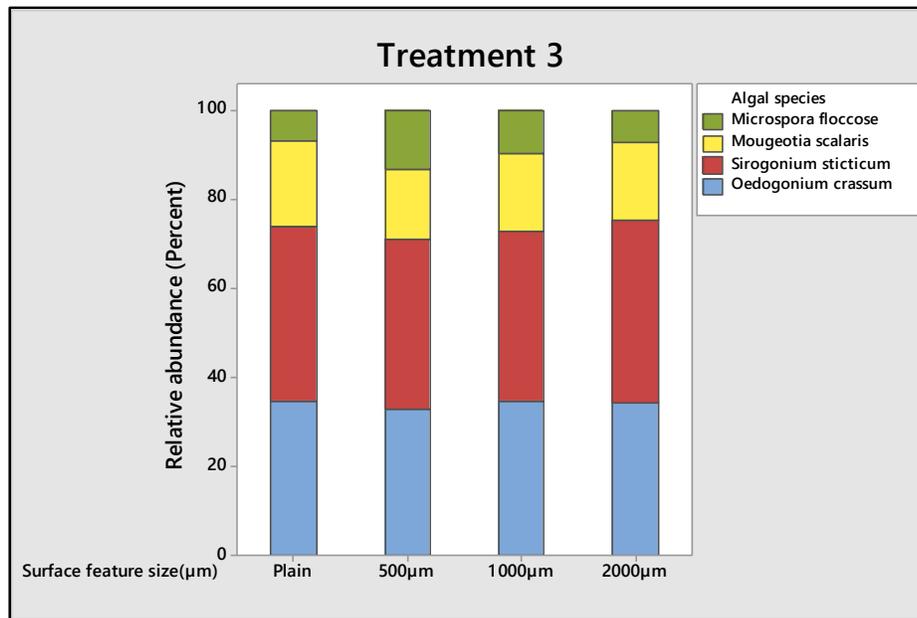
Figure 4.11: Relative abundance of four selected algal species at different surface topographies during Treatment 3.

4.5 Relative abundance of all four algal species at each surface topography in different nutrient treatments

4.5.1 Plain surface

Figure 4.12 illustrates the relative abundance of four selected algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) on plain surface under controlled environmental conditions. It can be observed that for all four species percent occurrence in overall algal biomass varies in each of the three treatments. At Treatment 1, the relative percent occurrence of three algal species, i.e., *Sirogonium sticticum*, *Mougeotia scalaris and Microspora floccose,* are competitive, but *Oedogonium crissum* was found to be least abundant. As the nutrient concentration increases, *Mougeotia scalaris and Microspora floccose* abundance increased in the system and the population of *Mougeotia scalaris* was reduced. Also, *Oedogonium crassum* was rare in the system (Table 4.2). But at higher nutrient

concentrations, the trend was totally opposite, as *Sirogonium sticticum and Oedogonium crassum* growth rates were increased and *Mougeotia scalaris* and *Microspora floccose* was less observed. This clearly indicates that growth rate of species was driven primarily by resource availability (nutrients) and there is competition between existing species at lower resource availability (Tillman, 1977; Fong et al., 1993). Also, at higher nutrient levels, species which are tolerable to high nutrient rates existed in abundance. Tilman (1977, 1985) first developed resource-ratio theory for phytoplankton, stating that changes in the environmental ratio of essential nutrients causes the change in existing community structure due to competition between taxa having different optimal nutrient ratios. Two species with different optimal nutrient ratios are able to coexist only if each species is a better competitor for the nutrient most limiting to the other species.
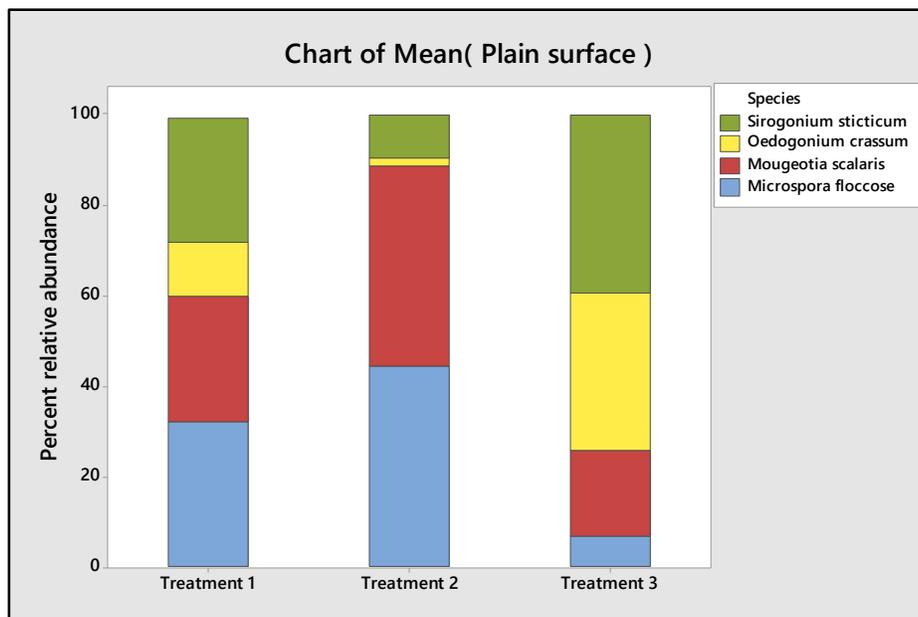
Figure 4.12: Relative abundance of four selected algal species on plain surface in all three treatments.

