Removal of pathogenic bacteria in algal turf scrubbers

By

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
Dec 10, 2016

Keywords: Algal turf scrubber, pathogen removal, wastewater treatment, ecological engineering, stormwater treatment

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Abstract

Algal Turf Scrubbers (ATS) are ecologically engineered systems that utilize the growth of filamentous algae on a submerged surface to remediate polluted water using sunlight as the main energy input into the system. Previous algal turf scrubber research has demonstrated the ability of these ecologically engineered systems to remove or degrade a variety of pollutants from water and wastewater including nutrients, metals and organic chemicals. It is therefore within reason that algal turf scrubbers may also be able to treat polluted water for pathogenic bacteria since the shallow water depth of these systems allow for significant exposure to ultraviolet (UV) light. Additionally, treatment may be a physical function of the algal turf itself, which in natural environments has been shown to trap sediment particles and pathogenic bacteria through the adhesion onto interwoven, mucilaginous algal filaments. Although similar algae-based treatment systems such as High Rate Algal Ponds (HRAPs) have been reported to significantly reduce the level of pathogenic bacteria in treated water, insufficient research has been conducted on pathogen die-off in ATS systems. The objective of this research is to measure the rate of removal of active bacteria in a lab scale ATS and assign significance to two variables identified to affect pathogen removal in algal turf scrubbers: the direct and indirect effects of UV light exposure and the physical sequestration of pathogens by algal turf. The experimental approach of this research utilizes four lab scale ATS reactors that attempt to separate the effect of these variables on the removal of the aquaculture pathogen *Flavobacterium columnare* and a non-pathogenic strain of *Escherichia coli* using treatment combinations of reactors with and without algal turf and UV

supplemented light. The results suggest that for both *F. columnare* and *E. coli* removal from the water column is primarily a physical function of the algal turf itself while UV light does play a role in enhancing the removal of *E. coli* but not *F. columnare*. Batch reactor experiments showed an average 3.6 log reduction in *F. columnare* and 2.6 log reduction with *E. coli* compared to experimental controls over a 24 hour period. The results suggest that larger ATS may significantly reduce bacteria concentration of treated water although consistency of treatment would likely vary.

Acknowledgements

I give thanks to my advisor Dr. David Blersch for the opportunity to research the subject presented in this study and Dr. Yi Wang and Dr. Cova Arias for their input and support on this research. Special thanks to Dr. Cliff Lange for substituting on my committee at the last minute. I would also like to thank the members of the laboratories of Dr. Yi Wang, Dr. Cova Arias and Dr. Mark Liles for their support as well. Special thanks to Walter Mulbry for a used equipment donation used in the experiments and Dr. Dongye Zhao for modeling consultation. I also deeply appreciate the support and resources of the Department of Biosystems Engineering at Auburn University.

I also want to thank my loving friends and family for being a part of my life journey. I only hope my journey ends knowing I had a positive impact on the earth and its life. As above, so below.

Table of Contents

Abstract ii

Acknowledgements iv

List of Tables ix

List of Figures x

List of Abbreviations xii

Chapter 1: Introduction 1

1.1 Ecological water and wastewater treatment 1

1.2 Microbial pollutants in water 2

1.3 Purpose of research 3

1.4 Objectives 3

Chapter 2: Literature review 5

2.1 ATS background information 5

2.2 Proposed mechanisms of pathogen removal in Algal Turf Scrubbers 8

2.2.1 Solar mediated disinfection 9

2.2.2 Physical adsorption of bacteria onto algal turf 12

2.3 Modeling pathogen removal in ATS 14

2.4 Research pathogens 17

2.4.1 *Flavobacterium columnare* 17

2.4.2 *Escherichia coli* 18

Chapter 3: Methods 21
3.1 Laboratory scale algal turf scrubber 21
3.1.1 Temperature control 22
3.1.2 Lighting 24
3.2 Bacteria cultures 28
3.2.1 Flavobacterioum columnare culture 28
3.2.2 Escherichia coli culture 29
3.2 Algal culture 30
3.4 Preliminary experiments 32
3.4.1 Algae growth under UV supplemented light 32
3.4.2 F. columnare die-off in wastewater 34
3.4.3 F. columnare removal with humic acid supplementation 40
3.5 Main Experiment 43
3.5.1 Experiment Design 43
3.5.2 Reactor operating parameters 44
3.5.3 Preparatory tasks conducted prior to each experiment 47
3.5.4 Running the experiment: sampling, processing and plating 49
3.5.5 Post experiment: harvesting and disinfection 51
3.5.6 Data Analysis 52
Chapter 4: Results and Discussion 53
4.1 Variation of water quality parameters 53
4.1.1 *F. columnare* removal with surge 53
4.1.2 *F. columnare* removal without surge 54

4.1.3 *E. coli* removal with surge 55
4.2 Biomass productivity of reactors with algae 56
4.4 Pathogen removal from water column 58
4.4.1 Removal of *F. columnare* in water column with surge 59
4.4.2 Removal of *F. columnare* in water column without surge 60
4.4.3 Removal of *E. coli* in water column with surge 62
4.5 Pathogen concentration in sampled algal turf 64
4.5.1 *F. columnare* concentration in sampled algal turf 64
4.5.2 *E. coli* concentration in sampled algal turf 66
4.6 Modelling of pathogen removal in ATS 66
Chapter 5: Summary and Conclusions 74
5.1 Summary 74
5.2 Limitations of study 80
5.3 Translation of study results to real world application 82
5.5 Conclusions 84
5.4 Recommendations for future work 86
References 88
Appendix A: Lab scale ATS design and validation 95
Appendix B: Growth curves for *F. columnare* and *E. coli* 105
Appendix C: SAS output 107
Appendix C.1: Subsidized AFDM difference between UV and No UV algal productivity ... 107
Appendix C.2: Water quality variation for *F. columnare* w/ surge and mature turf 108
Appendix C.3: Water quality variation for *F. columnare* w/ surge and harvest turf 111

Appendix C.4: Water quality variation for *F. columnare* no surge and mature turf 113

Appendix C.5: Water quality variation for *F. columnare* no surge and harvest turf 115

Appendix C.6: Water quality variation for *E. coli* w/ surge and mature turf 118

Appendix C.7: Water quality variation for *E. coli* w/ surge and harvest turf 120

Appendix C.8: Log reduction of *F. columnare* with mature turf and surge 121

Appendix C.9: Log reduction of *F. columnare* with harvested turf and surge 123

Appendix C.10: Log reduction of *F. columnare* with mature turf and no surge 125

Appendix C.11: Log reduction of *F. columnare* with harvested turf and no surge 127

Appendix C.12: Log reduction of *E.coli* with mature turf and surge..................................... 129

Appendix C.13: Log reduction of *E.coli* with harvested turf and surge 131

Appendix D: Media and buffer formulations.. 133

Appendix D.1: Modified Sheih 133

Appendix D.2: Buffer recipe 135

Appendix D.3: Gulliard’s F/2 recipe 136

List of Tables

Table 3.1 Flow parameters of the lab scale ATS used for experimentation 35

Table 3.2 Flow parameters of the lab scale ATS used for experimentation 47

Table 4.1 Water quality data for *F. columnare* removal under surge with mature algal turf 54

Table 4.2 Water quality data for *F. columnare* removal under surge with harvested algal turf . 54

Table 4.3 Water quality data for *F. columnare* removal, no surge with mature algal turf 55

Table 4.4 Water quality data for *F. columnare* removal, no surge with harvested algal turf 55

Table 4.5 Water quality data for *E. coli* removal under surge with mature algal turf 56

Table 4.6 Water quality data for *E. coli* removal under surge with harvested algal turf 56

Table 4.7 Kinetic data used for model evaluation of *F. columnare* experimental data 71

Table 4.8 Kinetic data used for model evaluation of *E. coli* experimental data 73

List of Figures

Figure 2.1 Photo of landscape ATS and illustration of colonized ATS substratum 7

Figure 2.2 ATS channel cross-section and conceptual mass transfer gradient 7

Figure 2.3 Conceptual representation of routes of solar mediated disinfection in water 11

Figure 2.4 Photos of algal filaments showing trapped organic matter and adhered sediment.... 13

Figure 2.5 Scanning electron microscope image of *F. columnare* 18

Figure 2.5 Scanning electron microscope image of *E. coli* 19

Figure 3.1 Data logger output of water temperature in lab scale ATS environment 23

Figure 3.2 Data logger output of water temperature for inline reactor water heater 24

Figure 3.3 Spectral output of Phillips TL741 fluorescent lamps 25

Figure 3.4 Photo of lamp configuration used in reactor light fixtures....................................... 26

Figure 3.5 Spectral irradiance of Heliovite UV lamps with and without UV filter.................... 27

Figure 3.6 Comparison of spectral irradiance of Heliovite UV lamps with sunlight 27

Figure 3.7 Photo of 40X magnification of algal culture used for the study............................... 31

Figure 3.8 Productivity comparison of algae grown with and without UV supplemented light 33

Figure 3.9 Dark die-off of *F. columnare* in three different waters 37

Figure 3.10 UV die-off of *F. columnare* in three different waters 37

Figure 3.11 Linear regression of F/2 concentration and *F. columnare* dark die-off 39

Figure 3.12 Linear regression of F/2 concentration and *F. columnare* UV die-off.................... 40

Figure 3.13 Removal of *F. columnare* with humic acid supplementation for mature turf 42

Figure 3.14 Removal of *F. columnare* with humic acid supplementation for harvested turf..... 42

Figure 3.15 A graphical representation of the treatments used for the main experiment........... 44

Figure 3.16 Measured flow rates of each reactor used for the main experiment........................ 46

Figure 4.1 Progressive growth of algal turf during 9 day harvest cycle 57

Figure 4.2 Nine day harvest productivity over the duration of the main experiment................. 57

Figure 4.3 Removal of *F. columnare* with a mature algal turf and surge.................................. 59

Figure 4.4 Removal of *F. columnare* with a harvested algal turf and surge.............................. 60

Figure 4.5 Removal of *F. columnare* with a mature algal turf and no surge............................. 61

Figure 4.6 Removal of *F. columnare* with a harvested algal turf and no surge......................... 61

Figure 4.7 Removal of E. coli with a mature algal turf and surge... 63

Figure 4.8 Removal of E. coli with a harvested algal turf and surge... 63

Figure 4.9 Concentration of *F. columnare* in sampled algal turf, surge.................................... 65

Figure 4.10 Concentration of *F. columnare* in sampled algal turf, no surge............................. 65

Figure 4.11 Concentration of *E. coli* in sampled algal turf, surge... 66

Figure 4.12 Model results of *F. columnare* removal as a function of adsorption to algal turf... 70

Figure 4.13 Mass balance graph of *F. columnare* in solution and adsorbed to algal turf........... 70

Figure 4.14 Model results of *E. coli* removal as a function of adsorption to algal turf............. 72

Figure 4.15 Model results of *E. coli* removal as a function of adsorption and UV die-off 73

Figure 5.1 Conceptual illustration of pathogen removal mechanisms in the ATS 74

Figure 5.2 Measured 24 hour dark die off of *F. columnare* at varying pH 77

Figure 5.3 Comparison of solar UV output with UV output of Heliovite lamp 83

Figure 5.4 Model output for *E. coli* removal using measured solar irradiance of full sun......... 84

List of Abbreviations

AFDM Ash Free Dry Mass

ATS Algal Turf Scrubber

BOD Biochemical Oxygen Demand

CFU Colony Forming Unit

DI De-ionized (water)

DO Dissolved Oxygen

EC Electrical Conductivity

EPS Extracellular Polysaccarides

HCl Hydrochloric Acid

HRAP High Rate Algal Pond

HRT Hydraulic Residence Time

LPM Liters Per Minute

MS Modified Sheih

PAR Photosynthetically Active Radiation

PBS Phosphate Buffered Saline

PFR Plug Flow Reactor

RMSD Root Mean Square Deviation

ROS Reactive Oxygen Species

RPM Revolutions Per Minute

Temp	Temperature
TOC	Total Organic Carbon
UV	Ultraviolet (Radiation)
UV-B	Medium-wave Ultraviolet Radiation (280-315nm)
UV-A	Long-wave Ultraviolet Radiation (315-400nm)
WSP	Waste Stabilization Pond

Chapter 1: Introduction

1.1 Ecological water and wastewater treatment

Innovative solutions to the problems of water and wastewater remediation must be developed that efficiently remove unwanted physical, chemical and biological constituents in water and wastewater while also consuming the minimum amount of energy input from anthropogenic sources. This must also be accomplished in a manner that will maintain the ecological integrity of the planet meaning a reduction in chemical and energy inputs and a responsible handling of residual organic matter and effluent water from treatment processes. Also, with the concept of sustainability ever more pertinent in society today, it is realized that solutions are needed to solve the dilemma of current and future needs for clean energy, food production and water resources. A new engineering approach for solving all of these problems through ecologically designed systems is gaining ground and holds the key to a sustainable future.

Ecological wastewater treatment is defined by Jenssen and Vatn (1997) as a systems of wastewater treatment that allows for the recycling of materials present in the waste stream with a minimal amount of environmental stress exerted on the surrounding environment as well as the planet as a whole. For wastes that are of an organic origin such as agricultural and municipal wastes, recycling simply means at some point the recovered materials are returned to the soil from which it was harvested. While conventional systems greatly reduce aquatic ecosystem stress through the removal of Biochemical Oxygen Demand (BOD) and nutrients from

discharged water, they are often reliant on chemical inputs and/or energy intensive. The reliance of conventional treatment systems on these high energy inputs indirectly impacts the environment through pollution generated from manufacturing, power generation and transportation. There are also other minor direct environmental impacts of conventional systems such as chemical laden sludge and chlorine disinfection by-products (Mantis et al., 2005). With ecological systems man-made inputs are kept at a minimum since most of the energy required for treatment is ultimately from solar radiation (Jenssen and Vatn, 1997).

This idea of ecologically engineered wastewater treatment is captured perfectly with conceptual model of Advanced Integrated Wastewater Pond Systems (AIWPS) developed during the 1960's in California which used High Rate Algae Ponds (HRAPs) to treat wastewater and produce an algae biomass feedstock for energy production through anaerobic digestion (Oswald and Golueke, 1960). This system demonstrated the concept that there is no such thing as a waste material as it can be used as a feedstock in another process to produce another commodity of value. The Algae Turf Scrubber (ATS) is another ecological treatment system that also utilizes algae as a workhorse similar to HRAPs while also producing a potential feedstock for biofuels. Although the ATS is primarily used for the treatment of stormwater and non-point source pollution, the ATS has also been studied for nutrient recovery from wastewater (Adey et al, 2011; Clarens et al, 2010; Wilkie and Mulbry, 2002).

1.2 Microbial pollutants in water

The most important water and wastewater constituent in regards to human health is undoubtedly pathogens. Although other contaminants such as metals can cause serious health effects in high concentrations and/or over long term exposure, the effect of pathogenic microorganisms has almost immediate health effects on the individual. Pathogenic

microorganisms in water and wastewater consist of bacteria, virus, parasitic worms and protozoans and are introduced into the water supply through fecal contamination (Bitton, 1999). Bacteria include the organisms that cause cholera and other gastro-intestinal illness, which can lead to death if untreated.

1.3 Purpose of research

The purpose of this research is to investigate the relationship between pathogenic bacteria in water and an algal turf similar to that employed in an algal turf scrubber for water pollution treatment. The approach for this research is to simulate the relationship between bacteria and turf algae through experimentation with a laboratory scale ATS in an artificial UV light environment. The lab scale algal turf scrubber set up simulates the operation of outdoor ATS systems but with the experimental control of a laboratory environment. By measuring the change of a known initial concentration of bacteria over a period of time the behavior of pathogenic bacteria in the ATS environment can be elucidated. It is expected that there will be some degree of removal of active cells from the water column that will occur through a variety of mechanisms.

1.4 Objectives

This study aims to answer whether or not the biological and operating characteristics of the ATS facilitate the removal of pathogenic bacteria from water and to distinguish among the influencing mechanisms of removal. To do this, the objectives are as follows:

1. Quantify the rate of removal of an initial high concentration of bacteria over time under two possible stages of algal turf development: a mature algal turf and a harvested algal turf. A mature turf defined as the physical state in which algal filaments are extended into the water column and stationary stage growth has occurred. A harvested turf defined as the state in which all algal growth into the water

column has been removed leaving only the basal algal cells attached to the substratum.

2. Quantify whether or not the use of a period wave pulse characteristic to ATS operation has any influence on the removal of bacteria from the water column and the concentration in sampled algal turf.
3. Quantify the removal of pathogenic bacterial species that are characteristic of two different environments: *Flavobacterium columnare* (aquatic/aquaculture) and *Escherichia coli* (enteric/domestic wastewater).
4. Establish a context for mathematically modeling the kinetics of pathogen removal in the ATS.

The above objectives were achieved using four laboratory scale reactors that are designed to separate the effects from the two main routes of removal in the ATS: the effect of UV and near UV light on bacteria inactivation and the physical removal of bacteria via algal turf.

Chapter 2 Literature review

2.1 ATS background information

The algae turf scrubber is an ecologically engineered treatment system that has emerged within the last few decades as a solar powered alternative for the remediation of water and wastewater. Originally developed by Walter Adey to mimic highly productive coral reef algal communities, the ATS has been demonstrated through numerous studies for the ability to remove nitrogen and phosphorus from treated waters (Adey et al., 1993; Adey et al., 2013; Mulbry and Kangas, 2015; Lui et al., 2016). The success of earlier trials led to the expansion of studies involving the utilization of waste waters from municipal and agricultural sources that proved to be just as successful (Mulbry et al., 2010; Mulbry and Kangas, 2015; Lui et al., 2016). Research has shown that these systems are able to remove heavy metals from waters through algal cell wall assimilation and precipitation through photosynthetic alteration of water chemistry (Craggs et al.,1996; Rothman et al., 2013; Adey et al., 1996). The alteration of water quality parameters (pH and DO) due to algal photosynthesis in the presence of the UV spectrum of sunlight are also thought to contribute to the breakdown of some organic compounds such as chlorinated hydrocarbons (Adey et al., 1996). More recent studies on ATS systems have also started to evaluate the potential for the utilization of algal biomass from the ATS systems as feed stocks for biofuels, organic fertilizers and neutraceuticals increasing the economic incentive of these systems for greater utilization in wastewater and water treatment (Clarens et al., 2010; Adey et al., 2013; 28, Grayburn et al., 2013).

Simply stated, an ATS is composed of a shallow flowing channel in which a substratum is installed in the channel bed (Figure 2.1). Over time the substratum is colonized by benthic (typically filamentous) algae species indigenous to the water or wastewater being treated and develop a turf. During colonization of the substratum, algae first develop basal cells termed hold fast that attach and secure the algae to the substratum in a flow environment (Peterson, 1996). The turf experiences growth similar to bacteria in which exists a lag phase followed by exponential growth. During exponential growth the algal filaments extend upwards into the water column to increase exposure to solar energy and nutrients (Borchardt, 1996). As the turf matures, a concentration gradient emerges in which sunlight, gas and nutrients decrease from the interface of the turf and bulk fluid flow downwards to the substratum (Figure 2.2). Periodic wave pulses or other sources of turbulent flow promote the agitation of algal filaments allowing for some degree of mixing and disruption of diffusion limitations (Borchardt, 1996; Stevenson, 1996). At this point the growth rate of the turf slows as it enters a stationary phase, and sloughing of the turf can occur as the basal cells weaken from starvation (Peterson, 1996). Typically in the ATS before the stationary phase of growth is reached, the algal turf is harvested and the growth process is reset. An inherent characteristic of the ATS is its variability in productivity and remediation as a function of time (seasonal changes) due to shifts in species and water quality.

Figure 2.1. The long shallow channel of a landscape scale ATS located in Patterson, California (left) and an illustration of substratum colonized by filamentous algae (right). Photograph (left) from Adey et al. (2011). Illustration (right) from Adey et al. (1993).

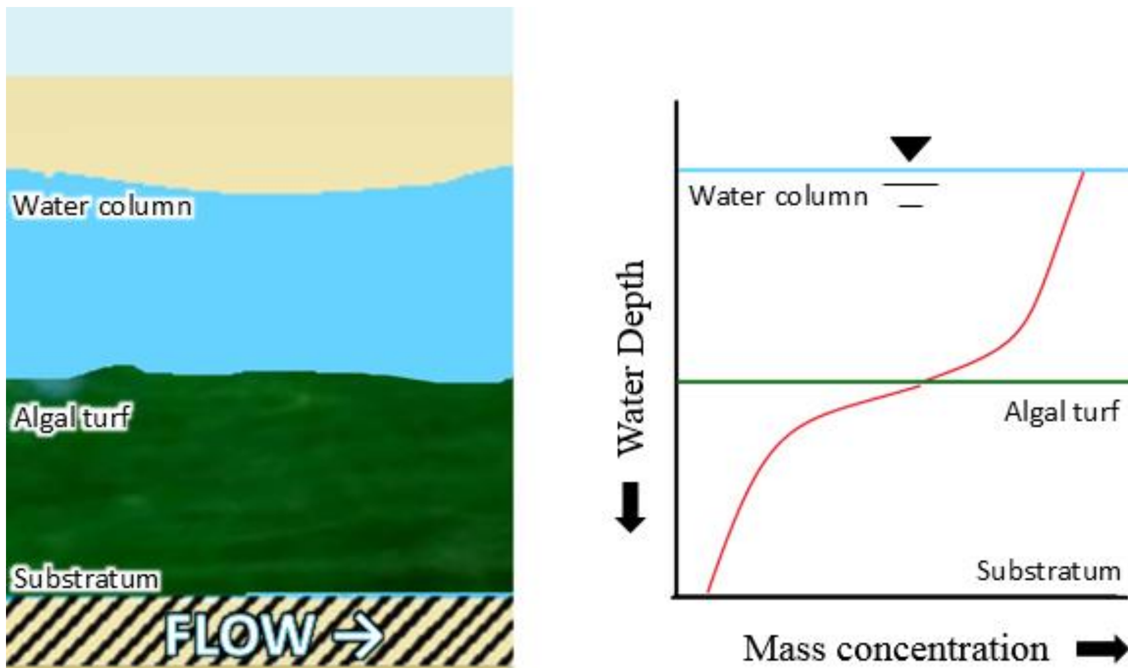

Figure 2.2 Illustration of a cross section of ATS showing shallow water column and mature algal turf (left) alongside a conceptual concentration gradient of carbon dioxide and dissolved nutrients (red line).