Table 4.2: Relative abundance of four algal species on plain surface in all three treatments

Treatment	*Sirogonium sticticum* (Mean ± S.E.)	*Oedogonium crassum* (Mean ± S.E.)	*Microspora floccose* (Mean ± S.E.)	*Mougeotia scalaris* (Mean ± S.E.)
1	(27.33%±1.80%)	(12.03%±1.02)	(32.18%±1.62%)	(27.51%±1.71%)
2	(9.56%±0.80%)	(1.99%±0.46%)	(40.84%±1.47%)	(43.61%±1.36%)
3	(39.28%±2.31%)	(34.75%±2.46%)	(8.86%±1.32%)	(19.13%±1.74%)

4.5.2   Substratum with 500μm radius hemispheres

Figure 4.13 and Table 4.3 illustrate the relative abundance of four selected algal species on the 500 µm substratum topography in all the three treatments under controlled environmental conditions. It was observed that occurrence of most of species was similar to the plain surface instead of *Microspore floccose*. At Treatment 1 and 2, relative percent occurrence of *Microspora floccose* was highest. At treatment 3, *Sirogonium sicticum* was the most abundant species (Table 4.3). From results it can be clearly observed that the substratum topography of 500 µm is best surface for *Microspora floccose* colonization at limiting nutrient concentrations, suggesting that this species is not competitive to nutrient enrichment.
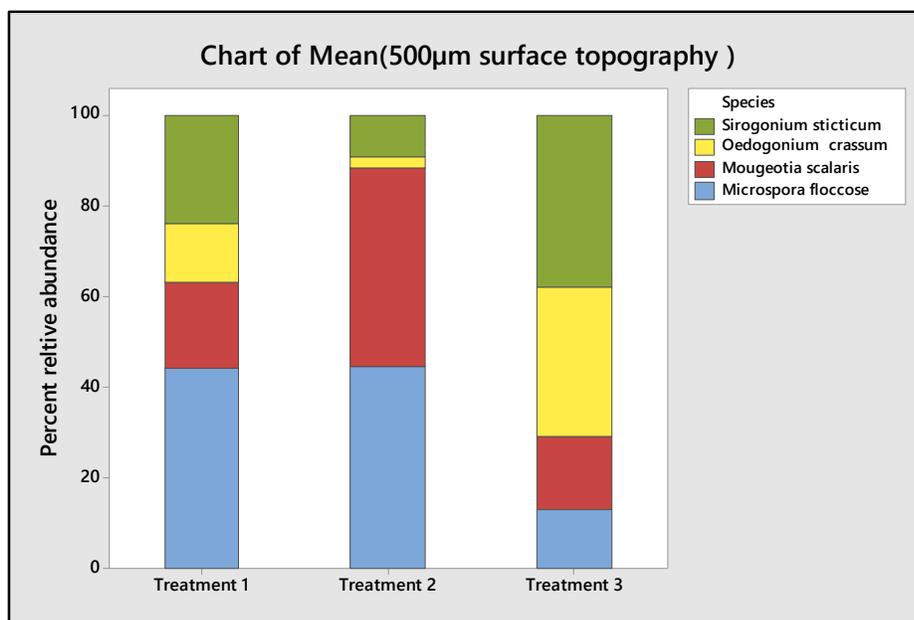
Figure 4.13: Relative abundance of four selected algal species at 500 in μm radius hemispheres surface in all three treatments.

Table 4.3: Percent relative abundance of four algal species on 500 μm surface topography in all three treatments.

Treatment	*Sirogonium sticticum* (Mean ± S.E.)	*Oedogonium crassum* (Mean ± S.E.)	*Microspora floccose* (Mean ± S.E.)	*Mougeotia scalaris* (Mean ± S.E.)
1	(23.81%±1.43%)	(13.18%±1.00%)	(44.34%±1.90%)	(18.67%±1.14%)
2	(9.23%±0.75%)	(2.26%±0.49%)	(44.49%±1.65%)	(44.02%±1.65%)
3	(37.97%±2.421%)	(32.98%±2.74%)	(13.21%±1.87%)	(15.84%±1.77%)

4.5.3 Substratum with 1000 μm radius hemispheres

Figure 4.14 illustrates the relative abundance of four selected algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) on the surface with 1000 μm substratum topography at different nutrient concentrations. It was observed that