Typically, most algal turf scrubbers utilize a surge mechanism that provides a periodic surge of water at the inlet of the channel which then attenuates as it travels to the outlet. This period wave pulse mimics the natural wave environment of lake and marine ecosystems in which algae inhabit (Adey et al., 2011). The periodic wave pulse is thought to increase algal

productivity since it disrupts laminar flow and creates turbulence in the water column. The turbulent flow conditions agitate the algal turf allowing for increased sunlight exposure and nutrient exchange that would otherwise be prevented by stagnation at the boundary layer under laminar flow (Stevenson, 1996). Filamentous algae are also found in streams which are absent of wave pulses but do contain pools and riffles that offer both turbulent and laminar flow environments. Since these are ecologically engineered systems, processes powered by sunlight drive the bulk of the remediation work. Typically the only other energy input into these systems is from a pump to raise water to the inlet of the channel and from harvesting the mature algae turf that can be accomplished solely through human effort.

2.2 Proposed mechanisms of pathogen removal in Algal Turf Scrubbers

Currently there has been little research investigating whether or not an algal turf or biofilm can potentially remove microbial pollutants from receiving waters (Schumacher et al., 2003). This along with the ability of the ATS to trap sediment from erosion presented a deficiency in the area of published research to explore these questions. Craggs et al. (1996) suggested that the short residence time of the ATS does not provide consistent disinfection, and treated water should be further processed using sand filtration with UV lamp disinfection, but this does not concern recirculating ATS systems. Schumacher et al. (2002) studied the ability of an algae biofilm system to reduce nutrients and fecal coliform bacteria in utilized activated sludge effluent. The researchers used a small, re-circulating algae biofilm reactor on municipal wastewater and demonstrated the ability of the system to reduce an initial concentration of 10^7 CFU/100 mL of *E. coli* to below 1000 CFU/100 mL after 4 hours of re-circulating flow in exposure to direct sunlight. However, the researchers did not use any experimental control and presented data from only 1 sample (n=1). The researchers also did not explore significance of

other possible mechanisms of removal within the ATS system such as how much if any bacteria is removed from adhesion to the algal biofilm and how much is removed due to solar UV disinfection.

At the beginning of this research it became necessary to identify the possible routes of pathogen removal from the water column in the ATS. Since there have been no studies on this subject in the past it was necessary to look to treatment systems similar to the ATS that have been studied. It was decided that wastewater treatment pond systems are a close analog to the ATS out of all known wastewater treatment systems including constructed wetlands. In particular the High Rate Algal Pond (HRAP) appears to be the closest candidate out of all treatment pond systems since its operation relies on a shallow water column depth and flowing water. After review of algal and treatment pond literature pertaining mostly to HRAPs and waste stabilization ponds (WSPs) the main mechanism of pathogen removal in these systems was identified as solar UV mediated disinfection from direct DNA damage and exogenous and endogenous photosensitizers (Curtis et al., 1992; Davies-Colley et al., 1999). However, the majority of algae that emerges in treatment pond systems are planktonic and not benthic. Since the ATS cultivates benthic algae, there may be an additional route of bacterial removal via physical sequestration by the algal turf.

2.2.1 Solar mediated disinfection

In natural systems and lab scale microcosms, filamentous green algae have been observed to harbor populations of *E. coli* and other coliform bacteria providing protection from the germicidal effects of UV light in addition to acting as a nutrient source as photosynthetic products are exuded from algal cells (Beckinghausen et al., 2014; Ksoll et al., 2007; McFeters et al., 1978; Cole, 1982). These studies would tend to suggest that the ATS would therefore not be

able to appreciably remove enteric bacteria from water; however, the design of the ATS system differs from that of natural systems and lab scale microcosms.

UV radiation from natural sunlight in the optimum disinfection range of 253.7 nm to 270 nm has been demonstrated to be an effective method for disinfection of water with disinfection rate affected by solar intensity, water depth and turbidity (Dessie et al., 2004; U.S. EPA, 1999). It has also been demonstrated that the inactivation of coliform and *E. coli* bacteria in lab experiments was mainly a function of light which could also be compounded under elevated pH and dissolved oxygen levels (Curtis et al., 1992; Reed, 1997, Parhad and Rao, 1974; Khaengraeng and Reed, 2005). All three of these variables are inherently characteristic of ATS systems.

As mentioned above waste stabilization ponds are a close analog to the ATS as far as understanding the influence of sunlight on pathogen inactivation. The mechanisms behind solar disinfection of drinking water (SODIS) involving the formation of Reactive Oxygen Species (ROS) in the presence of sunlight and dissolved oxygen can also be translated over to the ATS as well (Reed, 1997; Khaengraeng and Reed, 2005). Although the first study on treatment ponds acknowledged only temperature as the main factor influencing die-off of enteric pathogens, sunlight mediated disinfection has since been suggested as the most important influence in pathogen inactivation (Marias, 1974; Davies-Colley et al., 2000).

Solar UV disinfection can damage bacteria by three different routes: direct DNA damage, endogenous photosensitizer production and exogenous photosensitizer production (Maier et al., 2000; Curtis et al., 1992; Davies-Colley et al., 1999; Figure 2.3). Direct DNA damage takes placed in the UV-B range of 280-315 nm in which the components of DNA (nucleic acids and proteins) directly absorb the UV-B radiation. The absorption of UV-B radiation by cellular DNA

promotes the formation of lesions (pyrimidine dimers) that can become fatal or mutagenic to the organism but can be counteracted through cellular DNA repair mechanisms such as photoreactivation (Sinha and Hader, 2002). Only a small portion of UV-B radiation remains after it is filtered out by the Earth's atmosphere and what remains is easily attenuated through the water column in natural waters (Frost et al., 2005). The attenuation of UV-A through the water column is less rapid compared to UV-B suggesting it should provide a greater effect towards UV mediated disinfection.

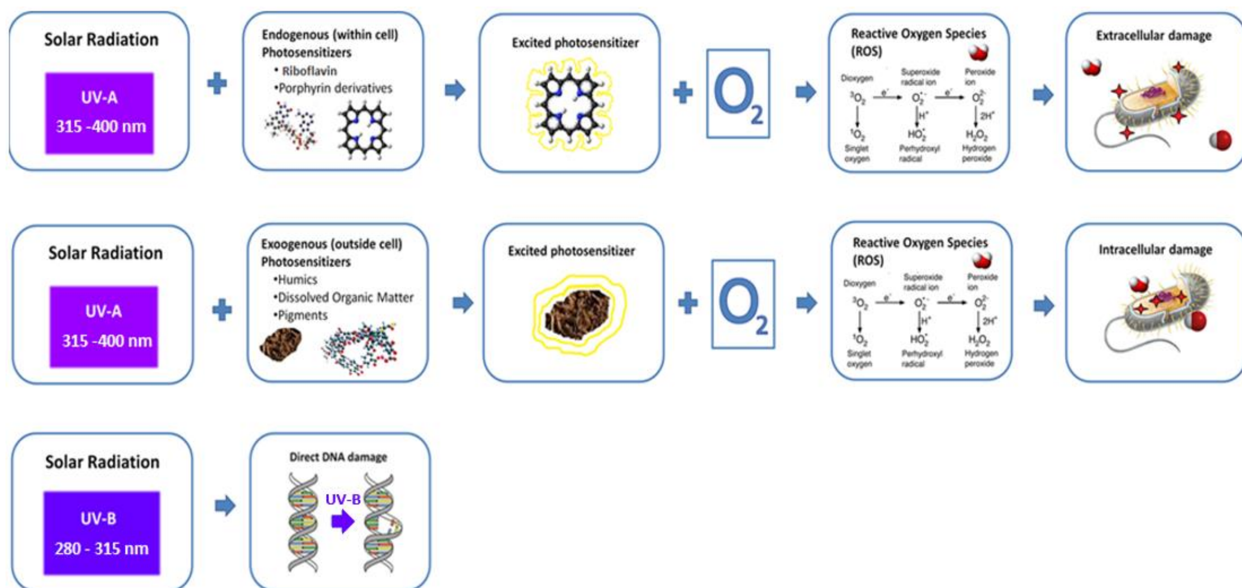

Figure 2.3. Identified possible routes of solar mediated disinfection in algal turf scrubbers.

UV-A radiation in the range of 315-400 nm causes indirect cellular damage through the formation of Reactive Oxygen Species (ROS) via photosensitizers both inside and outside of the bacterial cell (Sinha and Hader, 2002). ROS are produced when an excited photosensitizer reacts with molecular oxygen producing singlet oxygen, peroxide, superoxide, hydrogen peroxide or hydroxyl radicals. The production of ROS and the damaging effect of oxygen in these reactions are heavily dependent on the DO concentration in water (Reed, 1997; Maclean et al., 2008; Webb and Brown, 1979). Humic substances as well as photosynthetic pigments present in

natural waters have been identified as exogenous photosensitizers having the ability to absorb UV-A radiation and react to produce the ROS (Cooper et al., 1989; Curtis, 1992). Curtis (1992) stressed the importance of dissolved organic matter in the form of humic acids as the primary sensitizer responsible for disinfection in waste stabilization ponds. Photosensitizers are formed inside the cell itself when intracellular compounds absorb UV-A radiation and react with cellular oxygen to form ROS. Researchers have shown that intracellular compounds such as riboflavin, tryptophan and porphyrins are capable of UV excitation and ROS formation (MacKay et al., 1976; Lloyd et al., 1990; Maclean et al., 2008; Baier et al., 2006). Past research has suggested that photosensitizing reactions in the presence of UV-A radiation are dependent on and enhanced by cellular oxygen (Webb and Brown, 1979; Peak et al., 1983).

2.2.2 Physical adsorption of bacteria onto algal turf

Past studies on algal turfs in natural environments have reported that these filamentous algal communities trap sediments, in particular, fine size sediment particles (Neumann and Gebelein, 1970; Scoffin, 1970; Airoldi et al., 1996). Researchers studying the ATS have also observed the sediment trapping effect of the algal turf present in the systems although no studies have been conducted solely on this subject in relation to water quality improvement (D'Aiuto et al., 2015; Chen et al., 2015). In flowing water frictional forces at the interface of the turf and bulk fluid flow create a boundary layer condition in which a velocity gradient exists to dampen current velocity (Borchardt, 1996) The drag effect created from the boundary layer allows suspended particles to drop out of bulk flow and become temporarily or permanently trapped in the algal turf (Figure 2.4). Sequestration of trapped particles such as sediments occurs due to the interwoven algal filaments that form an irregular structure that discourages the dislodging of trapped particles (Neumann et al., 1970). This irregular structure of algal filaments may also

exude extracellular polysaccharides that allow fine grains to become cemented to the filaments (Stamski and Field, 2006). The filamentous network of algae is variable in density allowing for the trapping of both coarse and fine grain sediments; however, reports indicate that fine grain sediments are more easily trapped and retained over time (Airoldi et al., 1996; Scoffin, 1970).

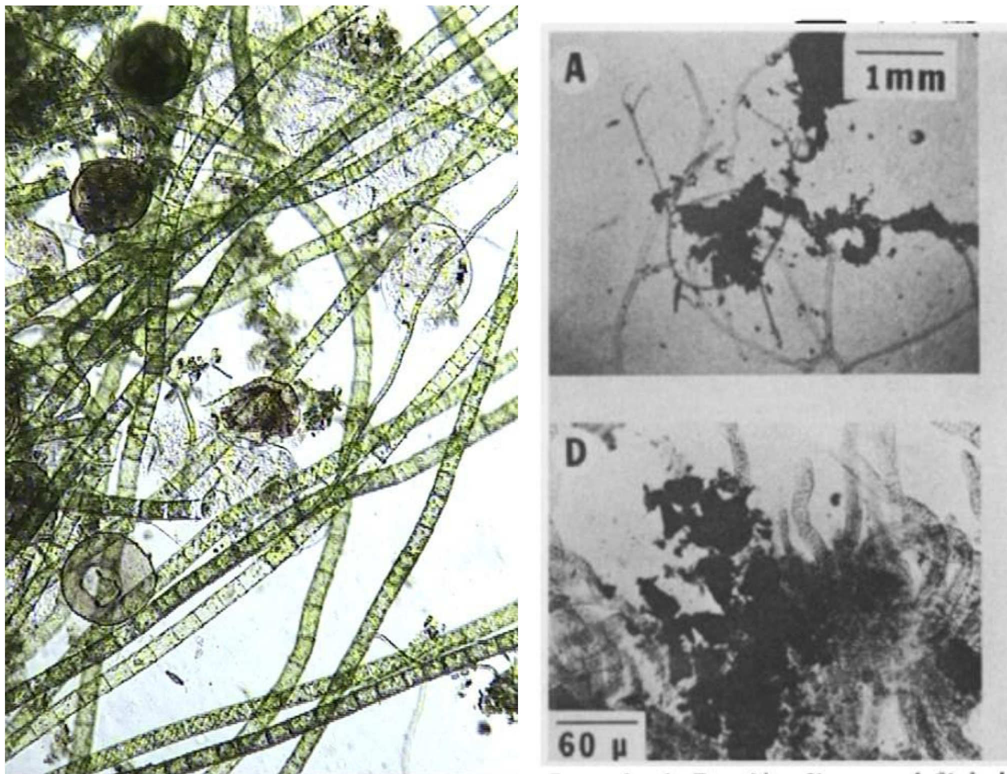

Figure 2.4. 10X magnification of organic matter trapped in the interwoven filaments of Oedogonium sp. sampled from the lab scale ATS used in this research (left).Sediment particles coating filaments of Cladophoropsis membranacea (A) and Lyngbya truncicola (D) from Neumann et al. (1970, right).

It is possible that bacteria that become trapped in algae may die off from resource limitation or form a biofilm on the filaments. In nature bacteria-algal communities have been observed in which the bacterial community is able to assimilate metabolites excreted by algae (Haack and McFeters, 1982; Ksoll et al., 2007). The freshwater filamentous algae *Chladophora* has also been observed to harbor enteric bacteria suggesting bacterial survival for a prolonged period of time from a supply of algal exudates and shading from UV radiation (Beckinghausen et

al., 2014). From the literature it can be speculated that bacteria can likely become trapped in algal filaments similar to fine sediment particles and should be considered as another main route of removal from the water column in the ATS. If this is the case it is also possible that bacteria can become resuspended into the water column due to turbulence created during a wave pulse from a tipping bucket. It can be expected then that the concentration of bacteria at the upstream end near the tipping bucket would be lower than at the downstream end of the ATS where water is less turbulent. In less turbulent sections of the ATS bacteria may also settle out of the water column and onto algae in full exposure to sunlight allowing for maximum UV disinfection.

2.3 Modeling pathogen removal in ATS

There are currently no mechanistic mathematical models for the prediction of pathogen removal in algal turf scrubbers. Schumacher et al. (2002) proposed a simple first order kinetic rate model for their study on pathogen removal of an algal biofilm but again this study ignored the separation and observation of removal mechanisms in the system. Their model used a modified kinetic constant that was a function of the surface area of the algal biofilm divided by the system volume. The authors also observed that the kinetic constant increased as a function of system pH for all tested pathogens including *E. coli*.

Since literature review indicates that pathogen removal may be due to both physical removal and solar mediated disinfection, a mathematical model should include terms that represent this activity. Physical removal is probably best represented using adsorption kinetics in which an equilibrium concentration of bacteria is achieved on the surface of the algal turf after a given amount of time (Daniels, 1980; Gupta and Rastogi 2008a). Solar mediated disinfection can be represented using models already developed for waste treatment ponds.

There are several proposed rate models describing the adsorption of bacteria onto various surfaces under different assumptions (Cowen, 1995; Daniels, 1980; Gupta and Rastogi, 2008). Daniels (1980) developed a rate equation for the adsorption and desorption of bacteria to a surface (ion-exchange resin) considering the process as diffusion controlled. The rate of adsorption is a function of the removal of bacteria from a well-stirred fluid, with removal occurring when the bacteria contact the surface of larger sized ion exchange resin particles. The rate model by Daniels (1980) was calibrated using optical absorbance to gauge bacterial adsorption over time yielding a graphical plot of log cumulative absorbance versus square root of time in which rate constants could be graphically solved. Other more complex and mechanistic models such as the Derjaguin, Landau, Verwey and Overbeek theory (DLVO) and Monte Carlo simulation were considered but were far too complex for the scope of this study (Bayoudh et al., 2009; Hsu, 1987; Margalit et al., 2013).

Gupta and Rastogi (2008a, 2008b) studied the kinetics of biosorption of lead (II) in solution by the green algae *Spirogyra* and *Oedogonium* in which they evaluated pseudo first order and pseudo second order rate models for best fit. Both first and second order equations were linearized for fitting of experimental data resulting in the estimation of kinetic coefficients for the models. For both species of algae Gupta and Rastogi found a best fit of data using the pseudo second-order kinetic model of McKay and Ho (1999). The presence of extracellular polysaccharides (EPS) present on the cell surface of brown algae contribute to the adhesion metals acting through multiple mechanisms including metal binding by functional groups and extracellular ion-exchange-like behavior (Davis et al., 2003). One shortcoming of the above models is that they were developed and studied for non-living adsorbates such as metals unlike bacteria, which are living organisms. Using the adsorption models to model bacteria assumes

that the bacteria behave similar to a molecule or ion, which may not be an accurate assumption since bacterial cells are not soluble. Bacterial cells are also subject to death, which may create error when measuring adsorption since cells that were adsorbed and died would not form a colony when plated.

The removal of bacteria in the ATS by UV radiation can be modeled according to previous research in treatment pond removal. Although numerous models exist for modeling pathogen removal in ponds, the model proposed first by Sarikaya and Saatci (1987) and later adapted for removal in high rate algal ponds by Craggs et al. (2004) will be evaluated since is an established model for pathogen removal in algal pond systems. This model assumes a first order disinfection process in which only the dark die off rate of bacteria, k_d (1/s) and the light dependent rate k_s (m^2/J) are considered (Equations 1 and 2). Light attenuation may also be factored into the equation but since the operating depth of the lab scale ATS is less than an inch it was neglected.

$$\frac{dC}{dt} = -k(t)C \tag{1}$$

$$k(t) = k_d + k_s G(t) \tag{2}$$

where C is the concentration of bacteria at time t and $G(t)$ is the total solar irradiance (J/m^2-s) at the water surface as a function of light exposure time. G accounts for all radiation incident on the water surface not just the UV-B and UV-A portions which contribute the most towards disinfection. The rate constant k_s is calculated from the slope of the linear plot of $\ln(C) + k_d t$ vs. $S(t)$. Combining equations 1 and 2 and integrating yields equation 3.

$$\ln(C) = \ln(C_0) - k_d t - k_s S(t) \tag{3}$$

where

$$S(t) = \int_{\theta=0}^{\theta=t} G(\theta)d\theta \tag{4}$$

is the total insolation or solar irradiance at the water surface integrated over time, C_0 is the initial concentration of pathogen and θ is the light exposure time of the water. For the case of this research the exposure time, θ, is considered as the amount of time a bacterial cell will be exposed to light in the reactor. Since the irradiance in the lab scale reactors does not vary with time, the irradiance $G(t)$ is constant as well as the exposure time θ.

2.4 Research pathogens

For the scope of this research it was important to select bacteria that are pathogenic or can be representative of a pathogenic bacterial strain. *Flavobacterium columnare* and non-pathogenic *Escherichia coli* were chosen since they are present in both natural and wastewaters. These organisms are also classified as Bio-Safety Level 1 (BSL1) organisms allowing for less stringent laboratory protocols required for experimentation. Both organisms are also easily cultured in the laboratory requiring inexpensive media for both culture and enumeration.

2.4.1 *Flavobacterium columnare*

F. columnare is a gram negative rod shaped aquaculture pathogen having cells that are 0.5 µm in diameter and 4-12 µm in length and capable of gliding movement through its environment (Thune et al., 1993; Figure 2.5). As reviewed by Groff and LaPatra (2000), outbreaks of Columnaris diseases that are usually the result of environmental stresses that weaken and expose fish to the disease are a result of conventional aquaculture production practices. Environmental stresses include high ammonia and nitrate concentrations, low dissolved oxygen, high stocking density and elevated water temperature above 15 °C. The disease affects only freshwater fish including catfish and tilapia, catfish being a more economically important species to the southeastern United States (Durborow et al., 1998; Amin et al., 1988).

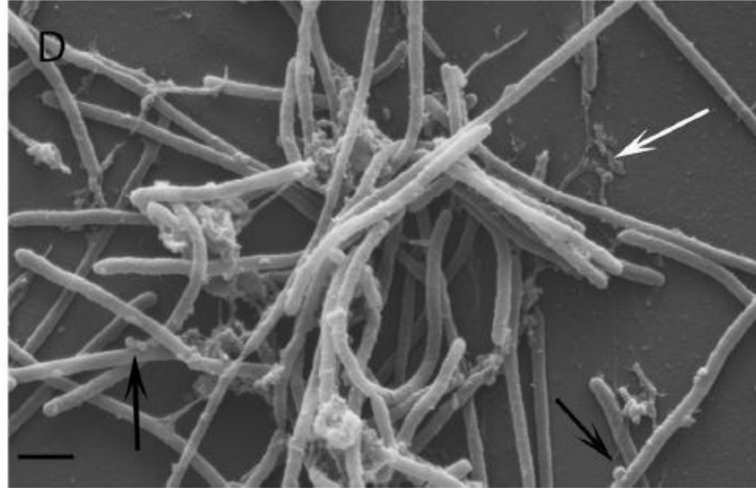

Figure 2.5. Scanning electron microscope image of the long rod shaped cells of F. columnare attaching to glass slides. Scale bar is 1 µm. Arrow bars indicate EPS secretion. Image taken from Cai et al. (2013).

The research of Cai et al. (2013) suggest that while water bodies are the main reservoir of *Flavobacterium columnare*, the environmental source of the pathogen probably originates from biofilm formation on surfaces within the water body. Not only will *F. columnare* form biofilm on natural surfaces but also the surfaces of man-made materials such as glass and plastic (Cai et al., 2013)

2.4.2 *Escherichia coli*

For monitoring of the efficiency of the water and wastewater treatment process indicator microorganisms, typically fecal coliforms and *Escherichia coli* (*E. coli*), are used since they represent the level of contamination of the water and the possibility for the occurrence of more serious pathogens. *E. coli* are an enteric, gram negative rod shaped bacteria typically 2.0 µm in length and 0.5 µm in width (Bionumbers, 2010). These bacteria area also consistently present in feces, which gives more reliability for detection in water and wastewater (Bitton, 1999). In particular, *E. coli* has been shown to be the most reliable indicator of fecal contamination since it appears in high concentrations in all mammals, detection methods are inexpensive and persists

long enough in the environment to allow for the use of standardized testing procedures (Edberg et al., 2000). *E. coli* is easily detectable on selective media that prevents background levels of environmental bacteria from creating too much noise on plated samples.

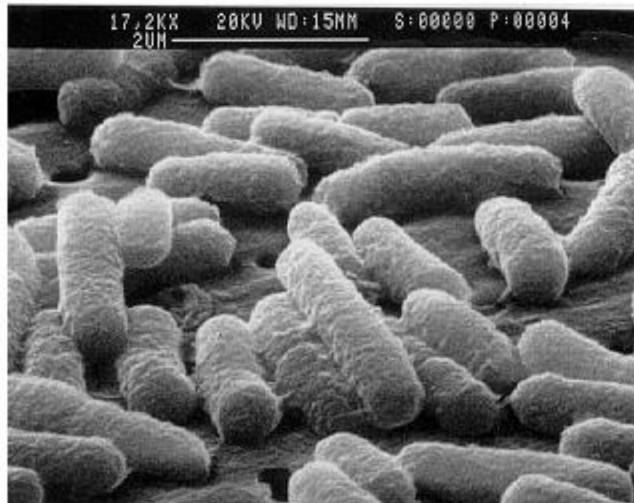

Figure 2.6. Scanning electron microscope image of E. coli cells. Scale bar is 2 µm. from The effects of K1F bacteriophage on the EV36 strain of E. coli, by Williard, C. (1998). Retrieved from http://www.optics.rochester.edu/workgroups/cml/me111/sp98-projects/courtney/.

Extensive research has been conducted on the removal of pathogenic microorganisms in water and wastewater using *E. coli* as an indicator organism. The catalog of research on *E. coli* and wastewater treatment allows for prioritization of variables for control during experimentation. Parhad and Rao (1974) showed that the growth rate of *E. coli* is negatively affected by high pH value independent of the presence of lab-cultured algae, *Chlorella*, in waste stabilization pond effluent. They first observed the relationship between pH of wastewater and the growth rate of *E. coli* and noticed at pH values above 9.2 *E. coli* growth ceased. They also observed in actual stabilization ponds that *E. coli* was never completely eliminated from the diurnal high pH variation as the cell count of *E. coli* recovered during the low pH of night. Curtis et al. (1992) observed the interaction of enteric pathogen die-off as a function of light influenced by pH, dissolved oxygen and humics in waste stabilization ponds, which experience elevated pH

and DO levels due to algal photosynthesis. Their observations also indicated a significantly greater die-off of fecal coliforms at pH values greater than 8.5 for treatments under sunlight and pH 9 for dark treatments. This information suggests pH control will probably be necessary to limit the influence of pH on the rate of die off in the treatments with algae.

Chapter 3: Methods

3.1 Laboratory scale algal turf scrubber

The investigation of the research question required scaling the ATS down and bringing inside the laboratory where environmental variables can be more closely controlled and easily monitored. The design of the lab scale ATS presented below attempts to replicate the design and operating characteristics of pilot and landscape scale ATS such as the periodic wave pulse and the long narrow channel. All together four lab scale ATS or reactors were created to separate the proposed mechanisms of pathogen removal stated in the previous sections. Two morphologically distinct pathogens were selected for experimentation and several preliminary experiments were also conducted in order to gauge some of the parameters of the main experiment.

At the time of this research there are no freely available plans for a lab scale ATS that emulate the high length to width channel ratio and the periodic wave pulse of pilot and landscape scale systems. One lab scale system that is used for both indoor and outdoor algae cultivation is the 1 m^2 fiberglass trough with a tipping bucket (Berner et al., 2015; Mulbry et al., 2010). These units provide a periodic surge through the tipping bucket but fail to emulate the high length to width ratio of landscape ATS as well as wave attenuation along the length of the ATS channel. Another lab scale reactor used is the incubator design, which does have a high length to width ratio for channels but does not provide the means for a periodic wave pulse (Zippel et al., 2007; Guzzon et al., 2008; Rains and Blersch, 2015). Also this design is not presented in a detail that allows for exact replication. It was therefore necessary to design and build a set of reactors that

would emulate the long narrow channels of larger scale ATS as well as the periodic wave pulse. It was decided that the design should be low cost and parts should be available at local hardware stores. The design was formulated around the idea of using a symmetrical residential vinyl rain gutter that would emulate the high length to width ratio of large scale ATS. Complete design details of the lab scale ATS along with a pilot growth study for validation of algal growth is provided in Appendix A. Briefly, the channel of the ATS measured 305 cm x 8.9 cm and set on a pivot that allowed for slope adjustment; the channel was set at a 2% slope by default. The substrate used for attachment of benthic algae was 1/8" UV stabilized polyethylene bat house netting (Industrial Netting, Minneapolis, MN) which was installed in each gutter. The illuminated substratum available for algal growth measured 244 cm x 8.9 cm (2172 cm^2).

3.1.1 Temperature control

The lab scale ATS was built and housed in the basement of the Corley building (Room 109) which at the time was not heater and only air-conditioned. For the main experiment it was necessary to maintain the reservoir water at 26 °C which could not be accomplished during colder weather without heating the reservoir water (Figure 3.1). An inline heater was built using a 25-watt EHEIM adjustable thermostat controlled aquarium heater (Model No. 3611090, EHEIM GmhH & Co.) to more efficiently transfer heat to the system water as it flowed from the pump to the reactor plumbing (Mullen, 2015). In conjunction with the heat from the fluorescent lighting, this method of heating was successful at maintaining temperature within ±2°C of 26 °C during cooler months. However, the inline heaters were sensitive to changes in the environment and had to be adjusted depending on the temperature of the basement at the time of the experiment. The inline heaters were also only effective within a narrow range of environmental temperatures, i.e., if the basement was below 16 °C the heaters were not capable of maintaining

the reactor reservoirs at 26 °C. However, the bulk of experimental data collection occurred after the coldest months of the year.

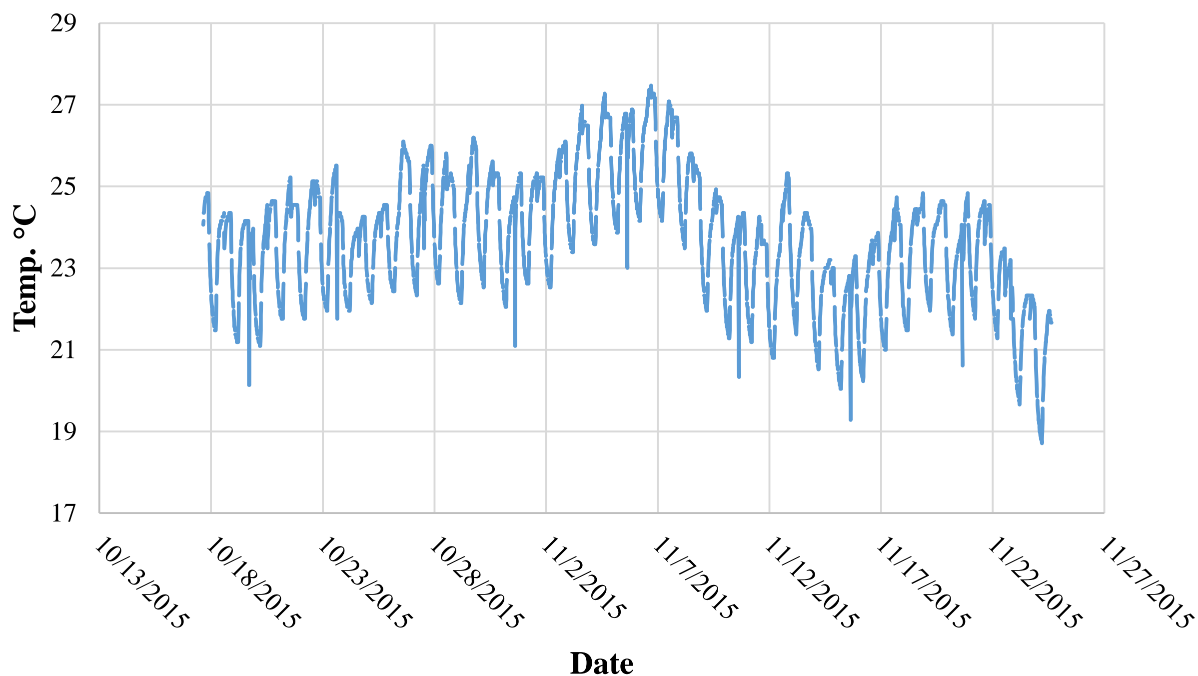

Figure 3.1. Data logger output of reservoir (water) temperature showing daily and week to week variations in reservoir temperature for a reactor before adding an inline heater.

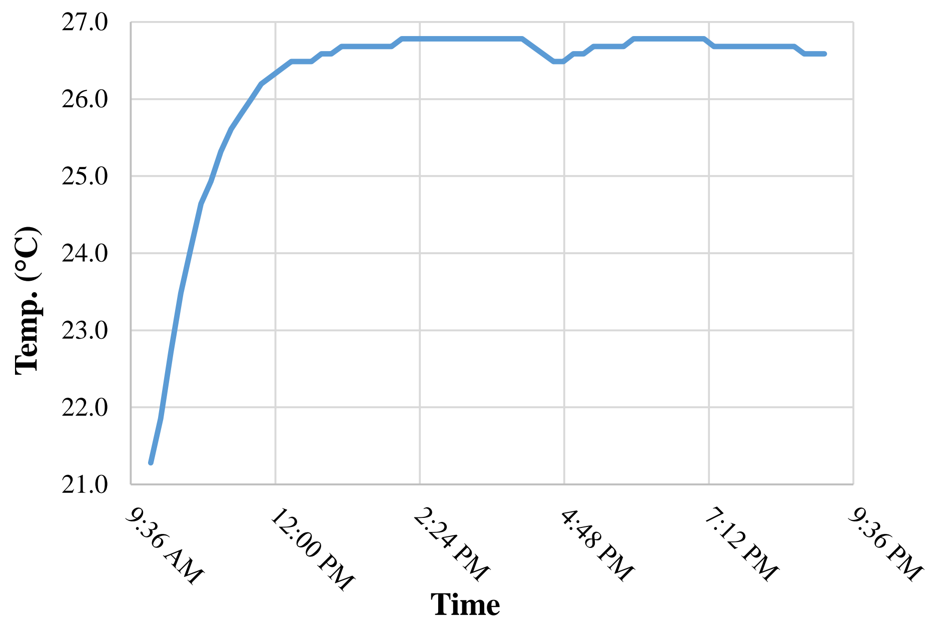

Figure 3.2. Data logger output of temperature data for a reactor reservoir after powering on an inline heater during February 2016. For this test, ambient water temperature was initially at 21°C. The inline heater maintained a steady state temperature in the reactor reservoir o 26.7 °C (±0.1°C).

3.1.2 Lighting

For this research a separate lamp configuration was used for growing the algae and for data collection during pathogen removal experiments. This was done to decrease project cost since the UV supplemented Sunsource lamps were more than double the lamps used to grow the algae. The configuration used for growing the algae prior to the experiment utilized 4 Philips 32W fluorescent Alto II TL741 lamps that provided the spectral output in Figure 3.3. All collected spectral data was measured using a Stellarnet Black-Comet UV-VIS spectrometer (Stellarnet, Inc.). Note that in Figure 3.3 the output of the Philips lamp provides an incomplete source of light by not providing for one of the peak absorbance wavelengths of both chlorophyll a and b. Although the Philips lamps do not provide the highest quality light for growing algae they are the most inexpensive option and have been used by researchers in the past (Tong et al., 2008).

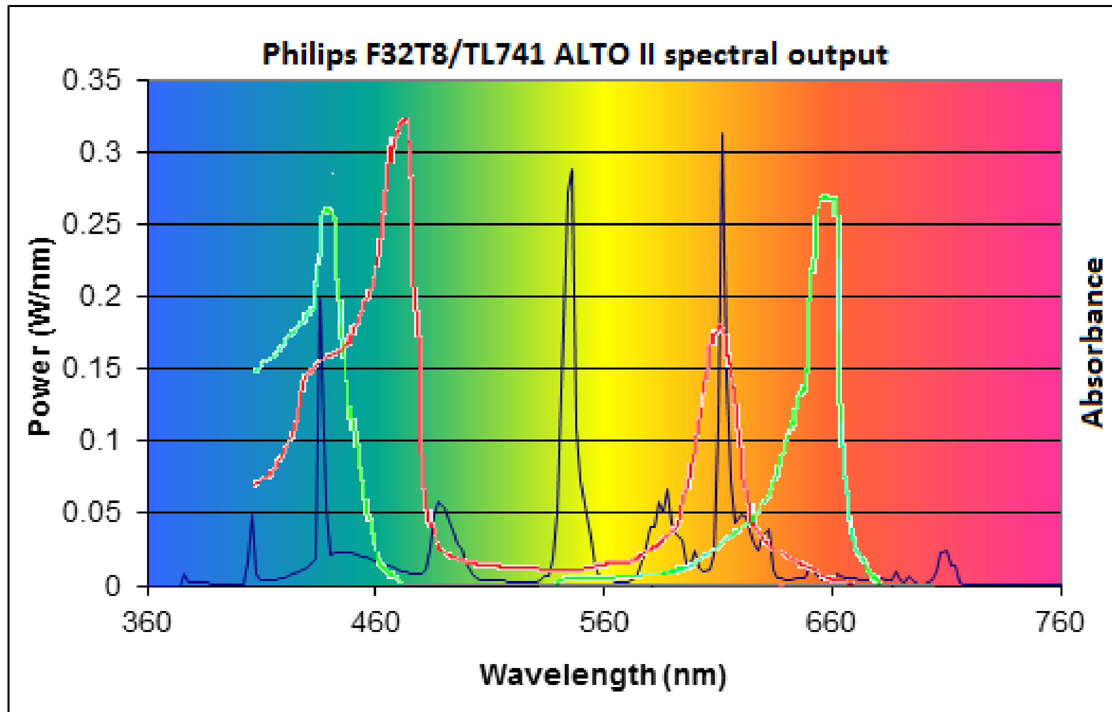

Figure 3.3. Spectral power output of the Philips 32W T8 TL741 lamps used to grow algae with approximate (not to scale) wavelength absorbance peaks of chlorophyll-a (green line) and chlorophyll-b (red line). Spectral data provided by Koninklijke Philips N.V.

UV light during the experimental trials was supplemented using Sunsource Heliovite UV 32W T8 fluorescent lamps (Everyday Green, LLC) that produced the spectral irradiance in Figure 3.5. Initially, a Zoo Med Reptisun UV-B 5.0 32W T8 lamp (Zoo Med Laboratories, Inc.) was evaluated for use since a Zoo Med Reptisun UV-B 10.0 32W T8 lamp was used by Beckinghausen et al. (2014) to study bacterial associations with *Cladophora*. The Reptisun 5.0 lamp provided the same UV output as the Heliovite lamp but with a lower PAR. The Heliovite lamp was chosen since it provided ample PAR so that photosynthesis would be optimal during the experiment.

The lighting configuration for each light fixture used during the experiment consisted of one Philips TL741 lamps and one Sunsource Heliovite fluorescent lamps (Figure 3.4). For treatments without UV, fluorescent tube filters were installed on the Heliovite lamps to remove

most of UV-B and a majority of UV-A light (FS10 UV light filter; Ergomart, Dallas,TX). However, the filter marginally reduced the output across the spectrum that would require light fixtures without filtered lamps to be raised an additional 0.6 cm above the reactor channel than filtered UV lamps in order to match spectral irradiance for the experiment trials (Figure 3.5).

Figure 3.4. View of a light fixture used to house the Phillips cool white lamp (top) and (filtered) Heliovite UV lamp (bottom).

The combined spectral irradiance of these two lamps provided the UV spectrum desired to simulate natural sunlight while also providing adequate PAR for algae cultivation; however in comparison to natural sunlight the lamps provided less output (Figure 3.6). This might suggest that the algae growth rate could be lower than natural systems as well as the disinfection rate due to UV radiation.

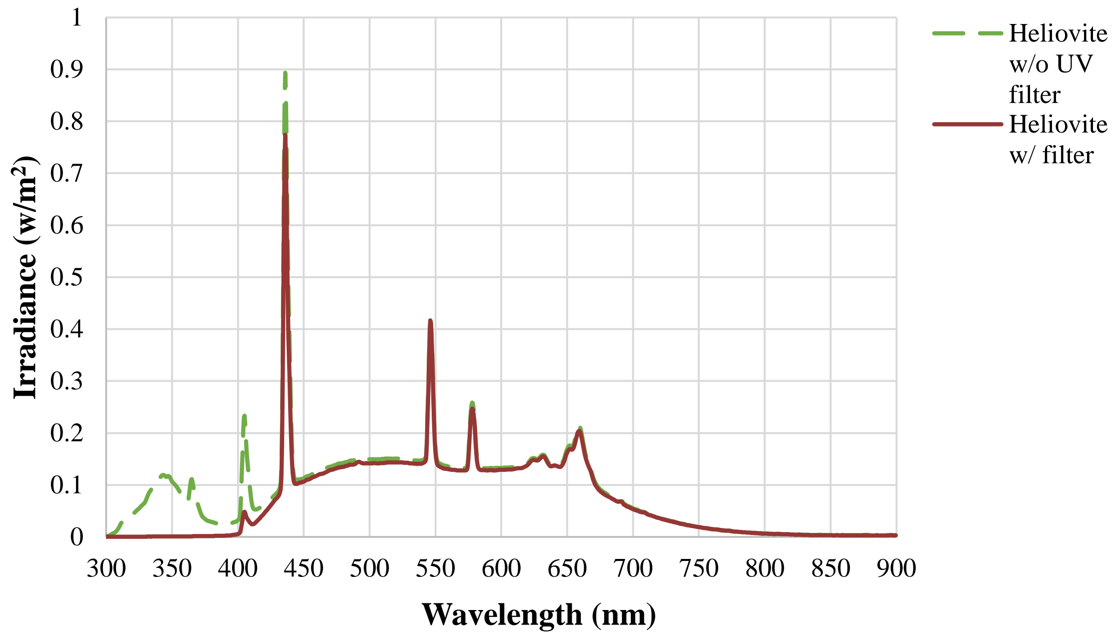

Figure 3.5. Spectral irradiance of UV supplemented and UV filtered light using combined Philips and Sunsource Heliovite lamps showing almost complete filtering of the UV-B and UV-A spectrum. Note: the irradiance measurement for filtered light was taken at 5 cm from the light source while the unfiltered light was taken at 5.7 cm from the light source in order to correct for power reduction from the UV filter.

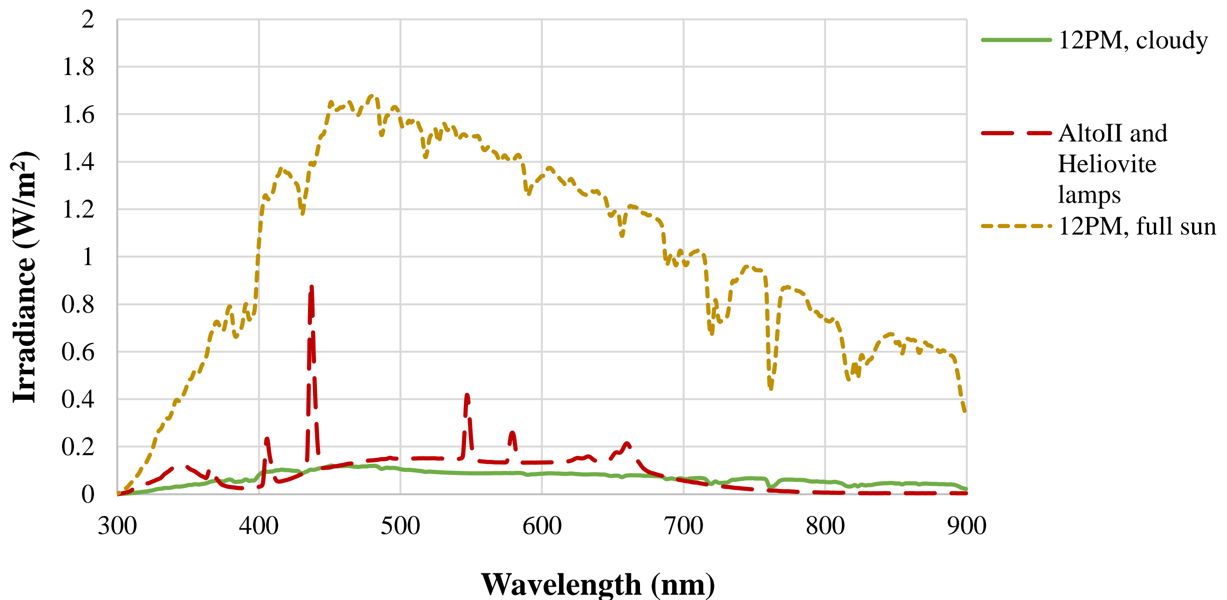

Figure 3.6. Comparison of spectral irradiance of the Sunsource Heliovite lamp configuration with full sunlight and cloud cover observed on a spring day at noon in the courtyard of the Corley building at Auburn University, Auburn, AL.

3.2 Bacteria cultures

After researching it was determined that *Flavobacterium columnare* would be the best aquaculture pathogen for experimentation since it can be grown and identified on inexpensive media (Modified Sheih) and produces a distinct golden colony when plated. These bacteria are also characteristically long and originate in aquatic environments suggesting a cellular adaptation to low levels of UV radiation. Most importantly *F. columnare* is a serious pathogen for catfish and tilapia and also has a strong tendency to form biofilm on both natural and manmade materials (Cai et al., 2013).

It was also decided that in order to relate to pollutant remediation of natural water and wastewater treatment, *E. coli* should also be tested for removal. Non-pathogenic E. coli strains can serve as an analog to the behavior of pathogenic bacteria found in nature and have previously been used to study removal during water treatment (Zhang and Oyanedel-Craver, 2013). Similar to *F. columnare, E. coli* was selected for its ease of cultivation, enumeration and relevance to water resources.

3.2.1 Flavobacterioum columnare **culture**

Flavobacterium columnare was chosen as an organism of study for the experiment for several reasons. First, Columnaris disease, which is caused by *F. columnare*, is an important freshwater aquaculture pathogen and one of the leading causes of mortality in farmed catfish in the southeastern United States highlighting its significance to an agricultural product of the region (Durborow et al., 1998). Second, *F. columnare* is a biosafety level 1 (BSL 1) organism posing no exposure risk to human health due which is important since the ATS reactors would produce aerosols due to splashing of water in the system. This organism is also easily discerned

from other bacteria when plated on Modified Sheih agar media resulting in a distinct golden colony (Decostere et al., 1997).