the occurrence of most of species was similar as the plain surface and 500 μm substratum topography at lower nutrient concentrations, but at higher nutrient concentrations, algal species having high optimum nutrient ratios was abundant regardless of surface topography (Table 4.4).
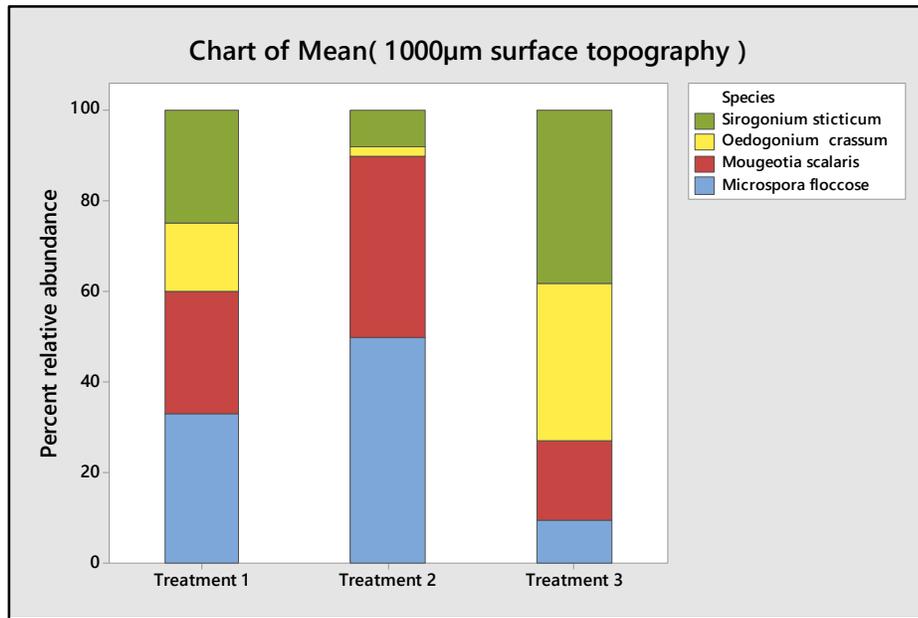
Figure 4.14: Relative abundance of four selected algal species at 1000 μm radius hemispheres surface in all three treatments.

Table 4.4: Percent relative abundance of four algal species on 1000 μm surface topography in all three treatments.

Treatment	*Sirogonium sticticum* (Mean ± S.E.)	*Oedogonium crassum* (Mean ± S.E.)	*Microspora floccose* (Mean ± S.E.)	*Mougeotia scalaris* (Mean ± S.E.)
1	(27.87%±1.55%)	(15.00%±0.97%)	(33.03%±1.51%)	(27.10%±1.91%)
2	(8.06%±0.62%)	(2.09%±0.47%)	(49.80%±1.58%)	(39.98%±1.93%)
3	(38.23%±2.20%)	(34.51%±2.26%)	(9.69%±1.44%)	(17.57%±1.56%)

4.5.4 Substratum with 2000 μm radius hemispheres

Figure 4.15 illustrates the relative abundance of four selected algal on the surface with 2000 μm substratum topography in all the three treatments under controlled environmental conditions. It can be observed that *Microspora floccose* was observed to be the most abundant species at lower and medium nutrient concentrations on 2000 μm surface. But similar to other surface topographies, at higher concentrations *Sirogonium sticticum* and *Oedogonium crassum* were the most abundant spices regardless of the surface topography and *Mougeotia scalaris* was the least abundant.
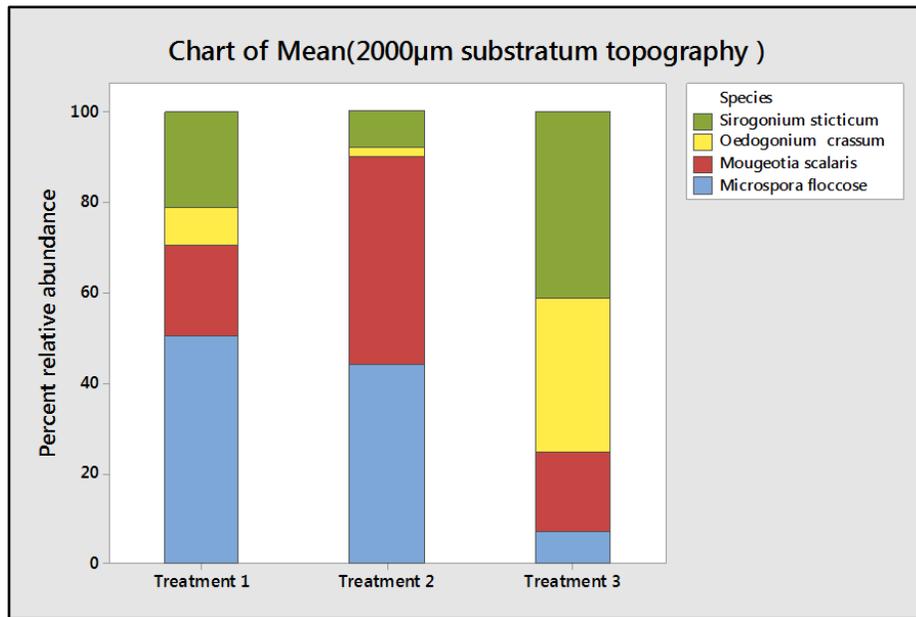
Figure 4.15: Relative abundance of four selected algal species at 2000 μm radius hemispheres surface in all three treatments.

Table 4.5: Percent relative abundance of four algal species on 2000 µm surface topography in all three treatments.