The isolated culture of *Flavobacterium columnare* BG27 was obtained from the laboratory of Dr. Cova Arias at Auburn University and continuously passed between 50 mL disposable centrifuge tubes every two days. These cultures were maintained on approximately 7.5 mL of Modified Sheih (MS) broth in a 28 °C incubator using a shaker plate to provide continuous mixing at 150 RPM. The cultures were passed every two days and periodically streaked for isolation to check for contamination. Glycerol stocks of the culture were maintained at -80 °C in case of culture contamination or failure. Periodically the continuous cultures were disposed of and new cultures started from the glycerol stocks in order to maintain genetic integrity of the strain.

3.2.2 Escherichia coli culture

E. coli DH10B was obtained from the laboratory of Dr. Mark Liles at Auburn University. This strain of *E. coli* is non-pathogenic and designated as a BSL 1 organism allowing for use during the experiment. *E. coli* cultures were maintained in a similar manner to the *F. columnare* cultures. The *E. coli* culture was continuously maintained on LB agar plates streaked from overnight Luria-Bertani (LB-Luria) broth cultures. Glycerol stocks from a broth culture grown from the original agar plate provided by the laboratory of Dr. Mark Liles were maintained at -80 °C.

The advantage of using this strain of *E. coli* was its resistance to the antibiotic Streptomycin allowing for an additional level of screening of background bacteria in plated samples. The use of the Streptomycin in conjunction with MacConkey agar, which is selective for enteric bacteria, allowed for removal of a majority of background bacteria in sampled

reservoir water. The DH10B strain does not ferment lactose and did not allow for differentiation on the MacConkey agar but this was not an issue due to the almost non-existent presence of other coliforms. The MacConkey agar does not allow for complete recovery of all bacteria in a sample. It was observed that serially diluted lab cultured *E. coli* plated on MacConkey agar had an average of 0.63 (± 0.21) orders of magnitude fewer colonies compared to the same culture plated on LB-Luria agar. This is most likely due to the limited availability of other carbon sources in the media compared to lactose, which DH10B is unable to utilize.

3.2 Algal culture

The algal culture used to colonize the reactors was acquired from an outdoor pilot scale ATS unit receiving wastewater from an indoor bio-floc tilapia system at the North Auburn Fisheries Unit. Sections of the plastic bat netting used in the lab scale ATS were placed in the pilot scale ATS for several weeks until the sections of netting were colonized by filamentous algae. These sections were then transferred to a reactor in the lab, and the algae were allowed to colonize the full length of illuminated netting in the lab reactor (244 x 7.6 cm grow area). The mixed culture of algae was maintained using Proline F/2 algae food (Pentair Aquatic Eco-systems, Inc., Cary, NC) in dechlorinated municipal tap water instead of sourceable aquaculture wastewater. This was done to avoid the fluctuations in nutrient concentrations that had been seen in sampled wastewater as well as avoiding the hassle of transporting gallons of wastewater to the lab. Proline F/2 is the same formula developed by Guillard and Ryther (1962) that provides approximately 55 mg/L nitrate (NO_3-N) and 3.44 mg/L phosphate (PO_4-P) at the recommended concentration.

At the start of the main experiment for pathogen removal the substratum had already been colonized for months. Haphazard sampling of the substratum of both reactors showed that both

reactors had the same major species of algae. Light microscopy was used to identify the major filamentous species present in the samples as well as suspended algae species using a guide for freshwater algae identification (Prescott, 1978). The filamentous algae were dominated primarily by *Oedogonium*, *Rhizoclonium* and *Sirogonium* (Figure 3.7). Major suspended algae species were identified as *Scenedesmus, Closterium* and *Oocystis.* There were several other suspended algal species present in lesser numbers but were not identified.

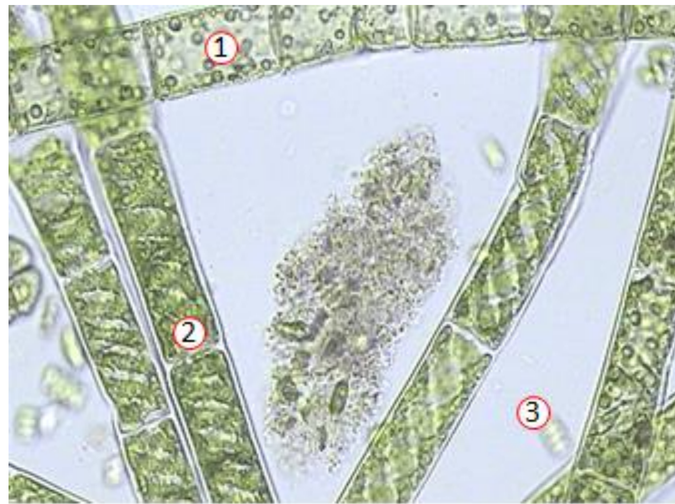

Figure 3.7. 40X magnification of sampled algal culture showing Oedodgonium (1), Sirogonium (2) and Scendesmus (3).

Although F/2 was the easiest and most economical media for growing algae in the lab it should be noted that F/2 is probably not the best media for the growth of benthic algae since it was developed for the cultivation of marine planktonic algae. During the study ‘greening’ of the reservoir water was periodically witnessed due to the growth of suspended algal species but did not appear to drastically inhibit benthic algal growth. Measures to halt suspended algal growth were not successful and included large hydraulic residence time in the reactor and lowered nutrient concentrations. The best management strategy for suspended algae was to periodically replace all of the system water. Also it was necessary to periodically flush the gutter and netting with dechlorinated tap water to remove a buildup of epiphytic or attached suspended algae and

organic matter that would coat the benthic algae. Briefly, the dechlorinator was a 0.1M solution of sodium thiosulfate crystals (Proline dechlornator sodium thiosulfate, Pentair Aquatic Eco-Systems, Inc.) that was kept refrigerated when not in use. If this was not done benthic algal growth would be drastically reduced since light and nutrient diffusion was decreased due to blockage from the algae and organic matter.

During the main experiment for pathogen removal a bleach solution was regularly used to disinfect the reactors at the end of the experiment for residual bacteria. This also served to disinfect the reactors of any suspended algae and algal spores greatly inhibiting the growth of suspended algal species during the nine-day turf growth cycle. Although suspended algae were still present in algae samples were observed using a light microscope, the reservoir water would remain relatively clear during the 9-day growth period for the algae. No optical absorbance data was collected on the daily change in water clarity.

3.4 Preliminary experiments

Several preliminary experiments were conducted to gauge some of the parameters to be used during the main experiment. These preliminary experiments were used to determine whether the UV light source would inhibit algal growth, what water would be used for the experiment and effects of water quality parameters on die-off. All data were analyzed using ANOVA with Tukey's multiple comparisons to detect variances among treatments. Linear regression was also used to gauge the relationship in concentration of nitrate and die-off. All statistical analyses were conducted using an alpha of 0.05.

3.4.1 Algae growth under UV supplemented light

UV-B and UV-A may have the ability to inhibit algal metabolism and possibly damage cellular structures; however, some algal species do have protective mechanisms against UV

radiation and may adapt to higher intensity light environments (Hill, 1996). This inhibition might affect the efficiency of pathogen removal since less oxygen would be produced by the algae leading to reduction in the evolution of photosensitizers. To better understand whether or not the algal turf in the algae+UV treatment would be affected by the UV supplemented light, a growth experiment was conducted over five harvests (Figure 3.8).

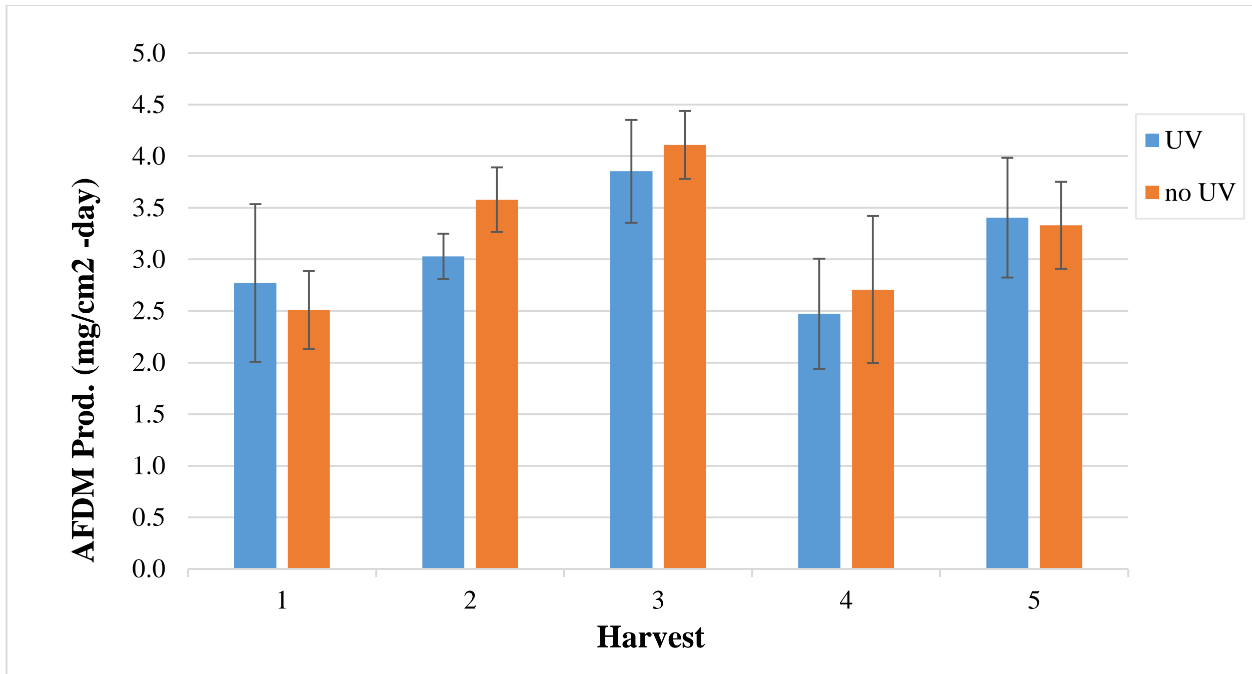

Figure 3.8. Mean and standard deviation of Ash Free Dry Mass (AFDM) productivity of harvested algae between algae grown under UV supplemented light and filtered UV light. Error bars represent standard deviation of productivity from algal turf sampled in triplicate during each harvest.

The treatment light fixtures used one Philips cool white lamp and one Sunsource Heliovite UV lamp. The control utilized the same configuration only the UV lamps were housed in filters to remove the UV portion of light. The algae was grown under a 16/8 diurnal for five days and received one half of the recommended concentration of Proline F/2 media in dechlorinated tap water. At each harvest the two reactors were harvested and processed for Ash Free Dry Mass (AFDM) and % Ash according to Steinman et al. (2007). Data was translated to a productivity value in mg/cm^2-day. To remove harvest-to-harvest variation due to the effect of

prior harvest or environmental variables, Equation 5 was used to calculate the subsidized value of UV productivity for statistical analysis using a one sample T-test ($H_0 = 0$). The T-test results indicated that the subsidized UV productivity was not significantly different that the control productivity without UV (P = 0.4790; Appendix C.1)

$$Subsidized\ AFDM = \frac{NO_UV - UV}{NO_UV} \tag{5}$$

3.4.2 F. columnare die-off in wastewater

It was determined that F/2 would be the best growth medium for use during the experiment since it would allow for consistent water quality parameters which could not be guaranteed using raw aquaculture wastewater. Also, the background bacteria level in raw wastewater would more than likely increase the difficulty of distinguishing *F. columnare* colonies at lower dilutions and could introduce wild strains of *Flavobacterium*. Preliminary experiments were conducted to test whether or not F/2 supplemented water would enhance the die-off of bacteria over aquaculture wastewater which could exaggerate removal of *F. columnare* during experimental trials. Testing required measuring the die-off rate of *F. columnare* between solutions of F/2 at manufacturer's recommended with dechlorinated tap water concentration and aquaculture wastewater under dark and UV supplemented light conditions. Also, it was considered that the organic materials generated by the algae might also influence die off so an additional solution consisting of F/2 and recirculated water from the reservoir of the gutter reactor was used. In total three 'wastewaters' were compared: F/2 in dechlorinated tap water, F/2 in water taken from the laboratory scale ATS and raw aquaculture wastewater from the tilapia greenhouse at North Auburn Fisheries Unit. Water quality parameters for the tested waters are presented in Table 3.1. Total organic carbon was measured using a total organic carbon analyzer

(TOC-L, Shimadzu Corp., Kyoto, Japan). The remaining parameters were measured using a YSI 9300 photometer (YSI, Inc.).

Table 3.1. Water quality parameters of the three waters tested for dark and UV die-off of F. columnare.

	Settleable solids (mL/L)	Turbidity (NTU)	TOC (mg/L)	NO_3-N (mg/L)	PO_4-P (mg/L)	Alkalinity $CaCO_3$ (mg/L)	Hardness $CaCO_3$ (mg/L)	Total Iron (mg/L)
AWW	45	200	28	312	33.0	56	130	0.40
F/2+algae	-	123	47	55	3.4	100	75	0.43
F/2	-	0	-	55	3.4	90	75	0.43

The following culturing method was used for die-off experiments. A modified Shieh agar plate was streaked for isolation using a 48-hour stock culture. This plate was then incubated for 48 hours before an isolated colony was collected and used to inoculate 5 mL of MS broth in a 50 mL centrifuge tube. This culture was then grown for 24 hours to late exponential phase growth at 150 RPM on a shaker table in a 28 °C incubator. After 24 hours, 1 mL of the culture was pipetted into a microcentrifuge tube and centrifuged at 3000g for 5 minutes to form a pellet (Arias et al., 2012). The pellet was then washed with a 1x phosphate buffered saline (PBS) solution before being resuspended in 6 mL of PBS. One mL of the bacteria suspension was then used to inoculate 9 mL of a given wastewater tube.

The spiked wastewater tubes were placed in a photo-incubator (VWR Signature Diurnal Growth Chamber Model 2015, VWR International, LLC) at 28 °C for the duration of the experiment (16 hours for the UV trial, 35 hours for dark trial). For dark die-off conditions, no lighting was provided. For UV supplemented light conditions, two Sunsource Heliovite UV lamps and 2 Phillips TL741 lamps were installed in the photo incubator. Also, the plastic 50 mL centrifuge tubes used in the dark die off experiments were replaced with glass test tubes to allow for light transmission to the wastewater. In hindsight, quartz glass tubes should have been used

to limit the reduction in transmittance of UV light through the glass. The test tubes were aligned side by side in a test tube rack and placed 2 inches from the photo incubator light source similar to the distance of lamp placement in the lab scale ATS during the main experiments.

The dark and UV die off of *F. columnare* was measured for each of the three wastewaters by serially diluting each periodic sample and plating in triplicate on MS agar plates (Figures 3.9 and 3.10). Figures 3.9 and 3.19 show that over time the concentration of active bacteria in the aquaculture wastewater declines at a faster rate compared to the F/2 treatments. The treatment using solely F/2 had the slowest die-off among all three treatments. Overall the solutions using F/2 algae food did not enhance the die-off of *F. columnare* compared to aquaculture wastewater and served to maintain growth for a short amount of time after the start of the experiments. While the F/2 media does not enhance the die-off rate of *F. columnare* compared to the aquaculture wastewater it still cannot be considered as having similar kinetics. This suggests that the results of the main experiment using F/2 will show an overall lower log reduction in bacteria than what might be possible with actual wastewater. Again, the main reason to use F/2 was to allow for a lower variance in water quality parameters that cannot be achieved using real wastewater.

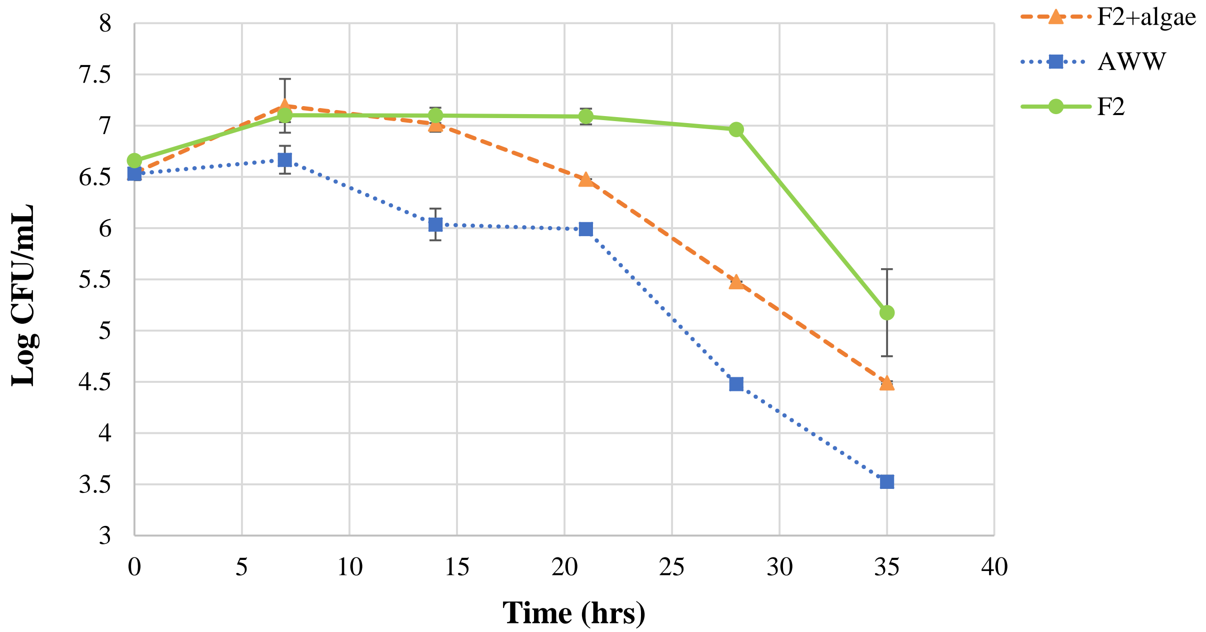

Figure 3.9. F. columnare die-off among three wastewaters under dark conditions. AWW is aquaculture wastewater, F2 is dechlorinated tap water with F/2 algae food and F/2+algae is F/2 algae food added to dechlorinated water containing organic matter from algae. Error bars are standard deviation (n=3)

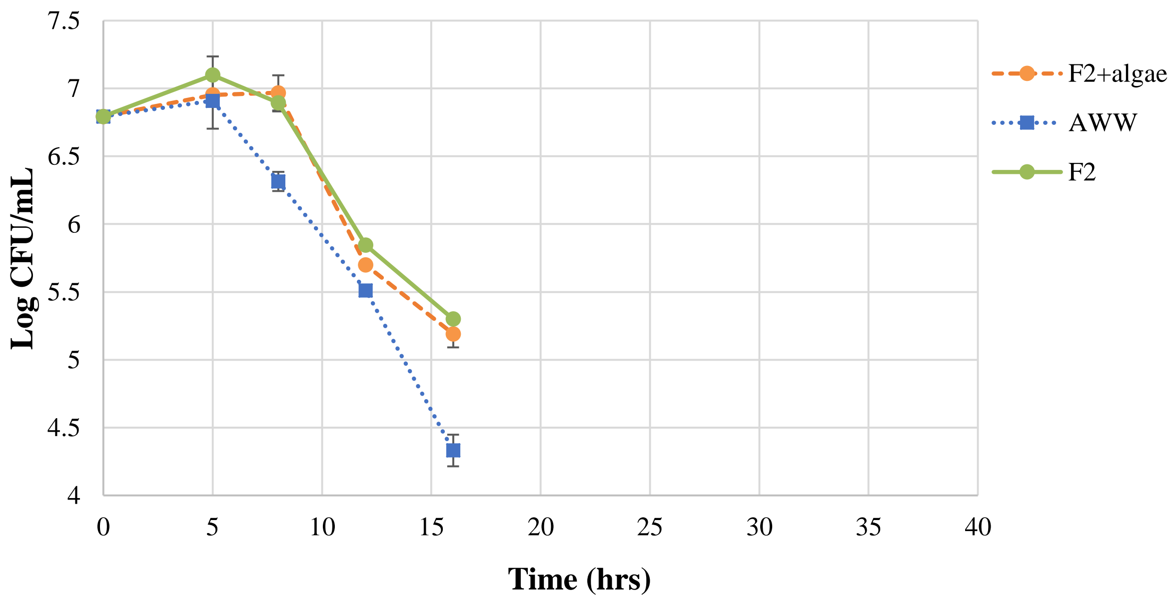

Figure 3.10. F. columnare die-off among three wastewaters under UV supplemented light conditions. AWW is aquaculture wastewater, F/2 is dechlorinated tap water with F/2 algae food and F/2+algae is F/2 algae food added to dechlorinated water containing organic matter from algae. Error bars are standard deviation (n=3).

It became apparent that during the experiment that the nutrient concentration will vary between algae and no algae treatments due to assimilation of the nutrients by the algae. Controlling for this in the experimental system would require dosing pumps dispensing the F/2 solution that would allow for a constant nutrient concentration matching no algae treatments. To avoid the excess cost and labor for an array of dosing pumps, laboratory experiments were conducted to observe *F. columnare* die off at varying nutrient concentrations while holding pH and conductivity constant. These experiments were conducted for UV supplemented light and dark die off conditions. The *F. columnare* spiked wastewater tubes were placed in a cardboard enclosure within the incubator to block reflected light in order to emulate the light source in the lab scale ATS. A salt solution to equalize conductivity (EC) was created by evaporating filtered reservoir water from the lab scale ATS that had not been dosed with F/2 algae food for several days resulting in uptake of all available nitrogen and phosphorus. Traces of nitrogen and phosphorus were not detectable in the solution at the detection limit (0.2 mg/L nitrate; 0.03 mg/L phosphate) using a YSI 9300 photometer (YSI, Inc.). The reservoir water was filtered to remove organic material and algae before drying at 105 °C for 24 hours. After drying for 24 hours, the residue was then combusted at 575 °C for three hours to burn off any remaining organics. The remaining ash was then resuspended in DI water and used to equalize the conductivity between treatments. The nutrient concentrations evaluated for this experiment were F/2 at 0, 0.3x, 0.6x, 0.9x, and 1.2x the recommended dosage of F/2.

Figures 3.11 and 3.12 indicate that there is no significant correlation between the reduction of *F. columnare* and the concentration of F/2 under both dark and UV supplemented light conditions (Dark: $R^2 = 0.0415$, $P = 0.4666$; UV: $R^2 = 0.0761$, $P = 0.3196$). F/2

concentration was then manually maintained in the algae reservoirs by adding an amount of F/2 every 6 hours equivalent to the uptake rate for the 6 hours.