Treatment	*Sirogonium sticticum* (Mean ± S.E.)	*Oedogonium crassum* (Mean ± S.E.)	*Microspora floccose* (Mean ± S.E.)	*Mougeotia scalaris* (Mean ± S.E.)
1	(21.01%±1.28%)	(8.67%±0.59%)	(50.34%±1.44%)	(20.01%±7.95%)
2	(8.22%±0.63%)	(2.22%±0.525%)	(43.91%±1.12%)	(46.06%±1.57%)
3	(41.06%±2.47%)	(44.34%±2.47%)	(7.03%±1.17%)	(17.57%±1.83%)

4.6 Simpson's Diversity index (SI)

Simpson's diversity index (SI) was calculated to measure the diversity across different surface topographical features at different nutrient concentrations.

Table 4.6 and Figure 4.16 represent the Simpsons Diversity index values and plot for each surface topography under all three treatments. Species diversity as measured by Simpson's diversity index (SI) varied with surface topography during low nutrient conditions, but was similar across all surface feature sections during medium and high nutrient conditions.

During Treatment 1, the highest diversity was observed in the 1000 µm radius features surface (SI = 0.734), whereas the lowest diversity was observed in the 2000 µm surface (SI = 0.660). Simpson's diversity index decreased from the plain surface to the 500 µm radius surface, then increased from 500 µm surface to the 1000 µm surface, and decreased again for the 2000 µm surface. More diversity was observed at plain surface and 1000 µm radius surface as species are more evenly distributed and all the species were in abundance. However, on the surfaces with 500 µm and 2000 µm radius surface, *Microspora floccose* was the major component of the biomass as compared to other competitive species which resulted in lesser

diversity. Simpson's diversity index was most variable at Treatment 1 because at lower nutrient concentration there is more interspecies competition for existence as the nutrient sources were limited (Tillman, 1977; 1982)

During Treatment 2, the diversity did not vary as a function of surface topography. Simpson's diversity index during Treatment 2 was the lowest overall and varied in the range of 0.582 to 0.587. The cause of this is likely due to resource competition: the availability of more nutrients can reduce the apparent competition between algal species, as compared to lower nutrient concentrations, such as in Treatment 1.

Diversity during Treatment 3 is greater as compared to Treatment 2, and Simpson's diversity index varied in the range of 0.683 to 0.700. Highest diversity was observed in 1000 μm radius hemisphere surface, and lowest diversity was observed in 2000 μm surface at high nutrient concentration. This was overall similar to Treatment 1, i.e., the lowest nutrient concentration.

Table 4.6: Simpson's diversity index for each surface feature section.

Surface Feature Section	Treatment 1	Treatment 2	Treatment 3
Plain	0.727	0.587	0.687
500 μm	0.693	0.585	0.699
1000 μm	0.734	0.582	0.700
2000 μm	0.660	0.585	0.683
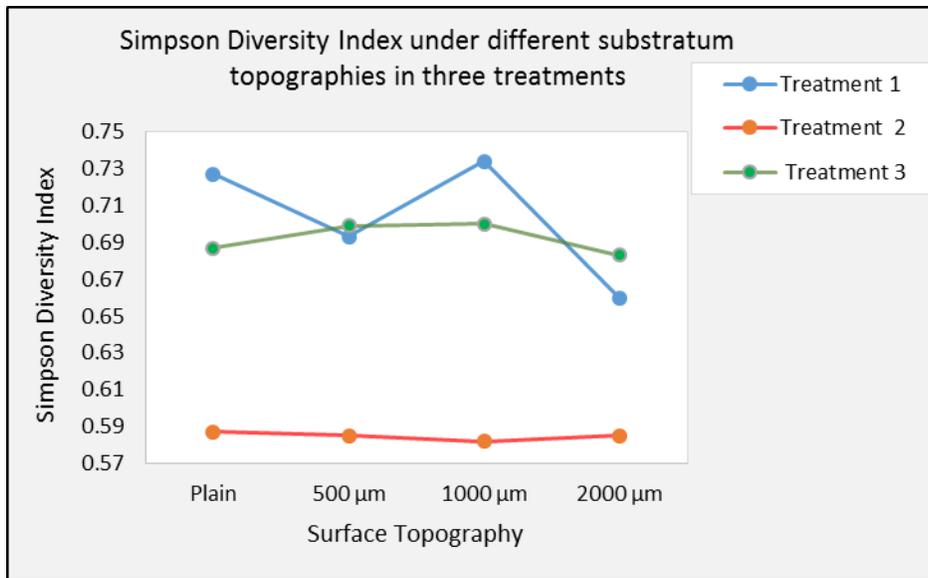
Figure 4.16: Simpson's diversity index versus surface feature section for each of the three treatments.

Chapter 5: Summary and Future Recommendations

5.1 Summary and discussion

The main objective of this study was to test the effect of surface topography on the biomass density and species selectivity under varying nutrient concentration. The entire experiment was divided into 3 treatments according to nutrient concentrations. Surface topography was designed using hemispheres of varying radius as surface features, keeping the plain feature as control. Experiments were conducted in a flow lane photo incubator, and 3 D printed tiles were replicated using clay. Required nutrients were provided using Proline F/2 algae food (Pentaire Aquatic Ecosystems). Three nutrient concentrations (low, medium and high) were selected. Environmental conditions (light intensity, temperature, and flow rate and flow velocity) were kept in control. Four common filamentous algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*) found in streams of Alabama were selected to be used in the experiment. A set of 20 tiles (5 replicates of each surface topography) was placed in pseudorandom pattern in the flow lane incubator during each treatment. The first harvest was done after 15 days, and after that three weekly harvests were done and used for analysis of biomass density, ash content and species abundance on different surface topographies in all the three nutrient concentrations.

The first objective of the study was to compare biomass density and percentage ash content under different surface topography in three nutrient concentrations. In this study it was

observed that nutrient concentration plays an important role in determining the growth dynamics of algae, and the effect of surface topography varied according to nutrient availability. Increase in nutrient concentration increases the algal biomass density abruptly, but the composition of algal biomass was dominated by diatoms as compared to the intended algal species at higher concentrations. It was observed that Treatment 3 has the highest biomass density, with a 186% increase as compared to Treatment 1 and a 136% increase as compared to Treatment 2 regardless of the surface topography. Biomass density on the surface having 2000 μm radius hemispheres was the greatest (15% more as compared to plain surface) in all the three nutrient concentrations. Results were similar to Hsieh and Wu, (2009) where, according to them, cultivation modes and nutritional management affect the growth rate and biochemical composition of algae. Substratum suitability is largely determined by environmental parameters such as flow regime and nutrient availability (Strathmann et al. 1981, Qian et al. 2000, Qian et al. 2003).

Biomass in the experiment was mainly composed of the four selected species. From Treatment 1 to Treatment 2, there was a species shift, and the populations of both *Microspora floccose* and *Mougeotia scalaris* increased abruptly. In the highest concentration (Treatment 3), however, *Oedogonium crassum* and *Sirogonium sticticum* were abundant when compared to other selected species. *Oedogonium crassum* species increased abruptly with increasing nutrient concentration, from Treatment 2 (2.16%) to Treatment 3 (34.67%). Most of the biomass at Treatment 3 was composed of diatoms, which may be more tolerant to higher nutrient concentrations, and salt build up also increases Si concentration in the water, a limiting factor for diatoms. The total number of selected algal species observed in Treatment 3 was less as compared to Treatment 1 and Treatment 2. The unidentified diatoms and unicellular algae

was the major source of biomass causing the abrupt rise in biomass density in Treatment 3. Nutrients have been considered as one of the major factors controlling the composition and abundance of phytoplankton community, and this community was dominated by diatoms due to the high nutrient levels (Wang et al., 2006).

Percent ash content was calculated to determine the inorganic content in the harvested algal biomass. High ash content leads to the inferior characteristic of algal biomass for many downstream utilization scenarios. It was observed that the biomass from medium nutrient concentration (Treatment 2) had the highest ash content (17.16%±0.71%), whereas the high nutrient concentration (Treatment 3) has lowest percent ash content (14.11%±0.32%) regardless of the surface topography. When looking at the effect of substratum topography, percent ash content was highest (16.46%±1.60%) on the plain surface, and the lowest (14.63% ±1.49%)) on the surface with a topography of 1000 μm radius hemispheres. Nutrient concentration and surface topography did not have a significant effect on percent ash content, however. The dominance of various species in the algal biomass may determine the ash content of biomass at different nutrient conditions, because nutritional management is known to affect biochemical composition of algal species (Hsieh and Wu, 2009). Nutrient variations can alter the lipid accumulation and triglyceride content in algal species (Xin et al., 2010; Rodofie et al., 2009) which might be a reason for variation in percent ash content under different treatments. The different nutrient concentration treatments also had different species population abundances, and their chemical composition may alter the ash content at different nutrient concentrations.

As for the effect of surface topography, the plain surface had highest percent ash content, and it generally decreased with increasing surface topography feature size. The surface with

1000 µm radius hemispheres has the lowest percent ash content (14.63%±1.49%). The average percent ash content in the algal biomass in all the samples in the experiment was 15.21% ± 0.73%. Surface topography did not significantly affect the percent ash content in the algal biomass. however change in nutrient concentration alters the species dominance in the algal sample, and at higher concentrations there was shift in species dominance, with an increase in diatoms and other unidentified unicellular algal species in harvested biomass that might alter the percent ash content.

The second objective of this study was to investigate the species selectivity under different surface topography over a range of nutrient conditions for the selected four filamentous algal species (*Oedogonium crassum, Sirogonium sticticum, Microspora floccose* and *Mougeotia scalaris*). Nutrient concentration had a major significant effect on the species selectivity, and the surface topography had a noticeable effect on species selectivity, although less so as compared to nutrient concentration. Similar results were observed by Strathmann et al. (1981) and Qian et al. (2000). According to those studies, the suitability of substratum for biofilm to colonize is largely determined by environmental parameters such as flow regime and nutrient availability. At Treatment 1 *Microspora floccose* is abundant (39.37 %). At Treatment 2 most of the biomass is composed of *Microspora floccose* (45.68%) and *Mougeotia scalaris* (43.50%). At Treatment 3 *Sirogonium sticticum* (39.14%) and *Oedogonium crassum* (34.14%) were most abundant. It is clear that the species populations in the experiment were driven by nutrient concentration to a large extent. Species dominance varies according to the nitrate and phosphate levels in the incubator water. It is also observed that at higher nutrient concentrations (Treatment 3), the overall species count for the selected algal species decreased, and unidentified unicellular algae including diatoms increased in abundance, leading to a

significant increase in biomass density. Munn et al. (2010) observed similar results, where the nutrient need for different algal species may affect the biomass production.