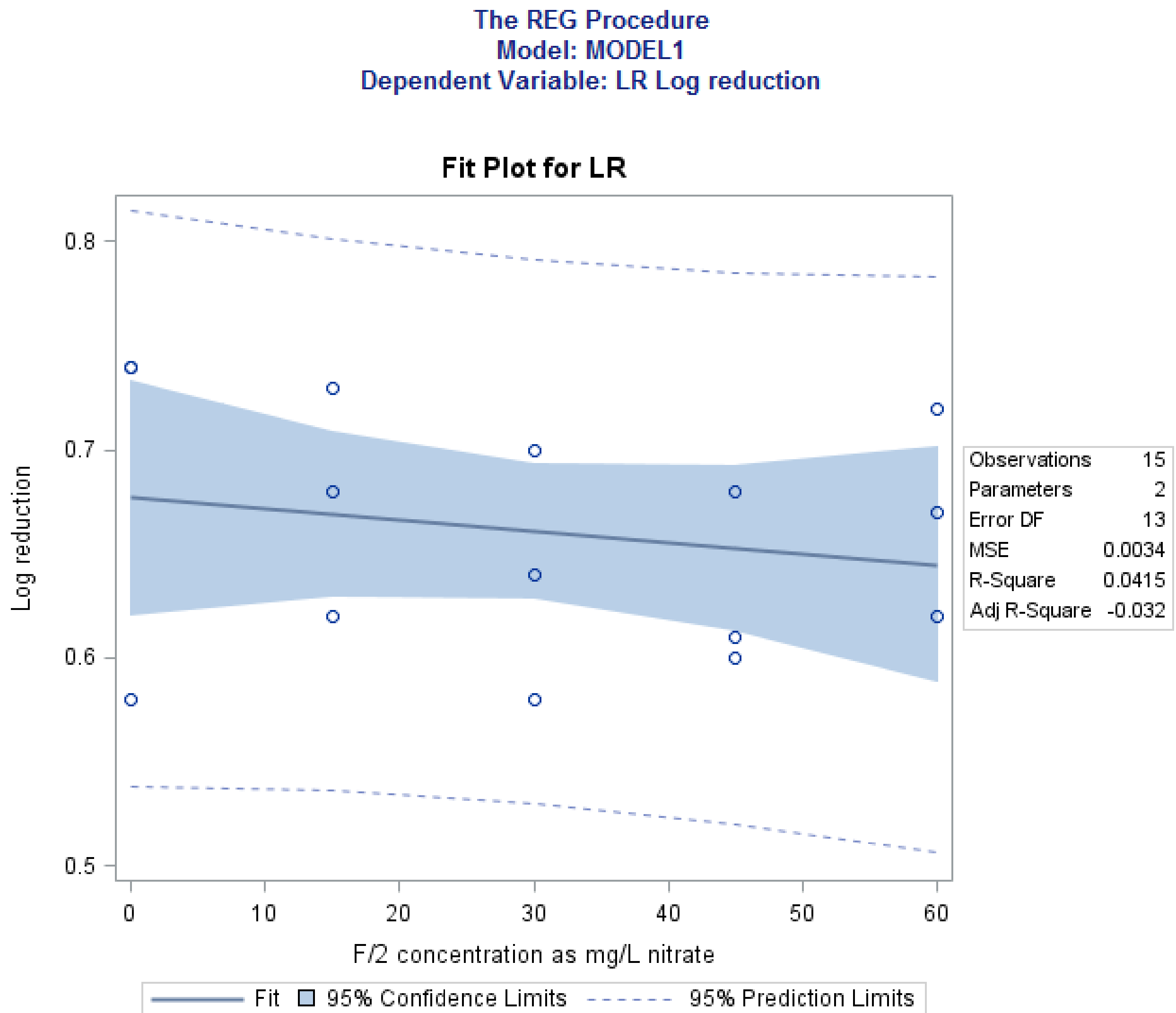

Figure 3.11. Log reduction of F. columnare over a 24 hour period in dark conditions as a function of F/2 concentration.

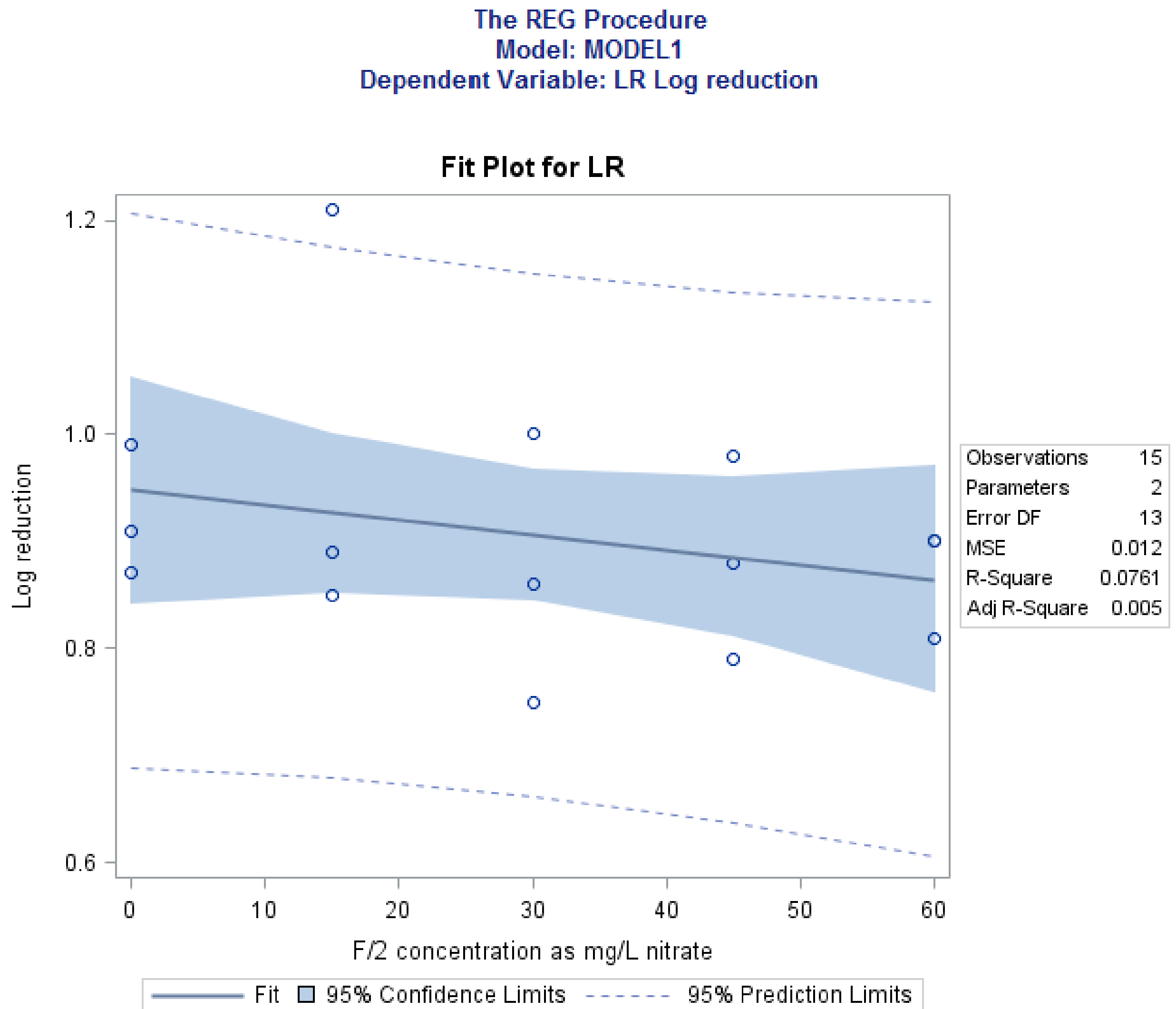

Figure 3.12. Log reduction of F. columnare exposed to UV supplemented light over a 12 hour period as a function of F/2 concentration.

3.4.3 F. columnare removal with humic acid supplementation

As stated by Curtis et al. (1992) the presence of humic acids in Waste Stabilization Ponds (WSPs) water are responsible for a majority of the solar mediated disinfection that is observed in these treatment systems via exogenous photosensitizers in the presence of UV-A. After completing the removal experiments with *F. columnare* it was decided to test whether or not the same response to humic acid could be seen in the lab scale ATS. A preliminary removal

experiment utilizing all four reactor treatments was conducted in which lab grade humic acid (Beantown Chemical, 130430-5G) was added at a concentration of 10 mg/L representative of surface water and wastewater (Asano et al., 2008; Rodrigues et al., 2009). All reactor operating parameters and procedures were followed exactly as the ones established for the main experiment except a humic acid stock solution was used to provide a concentration of 10 mg/L humic acid typical of surface waters (Rodrigues et al., 2009). Figure 3.13 shows that the addition of humic acid did not increase the overall removal for a mature turf exposed to UV light and retarded removal for a harvested turf under surge conditions (Figure 3.14). However, the UV treatment without an algal turf in Figure 3.13 did show a 1-log difference in removal compared to the treatment without UV after 6 hours. This may likely be due to sampling error since the same behavior was not observed for the treatments with algae and since the reduction appears to be a one-time event noting that no additional removal was seen after 12 hours. With this preliminary data it was decided that no further experimentation with humic acid supplementation would be conducted since the output of the UV lamp was likely not powerful enough to produce a significant effect.

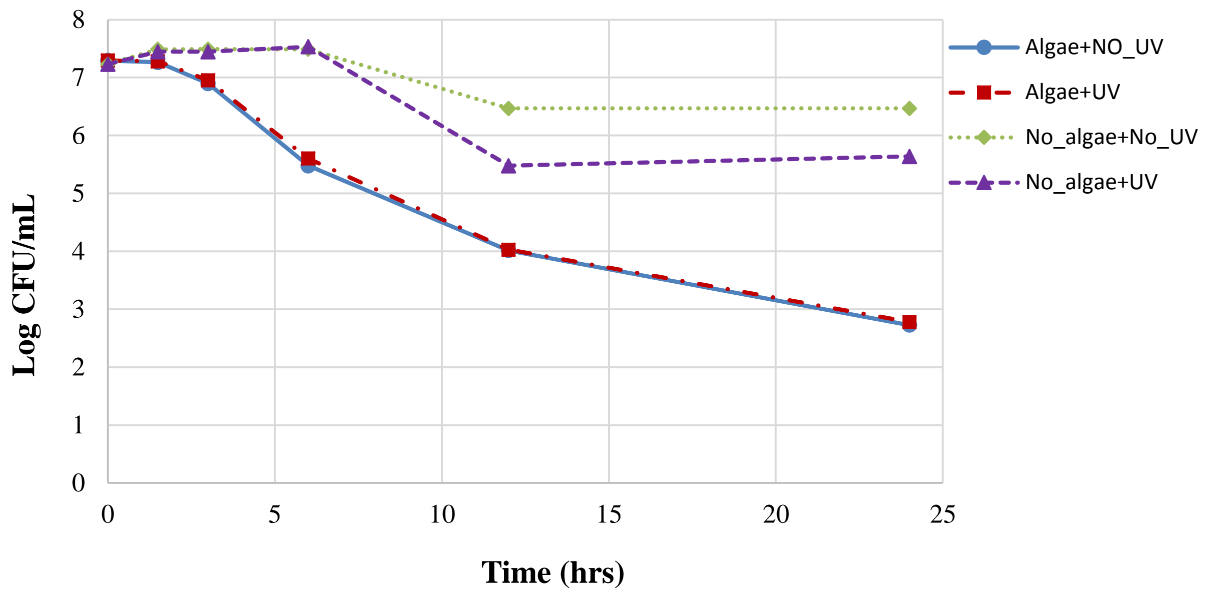

Figure 3.13. Log CFU/mL F. columnare vs time with humic acid supplementation for mature turf under surge conditions (n=1).

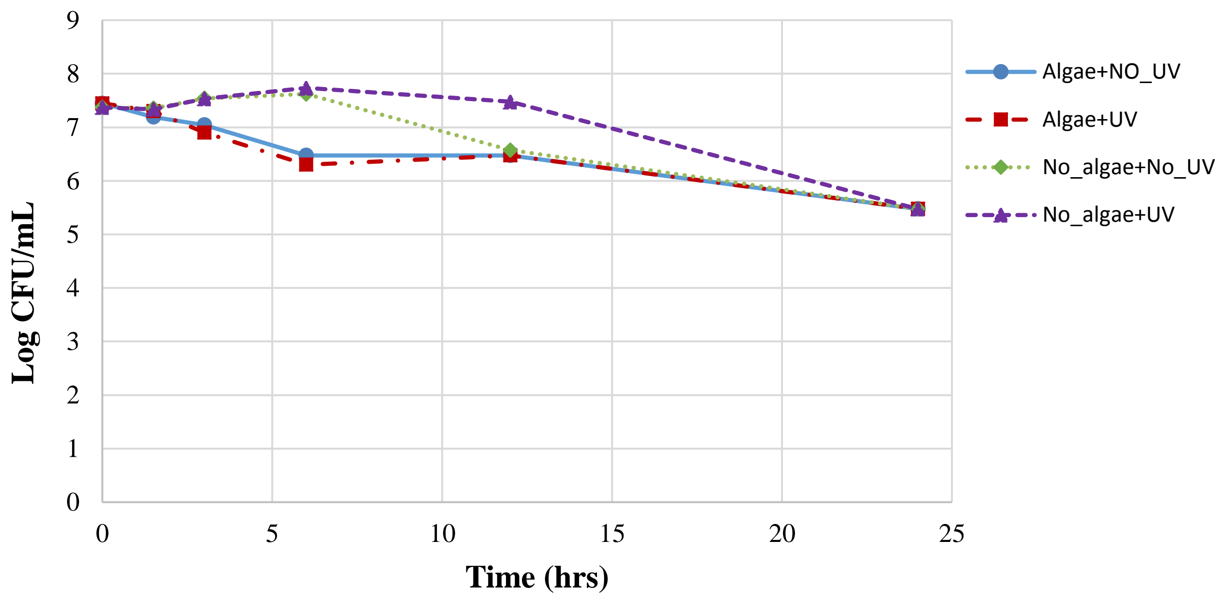

Figure 3.14. Log CFU/mL F. columnare vs time with humic acid supplementation for harvested turf under surge conditions (n=1).

3.5 Main Experiment

The main set of experiments was designed to observe the removal of the pathogen as a function of different ATS operating conditions. The following conditions were established for comparison:

1. Comparison of pathogen removal under surge (turbulent flow) and no surge (laminar flow) conditions.
2. Comparison of pathogen removal with a mature algal turf and a recently harvested algal turf.
3. Comparison of pathogen concentration in algal turf before and after each experiment.

3.5.1 Experiment Design

The main experiment was designed in order to separate the effect of the two proposed mechanisms of pathogen removal in ATS and provide experimental control. This design yielded a total of four reactors that used a combination of the presence or absence of UV light and an algal turf (Figure 3.15). The four reactors used in the study had one of the following combination of effects: algal turf present with UV supplemented light, algal turf present without UV supplemented light, algal turf absent with UV supplemented light and algal turf absent without UV supplemented light. Since it was not feasible to have replicates of the four reactors, experimental replication was carried out in time yielding one replicate per harvest cycle for both a mature and harvested turf. Altogether there were 15 harvests in which removal data was collected. Three additional harvests occurred in which only data for algal productivity was collected.

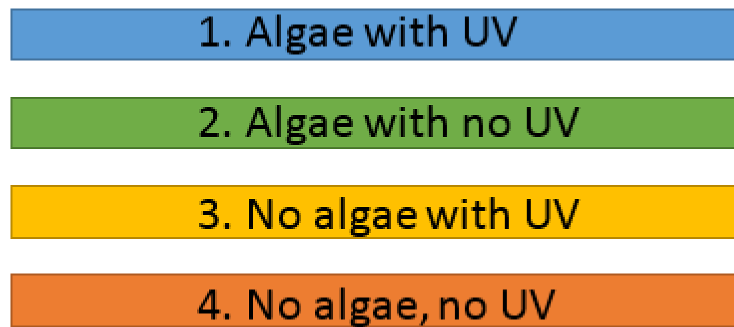

Figure 3.15. A graphical representation of the four treatments used for the main experiment. Each treatment is a combination of the absence of presence of an algal turf and UV light.

3.5.2 Reactor operating parameters

For the above objectives all algal turf was grown on 18/6 diurnal lighting over a nine day period in which the algae was harvested on the first day in the cycle. The measured photosynthetically active radiation (PAR) of the two cool white lamps used during turf growth averaged approximately 250 µmol m^{-2} s^{-1} (± 88 µmol m^{-2} s^{-1}) along the length of the lamp fixture. PAR was measured with an Apogee MQ-200 PAR meter (Apogee Instruments, Inc.). Daily maintenance of the algae during this nine-day period included maintaining an active reservoir volume of 7.6 L (total system volume of approximately 9.5 L) and daily addition of F/2 media at the manufacturers recommended concentration.

Reactor temperature was maintained at 26°C (±2°C) for the duration of the 24 hour experiment. This temperature was chosen since it is a typical range in which outbreaks of *F. columnare* can occur in aquaculture operations as well as being conductive for maintaining *E. coli* in the environment (Durborow et al., 1998; Blaustein et al., 2013). This temperature range was achieved during the colder months of 2016 with the inline aquarium heaters; however, the heaters were removed after spring since they were no longer thought to be needed. The flow rate of each reactor was adjusted to an average of approximately 6.0 LPM (± 0.7 LPM) by measuring the time required to fill a 1 L graduated cylinder at the channel outlet. Using ANOVA, variation

in flow rate among reactors was found to be insignificant (P=0.4465; Figure 3.16). This flow rate measurement included the flow from the base flow and surge device outlets. The difference between pre and post-harvest flow rates of reactors with algae varied only within ± 0.30 L/min and was ignored in any calculations.

Each surge device was configured to provide a periodic average surge volume of 0.83 L (± 0.01 L) every 17 seconds (± 0.5 seconds). An average of 54% of the overall system flow passed through the surge and the remaining through the base flow outlet. Measured flow rates of the reactors with the surge deactivated did not significantly vary from those of an activated surge. The height of water above the bottom of the channel during flow was on average 2 cm for a mature turf and 1 cm for harvested turf.

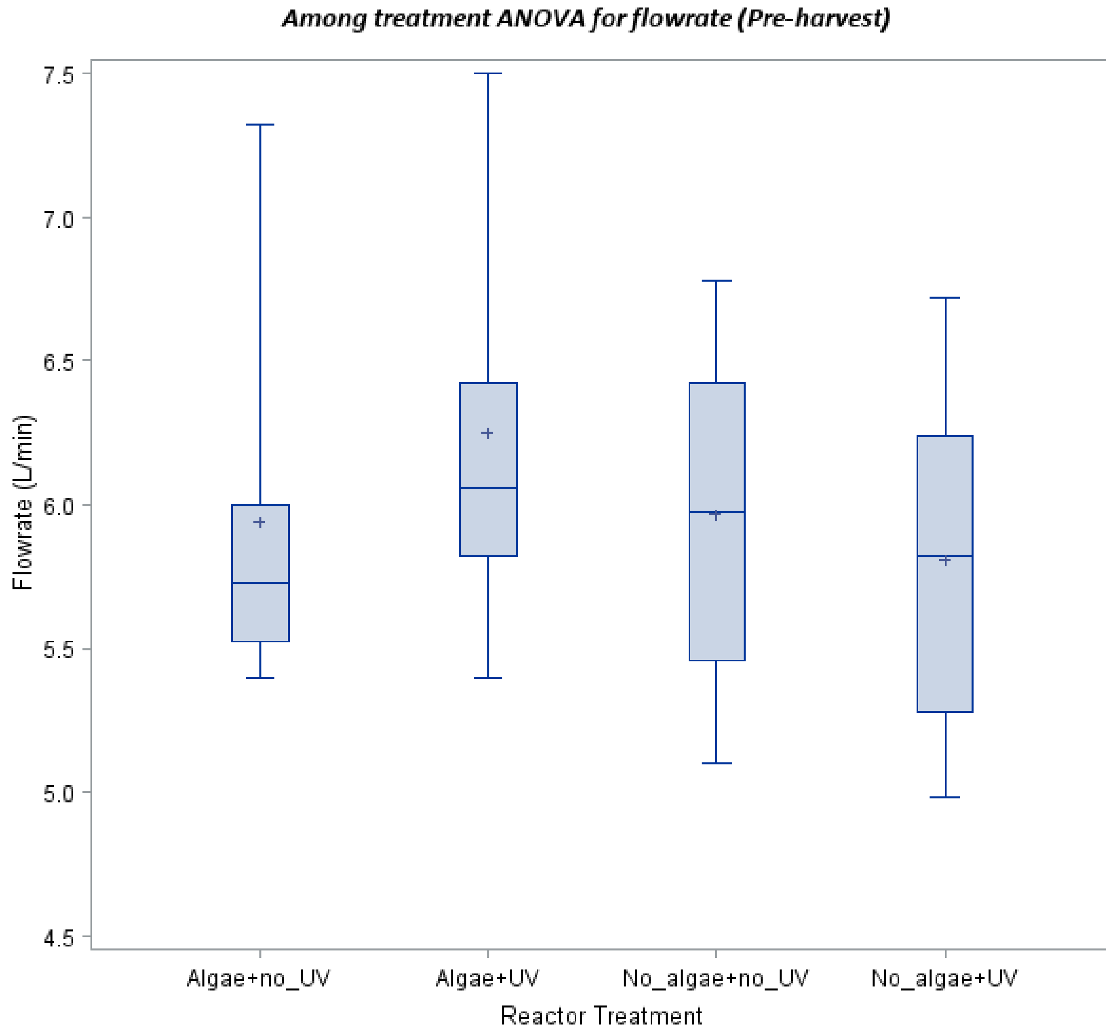

Figure 3.16. Flow rate of each reactor measured at the outlet of each channel (n = 5). Flow rate is combination of base flow and flow from periodic surge.

The reservoir hydraulic residence time (HRT) among all reactors, which was defined as the volume of water in the reservoir (with pump active) divided by the flowrate into the reservoir, was an average of 15 seconds (±0.5 sec). The HRT along with average velocity was used to calculate the total light exposure time for each reactor represented as light/dark ratio (Table 3.2). The average velocity was estimated by measuring the travel time of a buoyant foam disk to travel the length of the channel. The light/dark ratio is the total amount of time a fluid particle or bacterial cell was exposed to light compared to total experiment time. The reactor lighting for all treatments was on for the entire 24 hours of each experiment.

Table 3.2 Mean reactor flow parameters for each treatment (for a mature or harvested turf with or without surge) combination showing the estimated light exposure time for 24 hour experiment.

Reactor conditon	HRT (sec)	Reactor light exposure (sec)	Reactor dark exposure (sec)	Light exposure	Light exposure per 24 hours
Mature algae with surge	15	10.2	16.3	38.4	9.2
Harvest algae with surge	15	9.3	1.2	36.4	8.7
mature without surge	15	13.5	16.7	44.7	10.7
harvest algae without surge	15	11.1	1.4	40.3	9.7
no algae with surge	15	10.1	16.3	38.3	9.2
no algae without surge	15	12.7	16.6	43.4	10.4

3.5.3 Preparatory tasks conducted prior to each experiment

In order to maximize data collection within the nine day harvest cycle, experimental runs for removal under mature and harvested turf were executed within the same cycle. The experimental run under a mature turf occurred on day nine of the cycle and the harvested turf experiment occurred on day two. Bacterial cultures were grown in the Biological Systems lab of the Biological Engineering Research Laboratory (BERL) prior to the start of experiment. For *F. columnare* an isolated colony from a streak plate was transferred to 5 mL of MS broth in a 50 mL centrifuge tube. This culture was grown at 28°C and 150 RPM for 24 hours before 2.5 mL was transferred to a 250 mL flask containing 47.5 mL of MS broth. The 50 mL culture was again grown for 24 hours after which 10 mL was transferred to four 200 mL flask containing 190 mL of MS broth. This was the final scaling of the culture and was allowed to grow for 24 hours or until the start of the main experiment. For *E. coli* an isolated colony was used to inoculate 200 mL of LB broth and was allowed to grow for 12 hours at 37°C and 150 RPM until the start of the experiment.

All cultures were grown to the start of the stationary phase of growth, which was verified using growth curves for each organism under the above culture conditions (Appendix B). The

growth curves were created by measuring the change in optical density (OD600) of the 200mL over time using a UV-VIS spectrometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer, Inc.)

Approximately twelve hours before the start of the experiment a plastic tote was filled with 37L of tap water along with 4 mL of a 0.1M solution of sodium thiosulfate made from crystals of sodium thiosulfate (Proline dechlornator sodium thiosulfate, Pentair Aquatic Eco-Systems, Inc.) and heated to approximately 26°C using a 200W aquarium heater. Approximately one hour before the start of the experiment all reactors were drained and the water was replaced with the warmed dechlorinated tap water from the tote; buffers were also added to each reservoir to provide some pH control (Appendix D.2).

The control reactors without algae were filled with ~5.7 L of the warmed dechlorinated tap water. For the treatment reactors with algae the volume varied due to the presence of mature algal turf acting as a damper to velocity. This required a greater volume of water 7.5 L in the reservoirs when testing mature algal turf to maintain the same Hydraulic Residence Time (HRT) between the reactors with algae and the reactors without algae. To mitigate the difference of the total system volume of water between treatments it was also necessary to adjust the nutrient addition to reactors with mature algal turf as well as the volume of bacteria culture added to the reservoirs at the start of the experiment to achieve a consistent concentration among reactors. Experiments conducted on the harvested turf did not require these measures since the absence of a significant algal turf acted similar to the control reactors that did not have algae.

Before the pumps were restarted in each reactor, the algal turf was sampled to observe the background level of bacteria maintained in the algal turf. For experiments with surge this included haphazard sampling approximately 1mg wet weight of algae within 30 cm of the upstream and downstream ends of the channel. This was done to observe any longitudinal

difference in concentration in the algae that might be due to the turbulence induced by the surge device. The algae were haphazardly sampled in three places in each upstream or downstream section using forceps. Sampled algae was placed in a 1.5 mL microcentrifuge tube and set aside until the experiment had started. The same protocol was conducted for experiments without surge except the samples from the upstream and downstream sections were combined into a single sample. This was done since it was visually observed that the entire channel was under similar flow conditions due to absence of periodic surge (inflow from the pump was directed backwards at the front of the channel which acted to dampen turbulence). After sampling the algae the pumps were restarted and the reactors were allowed to recirculate for up to 30 minutes before the experiment began.

3.5.4 Running the experiment: sampling, processing and plating

Before spiking each reactor with bacteria the recommended standard concentration of F/2 algae food was added to each reactor reservoir. Afterwards a water sample from the reservoirs was taken to measure the background concentration of the pathogen in the reservoir before the start of the experiment. Sterile disposable transfer pipettes were used to sample approximately 1 mL of water from each reactor reservoir. After this was completed the bacterial flask cultures were removed from the incubator, combined and then divided into four clean 250 mL sample bottles at the appropriate volume for each reactor. The bottles were then placed in a marked, closed secondary container and transferred to Corley 109 for spiking the reactor reservoirs.

For the reactors with a mature algal turf, the reservoirs were spiked with 200 mL bacterial culture of *F. columnare* and the reactors without algae were spiked with 175 mL of the culture. For *E. coli* the reactors were spiked with the same volumes as *F. columnare*. For experiments on a harvested algal turf 175 mL of bacteria culture was used to spike all of the reactors regardless

of treatment. After spiking, the reservoirs were checked to make sure reservoir volumes were equal among reservoirs; any evaporated water was replaced with the warmed decholorinated tap water from the plastic tote until all reservoirs were at the same volume. Each reactor was then measured for pH, temperature and conductivity. Transfer pipettes were then used to sample 1 mL from each reservoir for the initial concentration of pathogen. Subsequent sampling events occurred at 1.5, 3, 6, 12 and 24 hours after the initial sample yielding 6 total samples per treatment. Each experimental trial lasted a total of 24 hours.

Experiments with *E. coli* followed the same general procedure as for *F. columnare* except pH was controlled on algal treatments using pH controllers (Jenco pH/ORP controller 3672, San Diego, CA) and peristaltic pumps (Masterflex, Cole-Parmer Instrument Co., Chicago, IL) to deliver 0.5M HCl on demand at a upper set point pH limit of 8.00. Although using an acid does increase chloride ion concentration that can impair algal growth, the overall condition of the algae was not expected to be affected since the buildup of ions was minor due to the 24 hour duration of the experiment. HCl has also been used in other studies on algal growth to control for pH (Tan et al., 2016; Granum and Myklestad, 2002).

All samples were processed and plated in the BERL lab. Algae samples were processed as follows. The algae sample was centrifuged for 5 minutes at 3000 g to remove excess water. Each algae sample was then carefully removed from the microcentrifuge tube and combined with 9mL of PBS in a small blender. The sample was then blended for 30 seconds in a food grade blender after which 1 mL of the homogenized sample was extracted and set aside. The remaining homogenate was disposed and the blender was washed with 70% Isopropyl alcohol followed by sterile DI water before processing the next algae sample. After all algae samples had been processed the blender container was disinfected with a 10% bleach solution. The blended algae

was then diluted and plated in triplicate in the Biological Safety Cabinet (BSC). Water samples were serially diluted in microcentrifuge tubes and plated in triplicate. Initially spread plate method was used for enumeration but was cumbersome due to the amount of plating (Maier et al., 2000). Halfway through the experiments with *F. columnare* the drop plate method was adopted for enumeration in order to save preparation and plating time as well as cut costs (Naghili et al., 2013). For the drop plate method, 10 µL drops were plated using a small volume pipetter. Plated samples of *F. columnare* on MS agar with Tobramycin were incubated at 28°C for 48 hours, and plated samples of *E. coli* on MacConkey agar (Becton, Dickinson and Companey (BD)) supplemented with Streptomycin were incubated at 37°C for 24-48 hours per manufacturer instructions (Arias et al., 2012).

3.5.5 Post experiment: harvesting and disinfection

At the end of the 24 hour experiment period the reactors were allowed to drain 10 minutes before manual harvesting of the algal turf by scraping. Harvested biomass was collected and dried in a Nesco FD-1040 Gardenmaster Digital Pro food dehydrator (The Metal Ware Corporation, Nesco/American Harvest) for 48 before weighing for air-dry weight (ADM). After harvesting all reactors were disinfected with household liquid bleach solution at 500 mg/L available chlorine for one hour as recommended by a published disinfection protocol for aquaculture (Yanong and Erlacher-Reid, 2012). Before bleaching algal reactors, the substratum was detached from the gutter, coiled and then placed in a bucket with dechlorinated tap water with an air stone. During disinfection the reactor channels and the foam inserts of the surge devices were scrubbed to remove any visible biofilm buildup. After bleaching for one hour each reactor was filled with fresh tap water with dechlorinator and circulated for an additional hour until no residual chlorine was detected using chlorine test strips (Hach, Loveland, CO). Plates

were enumerated for *F. columnare* after 48 hours of incubation and 24 hours of incubation for *E. coli*. Plates were counted for significant colonies of the pathogen tested (30-300 CFU).

3.5.6 Data Analysis

All data for the main experiment were collected in print before being transferred into an Excel spreadsheet (Microsoft, Inc.) for data management and processing. Data were then statistically analyzed using SAS 9.3 (SAS Institute Inc.). Analysis of variance (ANOVA) was used to test for significant differences among experimental treatments and Tukey's multiple comparisons test was used for post hoc analysis. All analysis utilized an alpha of 0.05.

Chapter 4: Results and Discussion

4.1 Variation of water quality parameters

4.1.1 *F. columnare* removal with surge

Table 4.1 and Table 4.2 show the observed mean and standard deviation of measured water quality parameters for removal under a mature and harvested turf with surge, respectively. For the mature turf experiments, the pH and conductivity (EC) of the algae treatments were statistically significantly different than treatments without algae using ANOVA (pH: $P < 0.0001$; EC: $P < 0.0001$). The water temperature did not significantly vary during the experiment ($P = 0.0716$). Appendix C.2 and C.3 displays all statistical significant grouping of variation among observed water quality variables using Tukey's multiple comparisons test.

The majority of the pH readings for the experiments with *F. columnare* under surge were off due to an old malfunctioning pH electrode that went undetected. Using linear regression of pH data collected with a new and accurate pH electrode and the old one, an linear equation was created to correct previously collected pH data. This equation was applied to all pH data collected before July 6, 2016. Dissolved oxygen was not measured during *F. columnare* removal experiments due to inadvertently overlooking the importance of measurement as it related to the study. This can be considered an important water quality parameter for solar mediated disinfection since dissolved oxygen is required for the production of ROS.

Table 4.1. Mean and standard deviation of measured water quality parameters for F. columnare removal under surge conditions with mature algal turf for the five experiments (n=5).

***F. columnare*: mature turf with surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	8.49	0.57	0.41	0.03	26.1	0.7	No_data	No_data
Algae+UV	8.56	0.55	0.41	0.03	26.1	0.5	No_data	No_data
No_algae+No_UV	8.02	0.49	0.47	0.06	26.2	0.7	No_data	No_data
No_algae+UV	7.96	0.43	0.45	0.06	25.8	0.6	No_data	No_data

Table 4.2. Mean and standard deviation of measured water quality parameters for F. columnare removal under surge conditions with harvested algal turf for the five experiments (n=5).

***F. columnare*: harvested turf with surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	8.61	0.50	0.45	0.04	26.3	0.6	No_data	No_data
Algae+UV	8.67	0.51	0.45	0.04	26.1	0.5	No_data	No_data
No_algae+No_UV	8.05	0.22	0.47	0.08	26.4	0.5	No_data	No_data
No_algae+UV	8.09	0.23	0.50	0.09	26.0	0.5	No_data	No_data

4.1.2 *F. columnare* removal without surge

The variation in water quality without surge for *F. columnare* was much the same as the experiments with surge (Tables 4.3 and 4.4). A significant amount of variance was detected among treatments for both mature and harvested turf experiments excluding temperature. Treatments with algae significantly varied for both mature and harvested turf experiments compared to treatments without algae ($P<0.0001$). Conductivity followed the same pattern with higher values seen with treatments without algae compared to treatments with algae for both mature and harvested turf experiments ($P<0.0001$). The difference in temperature was not significant with the mature turf experiments but was significant with harvested turf experiments

with the Algae+NO_UV differing with the No_algae+UV treatment but not the others (P<0.0170). Appendix C.4 and C.5 display the grouping of variation of all significant output using Tukey's multiple comparison.

Table 4.3. Mean and standard deviation of measured water quality parameters for F. columnare removal absent of surge with mature algal turf for the five experiments (n=5).

***F. columnare*: mature turf without surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	8.20	0.32	0.45	0.04	26.2	0.5	No_data	No_data
Algae+UV	8.31	0.34	0.44	0.03	26.1	0.5	No_data	No_data
No_algae+No_UV	7.61	0.24	0.61	0.09	26.2	0.5	No_data	No_data
No_algae+UV	7.67	0.27	0.59	0.07	25.8	0.5	No_data	No_data

Table 4.4. Mean and standard deviation of measured water quality parameters for F. columnare removal absent of surge with harvested algal turf for the five experiments (n=5).

***F. columnare*: harvested turf without surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	8.18	0.51	0.44	0.02	26.3	0.4	No_data	No_data
Algae+UV	8.26	0.50	0.44	0.02	26.1	0.4	No_data	No_data
No_algae+No_UV	7.67	0.25	0.54	0.06	25.9	1.0	No_data	No_data
No_algae+UV	7.71	0.24	0.51	0.08	25.7	1.0	No_data	No_data

4.1.3 *E. coli* removal with surge

The use of pH controllers and peristaltic pumps on treatments with algae allowed for a greater control on pH variation due to photosynthesis for the duration of the experiment. As stated before, pH control was needed with *E.coli* since research suggests that die-off accelerates above pH 8.5 (Curtis et al., 1999; Parhad and Rao, 1974). Dissolved oxygen (DO) was also recorded for experiments with *E. coli*. Measured water quality parameters are summarized for experiments with a mature and harvested algal turf in Tables 4.5 and 4.6 respectively.

For both set of experiments with a mature and harvested algal turf, pH and conductivity were not significantly different among treatments (mature: P=0.0638; harvested: P=0.9026). The mature turf experienced significant variation between treatments with algae and treatments without algae for both temperature and DO (Appendix C.6). No significant variation in DO was found with harvested turf experiments but temperature did significantly vary between treatments with and without algae (Appendix C.7). While the temperature variation was statistically significant it was well within the range of ±2 °C and should not have significantly affected results.

Table 4.5. Mean and standard deviation of measured water quality parameters for E. coli removal with mature turf and surge for the five experiments (n=5).

***E. coli* mature turf with surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	7.96	0.17	0.53	0.07	25.9	0.4	8.94	0.46
Algae+UV	7.98	0.19	0.52	0.06	26.0	0.3	9.00	0.27
No_algae+No_UV	7.86	0.27	0.51	0.07	25.5	0.2	8.86	0.34
No_algae+UV	7.85	0.27	0.52	0.06	25.5	0.2	8.74	0.36

Table 4.6 Mean and standard deviation of measured water quality parameters for E. coli removal absent with harvested turf and surge for the five experiments (n=5).

***E. coli* harvested turf with surge**								
	pH		EC (µS/cm)		Temp (°C)		DO (mg/L)	
Treatment	Avg.	Std.	Avg.	Std.	Avg.	Std.	Avg.	Std.
Algae+NO_UV	7.97	0.17	0.52	0.05	25.8	0.5	8.83	0.34
Algae+UV	7.94	0.23	0.51	0.04	25.8	0.6	8.86	0.30
No_algae+No_UV	7.92	0.29	0.52	0.06	25.5	0.5	8.84	0.29
No_algae+UV	7.93	0.26	0.52	0.06	25.4	0.5	8.78	0.38

4.2 Biomass productivity of reactors with algae

A substantial algal turf developed over the nine day harvest period given that all basal cells attached to the channel were removed during disinfection (Figure 4.1). The harvested biomass

was reduced to an average moisture content of 5.8% after drying for 48 hours in the food dehydrator for 48 hours. Figure 4.2 displays the overall harvest productivity of each reactor during the nine-day cycle.

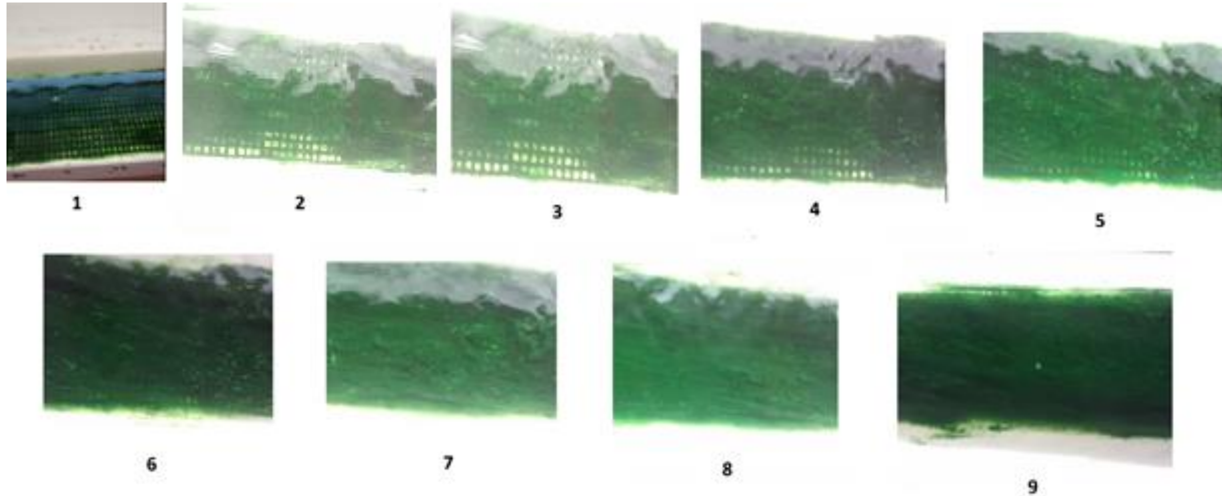

Figure 4.1. Progressive growth of algal turf during 9 day harvest cycle. Numbers correspond to days after harvest with the experiment with a harvested turf occurring on day 2 and mature turf experiment occurring on day 9.

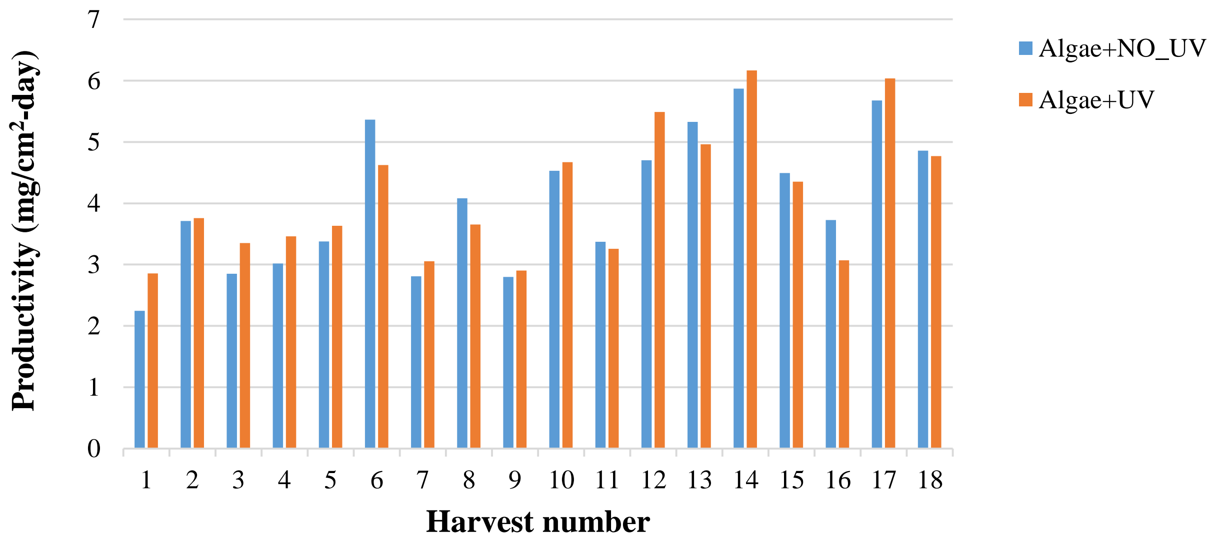

Figure 4.2. Productivity of algal treatment reactors for the duration of the experiments. Experiments using F. columnare occur from harvests 1-12 and E. coli 13-18.

Average measured pH, temperature and conductivity for the Algae+ UV reactor during the 9 day growth cycle (excluding days occurring on experiments) was 10.42 (±0.37), 25.6 °C (± 1.0 °C) and 0.42 µS/cm (0.10 µS/cm) respectively. The Algae+NO_UV pH, temperature and conductivity reactor averaged 10.38 (±0.34), 25.6 °C (±1.0 °C) and 0.42 µS/cm (±0.10 µS/cm) respectively. Biomass productivity over the 9-day growth cycle averaged 4.11 mg/cm^2-day (±1.06 mg/cm^2-day) for the Algae+NO_UV reactor and 4.04 mg/cm^2-day (±1.06 mg/cm^2-day) for the Algae+UV reactor. A one sample T-test using the calculated subsidized difference between the two reactors per equation 5 showed no significant difference in biomass productivity between the two reactors ($P = 0.5149$). Again, during the growth of the algal turf the treatments did not utilize the UV lamps and were not grown under a periodic surge.

4.4 Pathogen removal from water column

As a measure of overall removal effectiveness of each treatment, ANOVA was used to analyze variance in total log reduction (Equation 6) among the four treatments and Tukey's multiple comparisons test was used for post hoc analysis.

$$Log\ Reduction = \log_{10} \frac{B}{A} \tag{6}$$

where B is CFU/mL bacteria at the beginning of the experiment (t=0 hr) and A is CFU/mL bacteria at the end of the experiment (t=24 hr).

The introduction of *F. columnare* and *E. coli* cultures into the reactors undoubtedly resulted in colonization of the components of the reactors, and the reintroduction of the algal turf and substratum into the reactor treatments with algae likely reintroduced residual bacteria into the system. The disinfection protocol implemented after each experiment was intended to remove any residual bacteria and remove any biofilm formation on reactor components. Ideally

this would keep the level of residual bacteria relatively constant between experiments instead of gradually increasing.

4.4.1 Removal of *F. columnare* in water column with surge

The background level of *F. columnare* measured before the start of the experiments averaged 451 CFU/mL (±551 CFU/mL) for treatments with algae and 7 CFU/mL (±15 CFU/mL) for treatments without algae. The log reduction of *F. columnare* under the surge condition with both a mature (Figure 4.3) and harvested turf (Figure 4.4) was significantly different among the treatments (P<0.0001) with both algae treatments significantly different from the treatments absent of algae but not each other according to Tukey's multiple comparisons test (Appendix C.8 and Appendix C.9). Figures 4.3 and 4.4 show that the mature and harvested algal turf had a respective 3.7 log and 2.9 log greater reduction than treatments without an algal turf.