The variation in abundance of the selected algal species were more noticeable and significant at lower nutrient concentrations (Treatment 1) across different topographies than at higher concentrations (Treatments 2 and 3). At Treatment 3, the significance of surface topography was observed to be the least. This conforms to limiting factor theory that there is a competition between species for resources in nutrient limited conditions (Tillman, 1987). At medium and high nutrient concentrations, the greater nutrient availability reduced inter-species competition for nutrient resources. Thus, species that can tolerate high nutrient conditions would survive in the system. Overall, the selectivity for surface topography for the various species might be observed from effects at Treatment 1 (Table 5.1).

Table 5.1: Species preference over different surface topography at low nutrient concentration.

Species	Preferred substratum topography
Mougeoia scalaris	Plain surface
Oedogonium crassum	1000 μm radius hemispheres
Microspora floccose	500 μm and 2000 μm radius hemispheres
Sirogonium sticticum	500 μm and 2000 μm radius hemispheres

There is a complex relationship between the colonized surface and the colonizing algal species that includes various factors. For example, *Mougeotia scalaris* may be more abundant on plain surfaces because of smaller cell diameters (20-34 μm wide) and relatively thin walls (Akiyama et al., 1977), so that they cannot settle down between large surface topographic features. *Sirogonium sticticum* species have unbranched uniseriate filaments intertwined to

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form extensive skeins and have cylindrical cells (32-115 µm diameter), up to several times as long (Guiry, 2015). The diameter range of the cell matches with the area of interstitial spacing of 500 µm radius surface, which helps in a greater settlement of cells between two adjacent hemispheres. *Oedogonium crassum* has cylindrical filaments of diameter 30-33 µm, and usually have 2-4 celled spermogonia, usually requiring a wide area to settle down (John, 2002); it was observed here that the surface with 1000 µm radius hemispheres may have enough interstitial space to allow the cells to settle down.

Microspora floccose has cylindrical cells 14-18 µm wide (John, 2002; Harding, 1971). These small diameter cells can attach in smaller interstitial spaces, i.e., the surfaces of smaller radius topographies. At lower concentrations these cells were found to be abundant to both small and large topographical features (500 and 2000 µm surfaces), but when the nutrient concentration was shifted to medium and high, the 500 µm surface appears to be the best surface topography for *Microspora floccose* growth. At lower nutrient concentrations, according to limited resources theory, there might be a competition among species for existence (Tilman, 1977); *Microspora floccose* was the most abundant species at low nutrient levels (Treatment 1) and was more abundant in the system overall as compared to other algal species.

5.2   Conclusions

There are some conclusions from the above discussion.

1. The increase in nutrient concentration increased the algal biomass density abruptly, but the composition of algal biomass was dominated by diatoms as compared to the algae species at higher concentrations.

2. Surface topography affects the overall algal biomass density. The surface with 2000 μm radius hemispheres has highest biomass density.

3. Nutrient concentration affects the ash content percentage in algal biomass. The middle nutrient concentration (Treatment 2) had highest percent ash content. Surface topography does not affect the ash content of the algal biomass.

4. Nutrient concentration plays an important role in the recruitment and relative abundance of all tested filamentous algal species.

5. Surface topography significantly affects the recruitment of *Microspora floccose* in all the three treatments out of all four tested species.

5.3 Contribution of Research work and Future Recommendations

1. Knowledge of species selectivity by surface topography will help in the design of cultivation substratum for open environments where other environmental conditions are not under control to optimize biomass characteristics, possibly making the process cost effective.

2. The behavior of species in different nutrient conditions may contribute to increasing the wastewater treatment efficiency by the selection of species according to nutrient levels in wastewaters.

3. Future work can be done to determine the kinetic growth parameters of each tested species.

4. Future work can also be done with different shapes of surface features (squares, circles) to test the boundary layer formation, biofilm attachment and biomass characteristics under different surface topographical features.

5. Flow characteristics, flow velocity, boundary layer formations and surface free

energy of different surface topographies can be studied in detail to analyze the behavior of algal species in different flow environments.

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Note: References are according to the ASABE guidelines

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APPENDIX A

Preliminary data Results

Table A.1: Preliminary velocity measurement of selected four flow lanes of Incubator

Distance (m)	Flow Lane 1 Time(sec)	Velocity (m/sec)	Flow Lane 2 Time(sec)	Velocity (m/sec)	Flow Lane 3 Time(sec)	Velocity (m/sec)	Flow Lane 4 Time(sec)	Velocity (m/sec)
0.114	3.000	0.038	3.000	0.038	2.000	0.057	3.000	0.038
0.304	8.000	0.038	7.000	0.043	8.000	0.038	7.000	0.043
0.608	7.000	0.043	8.000	0.038	6.000	0.050	7.000	0.043

Table A.2 : Preliminary data for biomass measurement selected four flow lanes of Incubator