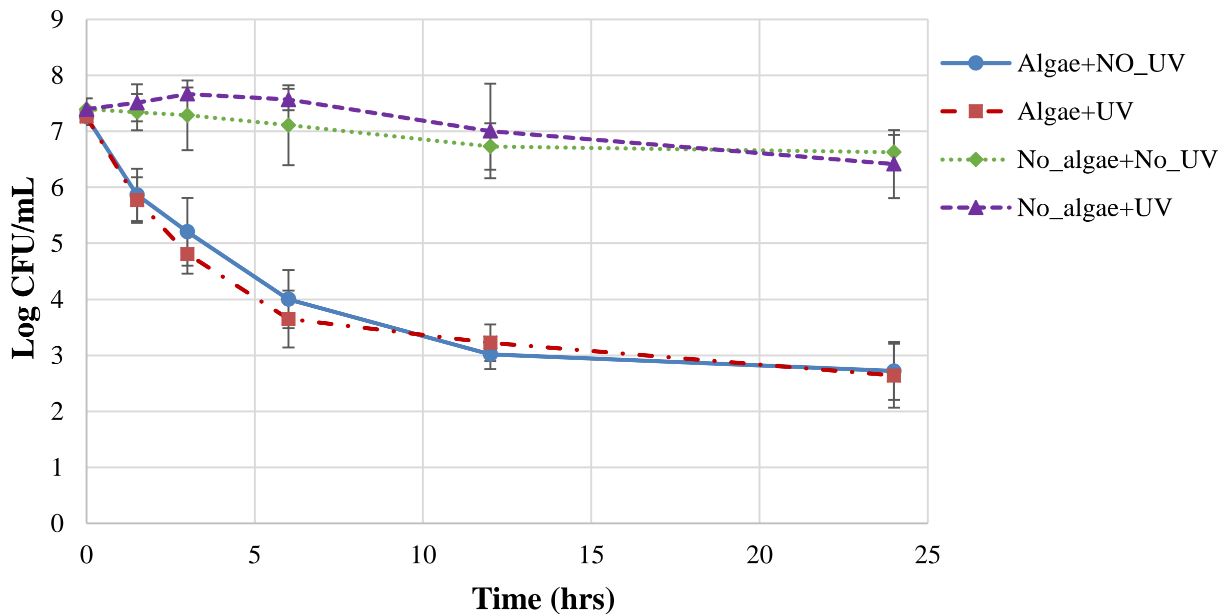

Figure 4.3. Mean log removal of F. columnare over time with a mature turf and surge. Error bars are standard deviation (n=5).

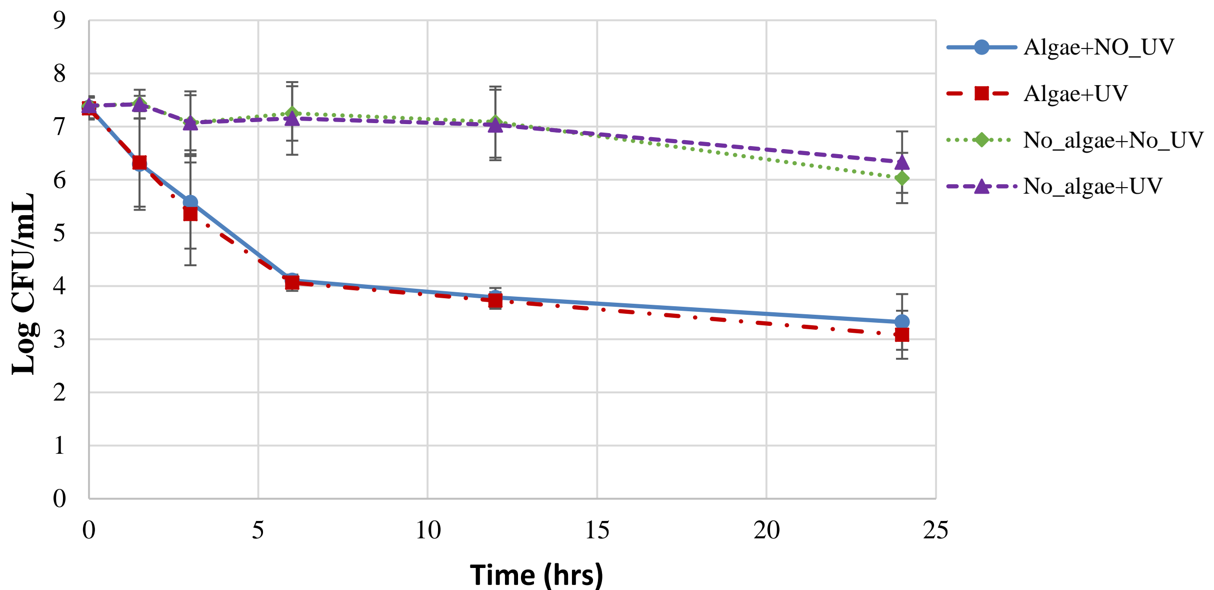

Figure 4.4. Mean log removal of F. columnare over time with a harvested turf and surge. Error bars are standard deviation (n=5).

4.4.2 Removal of *F. columnare* in water column without surge

The background level of *F. columnare* measured before the start of the experiments averaged 370 CFU/mL (±658 CFU/mL) for treatments with algae and 35 CFU/mL (±94 CFU/mL) for treatments without algae. The overall log removal curve for *F. columnare* experiments without a surge closely mirrors the results from experiments that did involve a surge. Statistically, the overall log reduction of *F. columnare* absent of surge with both a mature (Figure 4.5) and harvested turf (Figure 4.6) was found significantly different among the treatments (P<0.0001) with both algae treatments significantly different from treatments absent of algae but not each other according to Tukey's multiple comparisons test (Appendix C.10 and Appendix C.11). The treatments with an algal turf without surge showed a higher log reduction than the treatments without an algal turf. Overall the mature and harvested turf achieved a 2.9 and 3.0 greater log reduction over the treatments without an algal turf (Figure 4.5 and 4.6).

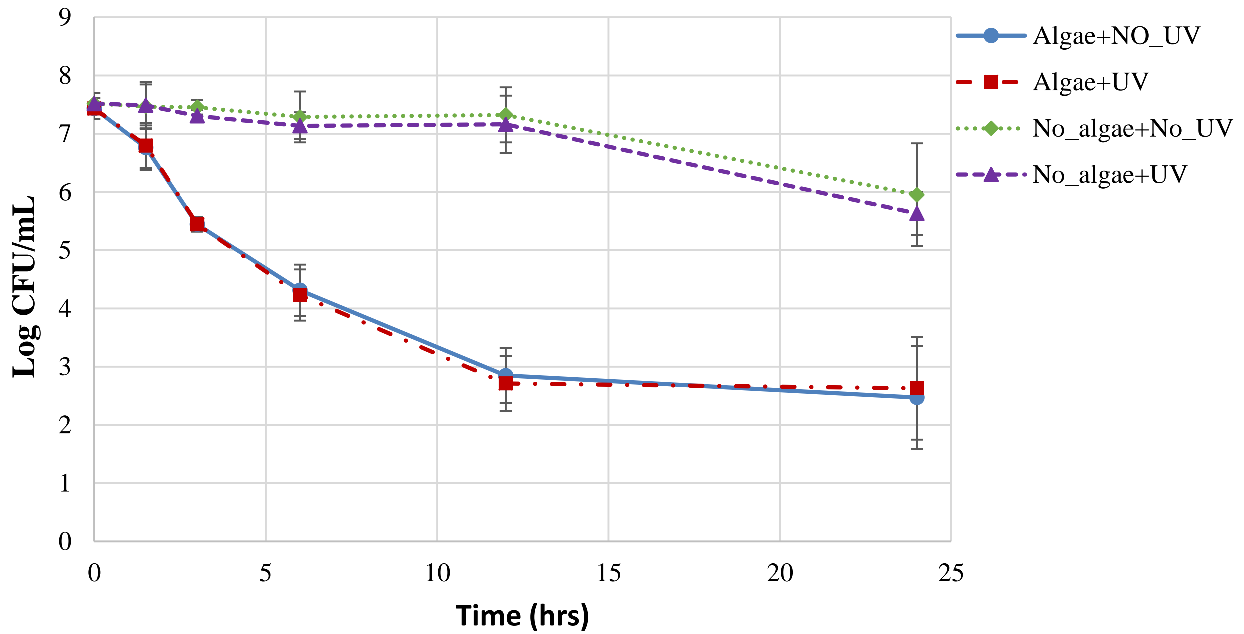

Figure 4.5. Mean log removal of F. columnare over time with a mature turf and no surge. Error bars are standard deviation (n=5).

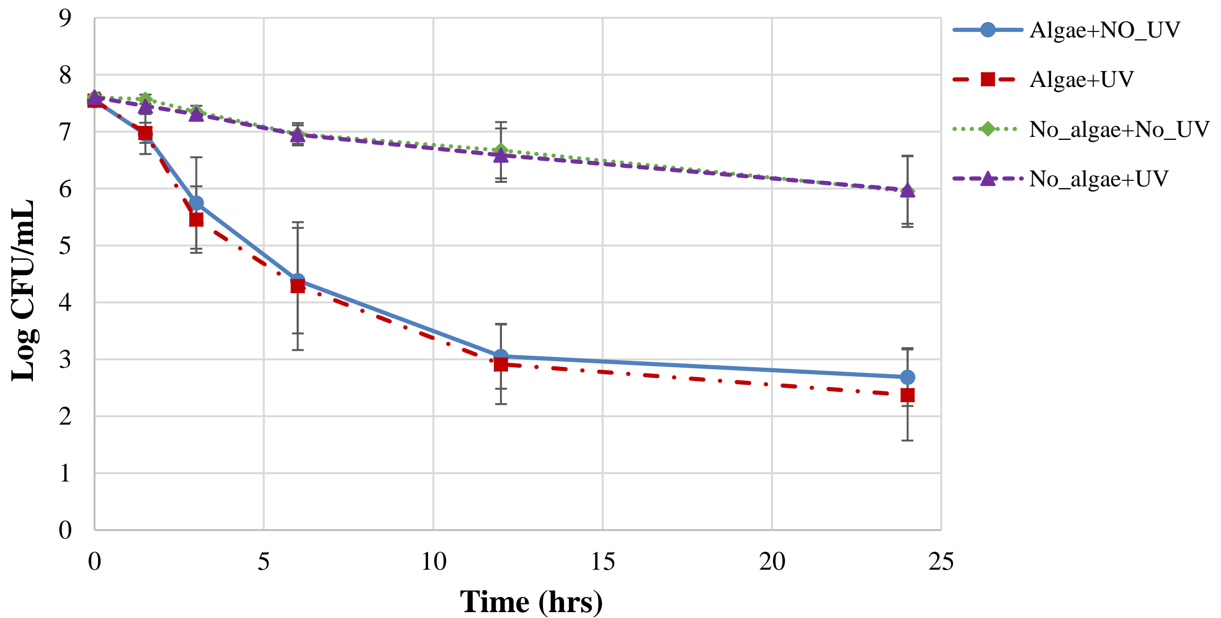

Figure 4.6. Mean log removal of F. columnare over time with a harvested turf and no surge. Error bars are standard deviation (n=5).

4.4.3 Removal of *E. coli* in water column with surge

The background level of *E. coli* measured before the start of the experiments averaged 657 CFU/mL (±648 CFU/mL) for treatments with algae and 1062 CFU/mL (±905 CFU/mL) for treatments without algae. Results for *E. coli* again show no observable difference in removal between a mature and harvested turf (Figure 4.7 and 4.8). The experiments with *E. coli* also show that the effect of UV appears to increase the removal of *E. coli* from the water column but the effect was not statistically significant between treatments with UV and without UV using ANOVA with Tukey's multiple comparisons test (Appendix C.12 and Appendix C.13). The algae treatment with UV achieved a 2.4 and 2.8 order of magnitude greater log reduction than the UV treatment without algae for a mature and harvested turf, respectively (Figure 4.7 and 4.8). Similar order of magnitude log reduction was also observed among treatments without UV. Between the treatments with algae, the UV treatment achieved a respective 0.9 and 0.7 order of magnitude greater log reduction than the treatment without UV for the mature and harvested turf. Overall, the difference in removal between treatments with algal turf and without algal turf was significant for both mature and harvested turfs (Mature: $P<0.0001$; Harvested: $P<0.0001$).

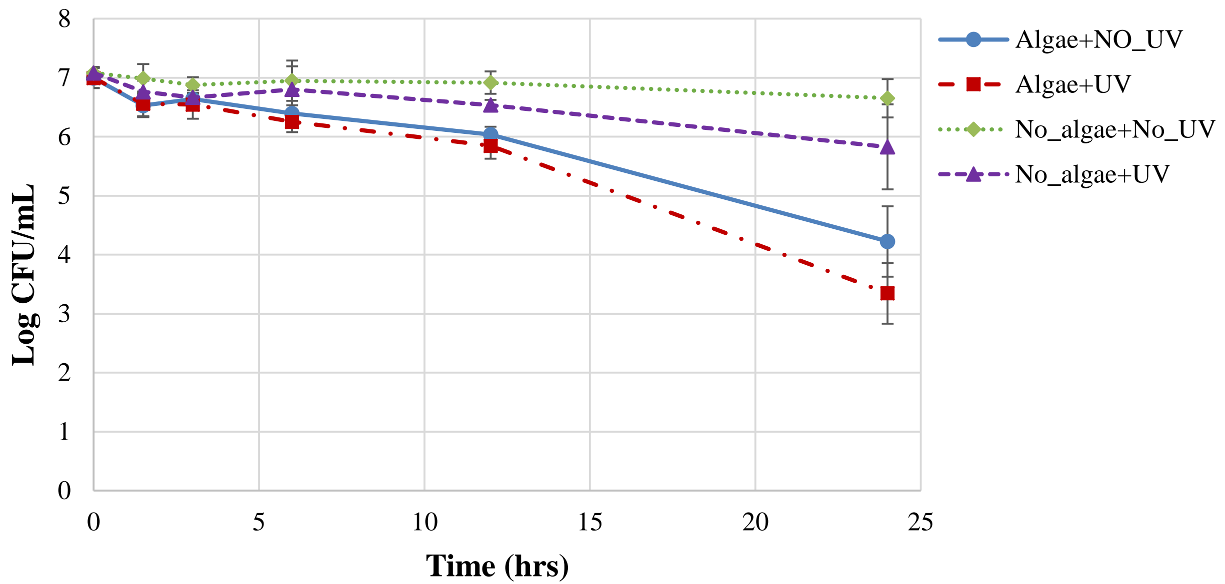

Figure 4.7. Mean log removal of E. coli with a mature algal turf and surge. Error bars are standard deviation (n=5).

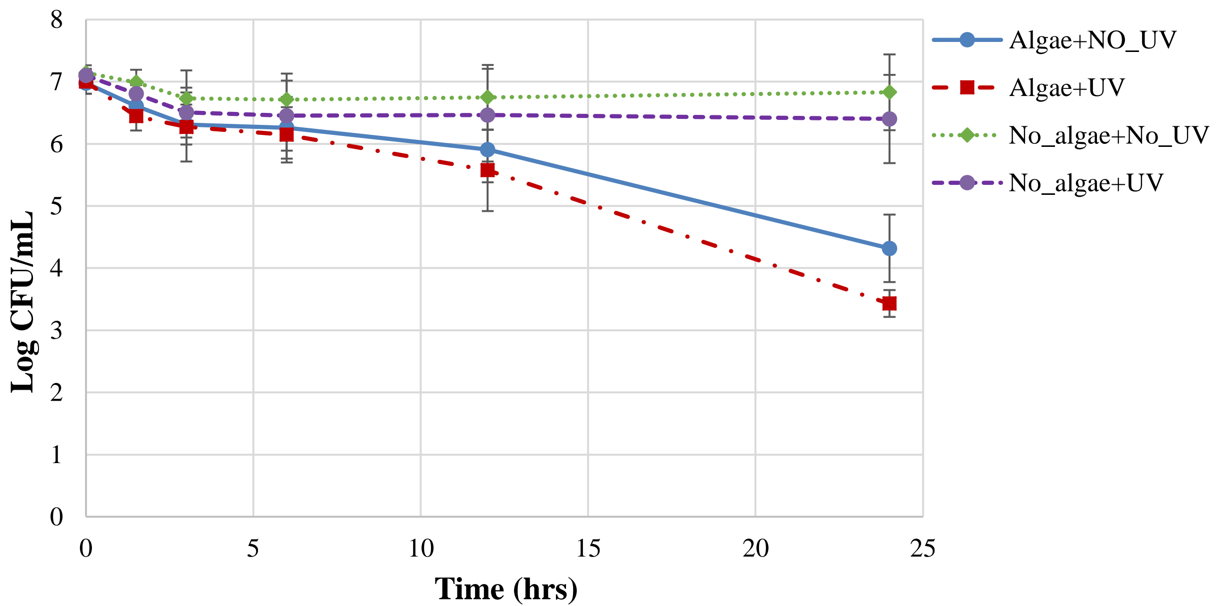

Figure 4.8. Mean log removal of E. coli with a harvested turf and surge. Error bars are standard deviation (n=5).

4.5 Pathogen concentration in sampled algal turf

The objective of the algae sampling protocol was to observe the number of bacteria that was attached to the algae at the beginning and end of the experiments. This was done by homogenizing the sampled algae in order to suspend any attached bacterial cells. The agar plates were enumerated for *F. columnare* after 48 hours of incubation and 24-48 hours of incubation for *E. coli*. Plates were counted for colony forming units of the pathogen tested (30-300 CFU); however there were several instances during enumeration for *F. columnare* that required counting colonies out of this range due to either too few colonies at the next dilution level, no colonies of the pathogen could be detected, or the background level of bacteria was too dense to discern *F. columnare* colonies.

4.5.1 *F. columnare* concentration in sampled algal turf

The measured concentration of *F. columnare* in the algal turf at the end of the experiment fit well with the estimated residual concentration of bacteria as a function of adsorption and dark die-off using a mass balance approach. From Figures 4.9 and 4.10 it can be seen that the concentration of *F. columnare* in the algal turf does not vary between algal treatments with and without UV for both experiments run with and without a periodic surge. There was no statistically significant difference among the increase in concentration among treatments and locations using ANOVA for surge ($P = 0.8000$) and no surge ($P = 0.6961$). Due to unforeseen circumstances with laboratory procedures data was collected for less than 5 samples.

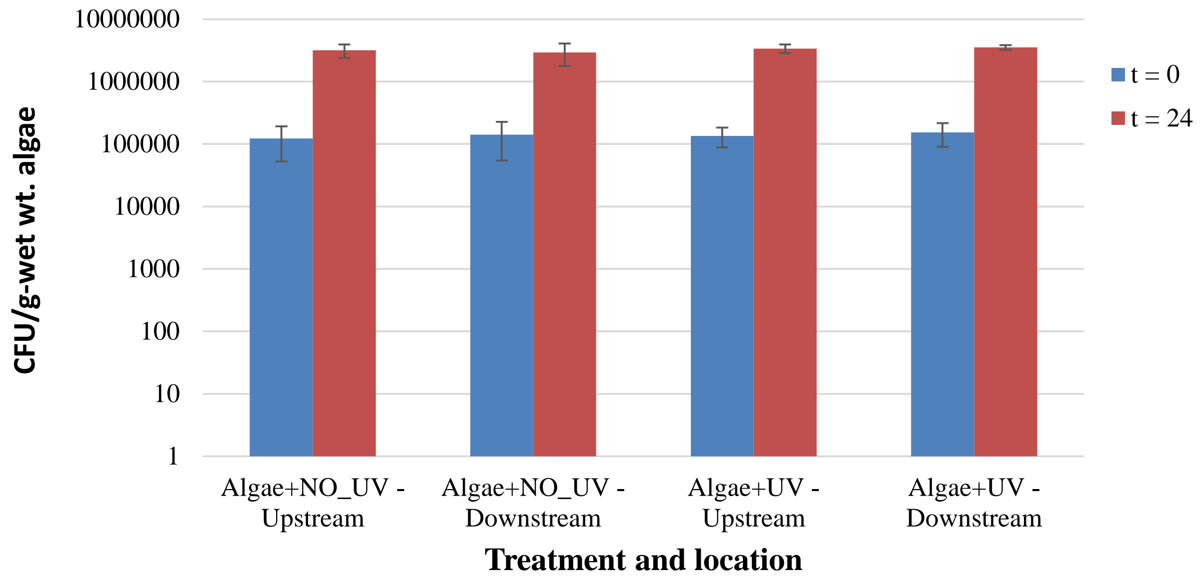

Figure 4.9. Mean F. columnare CFU per gram wet weight algae sampled at time before experiment (t = 0 hr) and time after experiment (t = 24 hr) for F. columnare with surge (n=3).

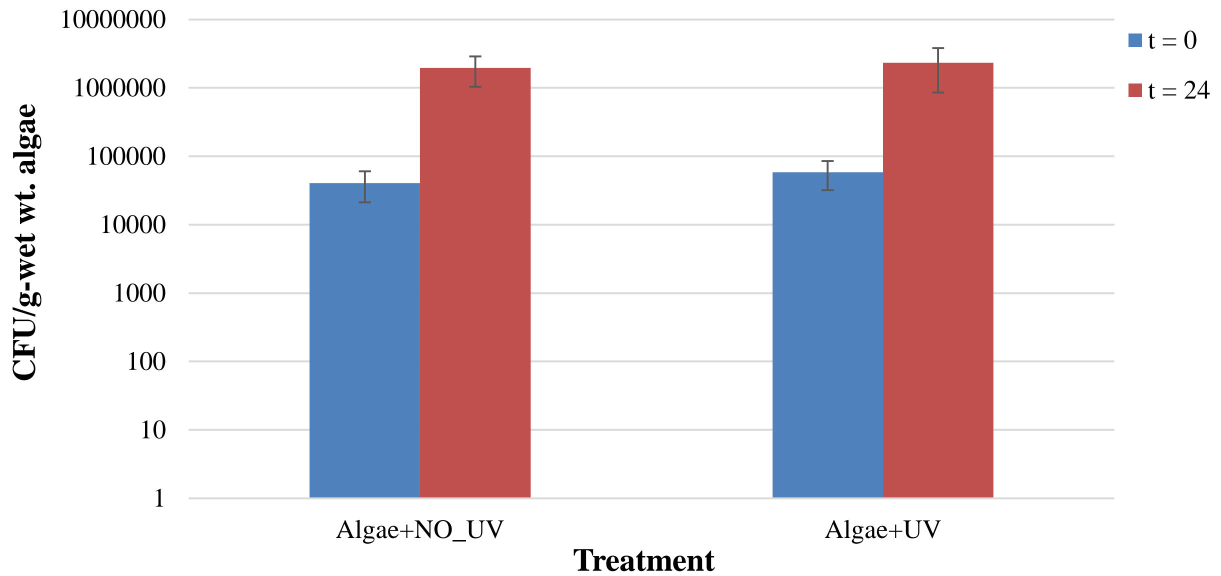

Figure 4.10. Mean CFU per gram wet weight algae sampled at time before experiment (t = 0 hr) and time after experiment (t = 24 hr) for F. columnare with no surge (n=4).