	Sample No	Average Biomass of three subsamples(grams)	SD
Harvest 1	Row 1	0.84	0.04
	Row 2	0.89	0.00
	Row 3	0.76	0.00
	Row 4	0.71	0.00
Harvest 2	Row 1	0.61	0.02
	Row 2	0.78	0.00
	Row 3	0.71	0.00
	Row 4	0.96	0.01
Harvest 3	Row 1	0.65	0.00
	Row 2	0.76	0.00
	Row 3	0.62	0.00
	Row 4	0.83	0.00

A.3: Illumination data on each ceramic tile at the start of the experiment

Light Intensity (µmol/m²-sec) Row 5	Light Intensity (µmol/m²-sec) Row 5	Light Intensity (µmol/m²-sec) Row 4	Light Intensity (µmol/m²-sec) Row 4	Light Intensity (µmol/m²-sec) Row 3	Light Intensity (µmol/m²-sec) Row 3	Light Intensity (µmol/m²-sec) Row 2	Light Intensity (µmol/m²-sec) Row 2
219	301	232	214	219	268	302	320
301	298	359	382	377	387	341	366
243	254	289	305	291	312	283	302
104	334	298	277	318	222	358	391
287	369	210	226	166	200	146	153
200	294	397	365	388	374	388	398
239	200	296	314	319	385	289	272
157	153	155	160	157	163	133	141
312	344	316	350	298	352	327	302
241	267	377	390	375	380	331	361
285	265	301	320	313	323	339	380
132	155	156	177	172	190	166	175
301	356	372	391	302	288	316	325
232	243	362	373	359	317	320	273
275	292	302	291	330	336	339	337
241	266	143	157	170	155	172	156
284	283	371	402	358	361	358	329
247	237	345	354	307	306	365	322
312	225	300	307	328	308	268	264

A.4 : ANOVA result for velocity data in preliminary experiment

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.244736735	3	0.081578912	1.715289	0.240727785	4.066180551
Within Groups	0.380478912	8	0.047559864			
Total	0.625215646	11				

APPENDIX B

Area calculation for clay tiles

.3.1 Area Calculation of all the four tiles having different substratum characteristics

3.3.1.1 Smooth Tile

Smooth tile has no surface topography and the surface area of the tile was calculated using

$$\text{Area} = a^2$$

Where a = side of square (cm^2)

Dimensions of smooth tile = 10.2 cm x10.2 cm = (4 in. x 4 in.)

Nominal surface area of tile = Actual surface area of tile = 104.04 cm^2 = 16 in.^{2}

Tile having surface topography

Tiles with surface topography having hemispheres of radius 500 μm, 1000 μm, and 1000 μm had different nominal and actual area as the surface was covered by hemispheres. Actual surface area of these tiles were calculated by subtracting the base area and adding the curved area of hemispheres to the nominal surface area of the tile

$$Sa = a^2 - 10201(\pi r^2) + n\ (\pi r^2$$

Where

Sa = Actual surface area of tile (cm^2)

A = nominal surface area of tile (cm^2)

N = number of hemispheres covering the surface

r = radius of hemispheres on the tile (cm)

Actual surface area of tile having 500 μm radius hemispheres

Tile having substratum characteristics of 500 μm (0.05 cm) radius hemispheres has 1020 hemispheres on the surface so area of the tile was 184.16 cm^2 (28.54 $in.^2$).

Actual surface area of tile having 1000 μm radius hemispheres

Tile having substratum characteristics of 1000 μm (0.10 cm) radius hemispheres has 2500 hemispheres on the surface so area of the tile was 182.57 cm^2 (28.30 $in.^2$).

Actual surface area of tile having 500 μm radius hemispheres

Tile having substratum characteristics of 2000 μm (0.20 cm) radius hemispheres has 625 hemispheres on the surface so area of tile was 182.58 cm^2 (28.30$in.^2$).

APPENDIX C

STATISTICAL ANALYSIS

C 1: Minitab Output of Nested ANOVA for Biomass density

```
Nested ANOVA: Biomass Density versus Concentration, Roughness

Analysis of Variance for Density

Source            DF        SS        MS        F        P
Concentration      2   38.4978   19.2489   70.594    0.000
Roughness          9    2.4540    0.2727    2.338    0.017
Error            168   19.5925    0.1166
Total            179   60.5444
```

C.1.1 Minitab output for pairwise comparisons between treatments using Tukey Kramer

```
Analysis of Variance

Source                  DF  Adj SS   Adj MS  F-Value  P-Value
Nutrient concentration   2   38.50  19.2489   154.54    0.000
Error                  177   22.05   0.1246
Total                  179   60.54


Model Summary

      S    R-sq  R-sq(adj)  R-sq(pred)
0.352926  63.59%     63.17%      62.34%


Means

Nutrient
concentration   N    Mean   StDev        95% CI
1              60  0.5540  0.1017  (0.4640, 0.6439)
2              60  0.6708  0.2082  (0.5809, 0.7607)
3              60  1.5882  0.5657  (1.4983, 1.6781)

Pooled StDev = 0.352926
```

Tukey Pairwise Comparisons

```
Grouping Information Using the Tukey Method and 95% Confidence

Nutrient
concentration    N    Mean  Grouping
3               60  1.5882  A
2               60  0.6708    B
1               60  0.5540    B

Means that do not share a letter are significantly different.
```

C.1.2. Pairwise comparison between surface topography at treatment 1.

Tukey Pairwise Comparisons

```
Grouping Information Using the Tukey Method and 95% Confidence

Substratum
Topography    N    Mean  Grouping
500          15  0.6128  A
0            15  0.5863  A
2000         15  0.5463  A B
1000         15  0.4704    B

Means that do not share a letter are significantly different.
```

C.1.3. Pairwise comparison between surface topography at treatment 2.

Tukey Pairwise Comparisons

```
Grouping Information Using the Tukey Method and 95% Confidence

SurfaceTopography   N    Mean  Grouping
2000               15  0.8437  A
500                15  0.7200  A B
0                  15  0.6280    B C
1000               15  0.4916      C

Means that do not share a letter are significantly different.
```

C.1.4. Pairwise comparison between surface topography at treatment 3.

```
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

SurfaceTopography   N   Mean   Grouping
2000               15  1.792   A
1000               15  1.657   A
0                  15  1.486   A
500                15  1.418   A

Means that do not share a letter are significantly different.
```

C.2 : *Micropsora Floccose*

C.2.1 Mintab output for Kruskal-Wallis Test: Species Count versus Treatment

```
Kruskal-Wallis Test on Species Count

Treatment   N   Median  Ave Rank      Z
1          180  13.000     321.1   5.33
2          180  19.000     397.3  13.36
3          180   1.000      93.1 -18.68
Overall    540            270.5

H = 370.47  DF = 2  P = 0.000
H = 371.49  DF = 2  P = 0.000  (adjusted for ties)
```

C.2.2 Minitab output for Kruskal-Wallis Test: Species Count versus Surface Topography

```
Kruskal-Wallis Test on Species Count

Surface
Topography    N  Median  Ave Rank      Z
   0         135   9.000     242.5  -2.40
 500         135  13.000     289.2   1.61
1000         135  11.000     261.8  -0.75
2000         135  14.000     288.4   1.54
Overall      540            270.5

H = 8.47  DF = 3  P = 0.037
H = 8.50  DF = 3  P = 0.037  (adjusted for ties)
```

C.3 : Oedogonium crassum

C.3.1 Kruskal-Wallis Test: Species count versus Treatment

```
Kruskal-Wallis Test on Species count

Treatment    N       Median  Ave Rank       Z
1          180  4.000000000     319.1    5.12
2          180  0.000000000     107.1  -17.21
3          180  6.000000000     385.3   12.08
Overall    540                  270.5

H = 312.20  DF = 2  P = 0.000
H = 316.74  DF = 2  P = 0.000  (adjusted for ties)
```

C.3.2 Kruskal-Wallis Test: Species count versus Surface Topography

```
Kruskal-Wallis Test: Species count versus Surface Topography


Kruskal-Wallis Test on Species count

Surface
Topography    N  Median  Ave Rank       Z
   0        135   3.000     254.7  -1.36
 500        135   3.000     274.7   0.36
1000        135   4.000     286.3   1.36
2000        135   3.000     266.4  -0.36
Overall     540             270.5

H = 2.97  DF = 3  P = 0.396
H = 3.01  DF = 3  P = 0.389  (adjusted for ties)
```

C.4 *Mougeotoa scalaris*

C.4.1 Minitab output for Kruskal-Wallis Test: Species count versus Treatment

```
Kruskal-Wallis Test on Species count

Treatment    N  Median  Ave Rank      Z
1          180   8.000     261.7  -0.92
2          180  18.500     425.6  16.33
3          180   3.000     124.2 -15.41
Overall    540             270.5

H = 336.64  DF = 2  P = 0.000
H = 337.54  DF = 2  P = 0.000  (adjusted for ties)
```

C.4.2 Minitab output for Kruskal-Wallis Test: Species count versus Surface Topography

```
Kruskal-Wallis Test on Species count

Surface
Topography    N  Median  Ave Rank      Z
   0        135   9.000     280.3   0.85
 500        135   7.000     259.6  -0.93
1000        135   9.000     269.7  -0.07
2000        135   9.000     272.4   0.16
Overall     540             270.5

H = 1.22  DF = 3  P = 0.749
H = 1.22  DF = 3  P = 0.748  (adjusted for ties)
```

C.5 Sirogonium sticticum

C.5.1: Minitab output for Kruskal-Wallis Test: Species count versus Concentration

```
Kruskal-Wallis Test on Species count

Concentration    N  Median  Ave Rank       Z
1              180   8.000     347.4    8.10
2              180   3.000     152.6  -12.42
3              180   7.000     311.5    4.32
Overall        540             270.5

H = 159.03  DF = 2  P = 0.000
H = 160.19  DF = 2  P = 0.000  (adjusted for ties)
```

C.5.2: Kruskal-Wallis Test: Species count versus Surface Topography

```
Kruskal-Wallis Test on Species count

Surface
Topography    N  Median  Ave Rank       Z
   0        135   6.000     271.2    0.06
 500        135   6.000     276.6    0.52
1000        135   6.000     264.3  -0.53
2000        135   6.000     269.9  -0.05
Overall     540             270.5

H = 0.42  DF = 3  P = 0.935
H = 0.43  DF = 3  P = 0.935  (adjusted for ties)
```