4.5.2 *E. coli* concentration in sampled algal turf

The results for initial and final concentration of *E. coli* in the sampled algal turf follow the same pattern seen with *F. columnare* (Figure 4.11). Again, little variation can be seen among treatments with and without UV as well as no distinct difference in concentrations between upstream and downstream samples. The increase in concentration of *E. coli* was not statistically significant among all treatments an locations using ANOVA (P = 0.3188). The use of MacConkey agar with Streptomycin allowed for the removal of a majority of background bacteria in the reactors.

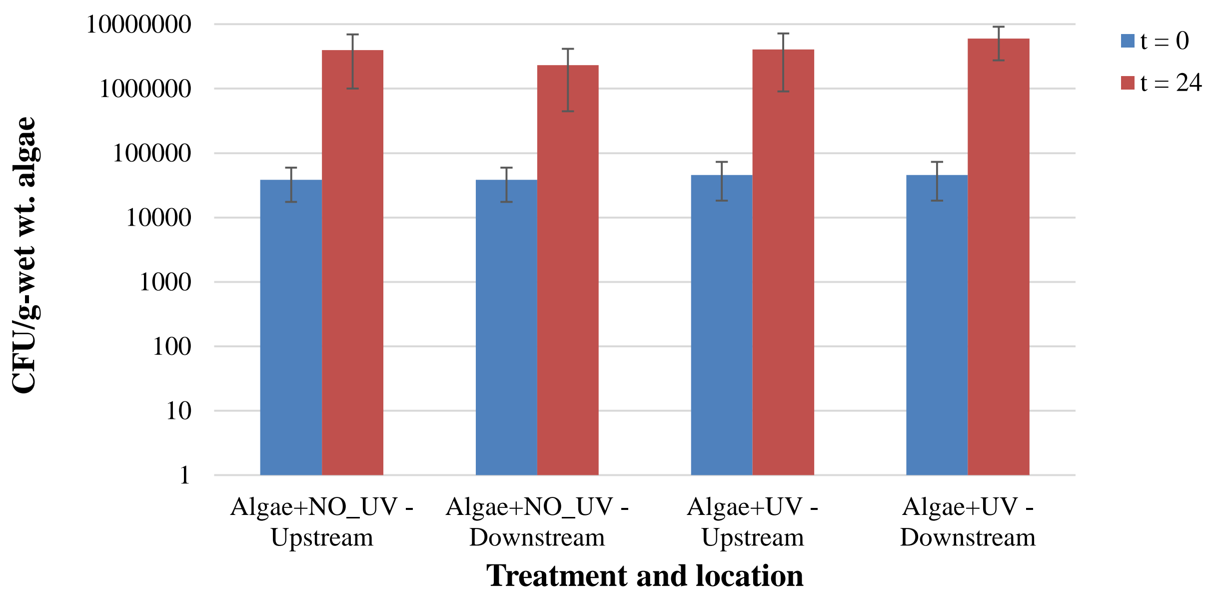

Figure 4.11. Mean CFU per gram wet weight algae sampled at time before experiment (t = 0 hr) and time after experiment (t = 24 hr) for E. coli with surge (n=4).

4.6 Modelling of pathogen removal in ATS

The models mentioned in the literature review were evaluated for fitting of the removal data and include the model proposed by Daniels (1980), the pseudo-first and pseudo-second order kinetic adsorption models and the first order solar disinfection model by Cragss et al.

(2004). The latter solar disinfection model was not applied to *F. columnare* removal since there was no significant difference in the removal between treatments with and without UV and any enhanced removal effect of higher UV irradiance was not tested.

For adsorption, the mass balance for a batch reactor was used to relate the concentration of bacteria in solution to the concentration of bacteria adsorbed to the algae (Equation 7).

$$(C_0 - C_t)V = q_t M \quad (7)$$

where C_0 is the initial concentration of bacteria in solution (CFU/mL), C_t is the concentration of bacteria in solution at time *t, V* is the total system volume (mL), q_t is the amount of bacteria adsorbed at time *t* (CFU/g wet weight algae) and *M* is total mass of algae in the system (g). For all batch reactor modeling of the lab scale ATS a volume of 7560 mL (total system volume for a mature algal turf) and mass of 180 grams wet weight of algae (average value calculated from harvest data) was used for the mass balance.

The linearized pseudo-first order kinetic model of Ho and McKay (1999) was first tested for fit with the data (Equation 8). Upon integration and substitution of Equation 7, the concentration of bacteria in solution at time t can be approximated (Equation 9).

$$\log(q_e - q_t) = \log q_e - \frac{k}{2.303}(t + t_0) \quad (8)$$

$$C_t = C_0 \frac{M}{V} q_e\left(1 - e^{k(t+t_0)}\right) \quad (9)$$

where q_e is the equilibrium concentration of bacteria adsorbed to the algae (CFU/g), *k* is the equilibrium rate constant of pseudo-first order sorption (1/hr), *t* is time (hr) and t_0 is a time constant (hr) used for adjustment of the intercept for the linearized plot of $\log(q_e - q_t)$ vs. t.

Equation 10 is the linearized pseudo-second order kinetic model of adsorption, which typically provides a better fit of data and does not require a measurement of q_e since it is estimated by the linearized plot (McKay and Ho, 1999). Upon integration and substitution of

Equation 7, the concentration of bacteria in solution at time t can be approximated (Equation 11).

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t \tag{10}$$

$$C_t = C_0 - \frac{M}{V} \frac{q_e^2 tk}{1 + q_e tk} \tag{11}$$

where k_2 is the pseudo-second order adsorption rate constant (g-CFU/hour). The slope of the linearized plot of $\frac{t}{q_t}$ vs. t yields k_2 and the intercept yields q_e.

The ion-exchange resin model of Daniels (1980) was also fitted using experimental data; however some of the model assumptions, such as the spherical shape of the adsorbent, do not quite match the adsorption geometry of the algal filaments in the lab scale ATS. Since the ion-exchange model above was the only bacterial adsorption model that could easily be applied to experimental data, it was included in this study. The model by Daniel (1980) evaluated with experimental data considers only adsorption and neglects desorption of bacteria (Equation 12).

$$\log \frac{A}{A_0} = kt + k'\sqrt{t} \tag{12}$$

where A is the optical density of bacteria at time t, and A_0 is the optical density at time 0. Both k and k' can be derived from curve fitting of the equation with experimental data but represent properties of the sorption system:

$$k = -4\pi\beta \mathrm{RND} \tag{13}$$

$$k' => 2kR/\sqrt{\pi \mathrm{D}} \tag{14}$$

where N is the number of adsorbent particles, β is an empirical constant relating n (number of bacterial cells) to A, D is the diffusion coefficient (cm^2/sec), and R is radius of adsorbent particles (cm). Since colony counts were used in place of optical density measurements, A

becomes C for the concentration of bacteria at time t (CFU/mL). Upon integration Equation 10 becomes Equation 13.

$$C_t = C_0 e^{\frac{1}{2.303}kt + k\prime t} \tag{15}$$

F. columnare was first modeled as a function of adsorption neglecting the effect of UV die off since no significant amount was measured. The bacteria in solution are constantly removed from solution by natural decay; therefore it was difficult to tease out the ratio of decay from bacteria in solution and adsorbed onto the algal turf. The above adsorption models are also developed for non-living particles that accumulate without die-off allowing for accurate measurement of mass adsorbed. For simplicity of modeling adsorption it was assumed that all bacteria removed from solution were adsorbed and accumulated on the algal turf. Natural die-off was included in the number of bacteria adsorbed to the algal turf (Figure 4.12). Kinetic data is presented in Table 6. A graphical mass balance of *F. columnare* in the system is presented in Figure 4.13.

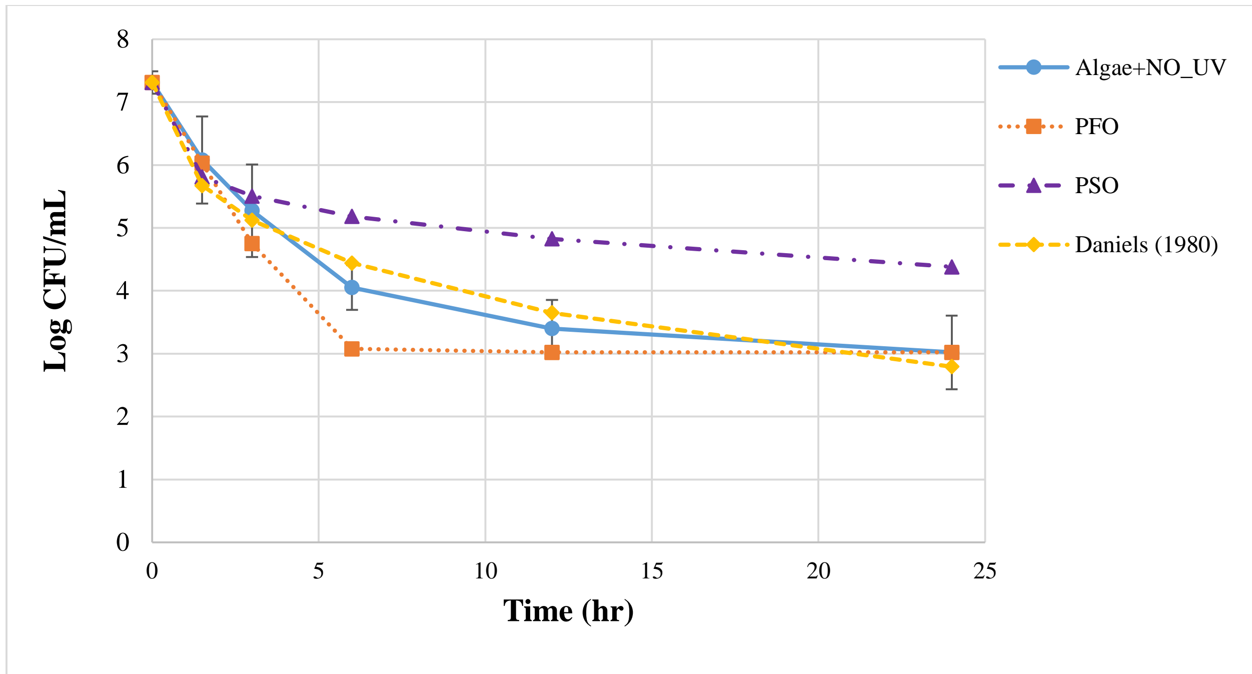

Figure 4.12. Results of modeling total removal of F. columnare from the water column. Algae+NO_UV is averaged from all measurements with surge. Models shown include pseudo-first order (PFO), pseudo-second order (PSO) and the model by Daniels (1980).

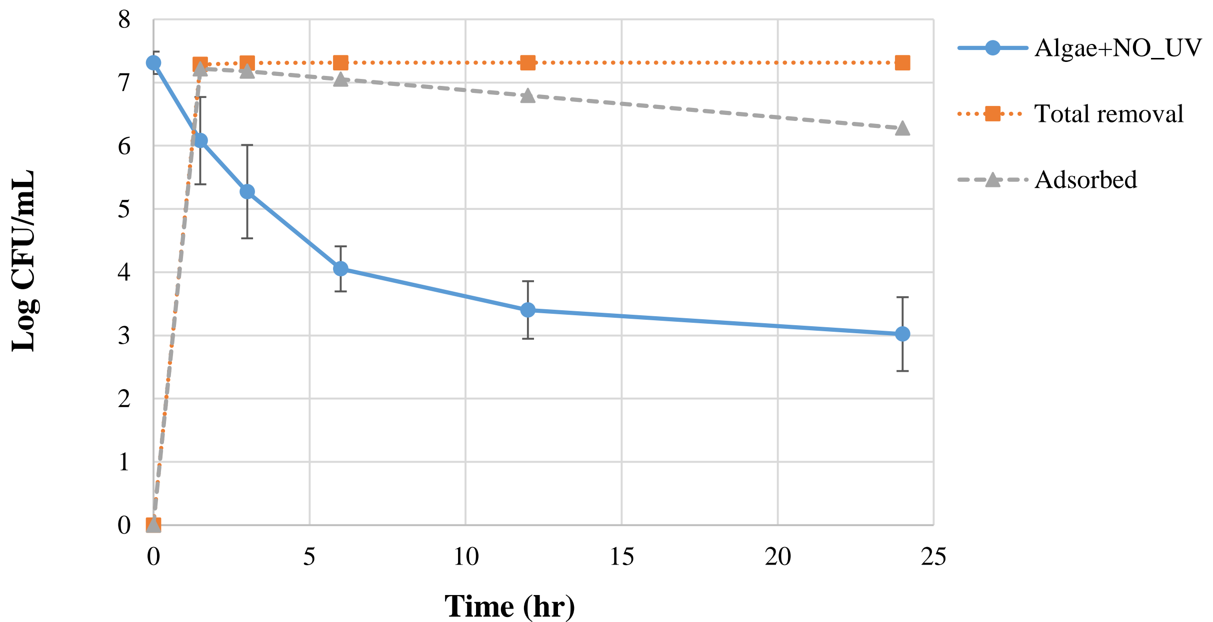

Figure 4.13. Mass balance on bacteria in the system over time. Total removal is the amount of F. columnare removed from the water column, Adsorbed is the number of F. columnare adsorbed to algae adjusted for dark die-off and Algae+NO_UV is the concentration of bacteria in the water column.

Table 4.7. Kinetic data used for model evaluation of F. columnare experimental data with Chi Square goodness-of-fit and Root Mean Square Deviation (RMSD).

Kinetic equation	q_e (CFU/g)	k (1/hr)	k' (1/hr)	t_0 (hr)	RMSD	Chi Square
PFO	8.61E+8	-2.07E+0	-	2.75E+0	0.9377	0.9532
PSO	8.62E+8	2.30E-8	-	-	0.4783	0.9949
Daniels (1980)	-	6.78E-3	-4.86E-1	-	0.2747	0.9998

For *E. coli* the same models for *F. columnare* were evaluated in addition to a simple first order kinetic model. Modeling for *E. coli* also included the first order model for solar mediated disinfection by Craggs et al. (2004). After evaluating all of the adsorption models it was determined that a simple first order kinetic model best fit the experimental data for the algae treatment absent of UV light (Figure 4.14). This model was combined with the first order model of Craggs et al. (2004) and plotted against the experimental data for the algae treatment with UV (Figure 4.15). The final equation for removal of *E. coli* as a function of UV light and algal turf is shown in Equation 14.

$$\ln(C) = \ln(C_0) - k_{ads}t - k_d t - k_s S(\theta) \quad (16)$$

where k_{ads} is the first order rate constant for adsorption, k_d is the dark die-off constant, k_s is the light disinfection rate coefficient and $S(\theta)$ is the insolation as a function of exposure time θ. The adsorption rate constant k_{ads} was determined from the linear plot of $\ln(C) - k_d t$ vs. t. An

insolation value of 0.41 MJ/m^2 was calculated from spectral data of the UV lamp configuration and used for *S* in equation 14. Kinetic data is presented in table 4.8.

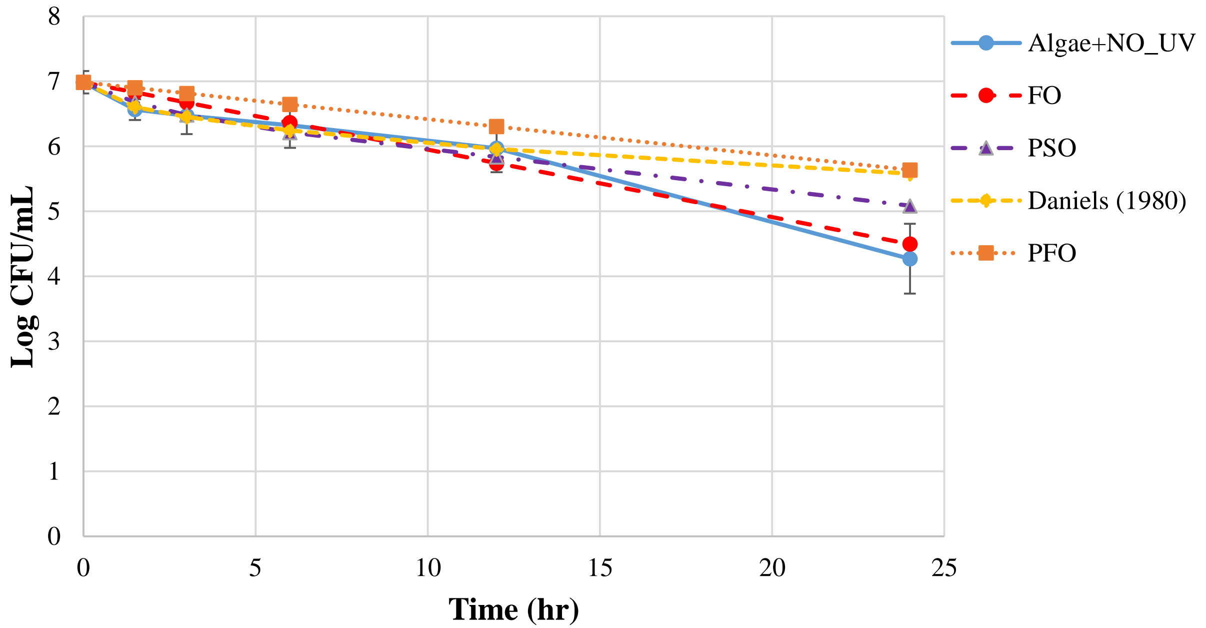

Figure 4.14. Results of modeling total removal of E. coli from the water column as a function of adsorption and dark die-off. Algae+NO_UV is averaged from all measurements with surge. Models shown include pseudo-first order (PFO), pseudo-second order (PSO), first order (FO) and the model by Daniels (1980).

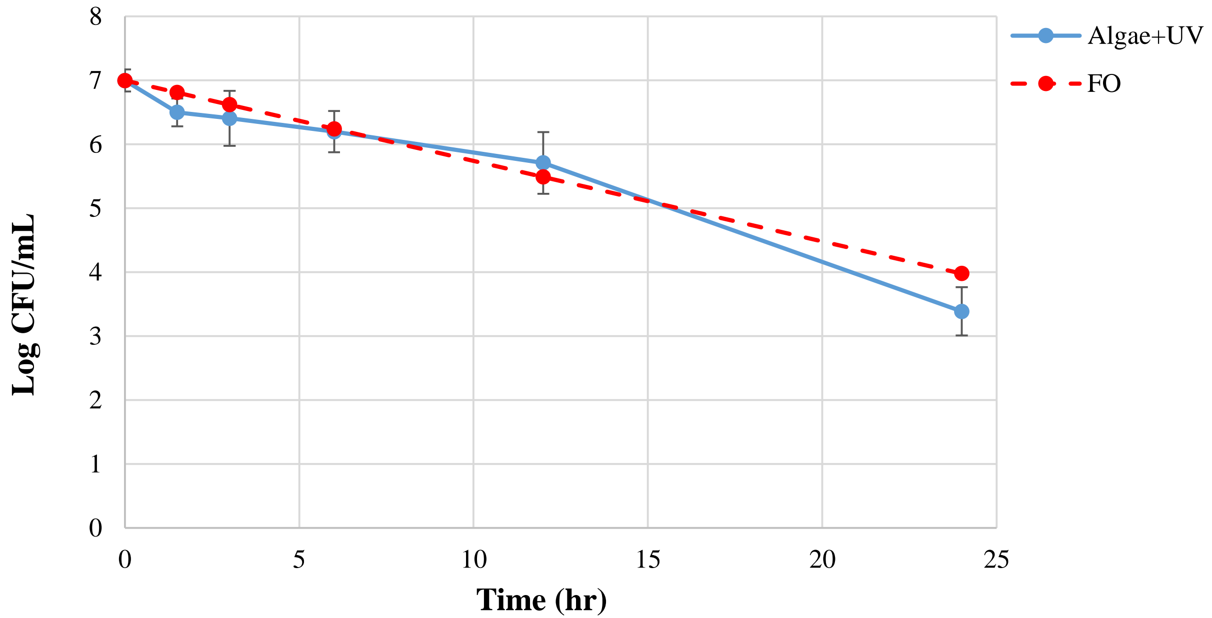

Figure 4.15. Results of modeling total removal of E. coli from the water column as a function of adsorption, dark die-off and UV exposure. Algae+UV is the average value of all measurements and FO is the first order kinetic model.

Table 4.8. Kinetic data used for model evaluation of E. coli experimental data with Chi Square goodness-of-fit and Root Mean Square Deviation (RMSD). FO (ads) is kinetic data and fit for first order adsorption only and FO (ads+UV) is kinetic data and fit for adsorption and UV removal.

Kinetic equation	q_e (CFU/g)	k (1/hr)	k' (1/hr)	t_0 (hr)	k_d (1/hr)	k_{ads} (1/hr)	k_s (m^2/J)	RMSD	Chi Square
PFO	4.05E+8	-1.31E-1	-	5.80E+0	-	-	-	5.936	0.9600
PSO	4.27E+8	1.46E-9	-	-	-	-	-	4.871	0.9722
Daniels (1980)	-	6.78E-3	-4.86E-1	-	-	-	-	2.929	0.9892
FO (Ads)	-	-	-	-	-2.52E-2	-2.14E-1	-	1.434	0.9976
FO (Ads+UV)	-	-	-	-	-2.52E-2	-2.14E-1	3.22E-1	0.543	0.9998

Chapter 5: Summary and Conclusions

5.1 Summary

The experimental data collected for this study does indeed show that the ATS has a capacity to remove bacteria from the water column. The effect of the two mechanisms of removal explored in this study, adhesion to the algal turf and solar mediated disinfection, were observed although the effect of solar mediated disinfection (presence of UV) was not statistically significant (Figure 5.1).

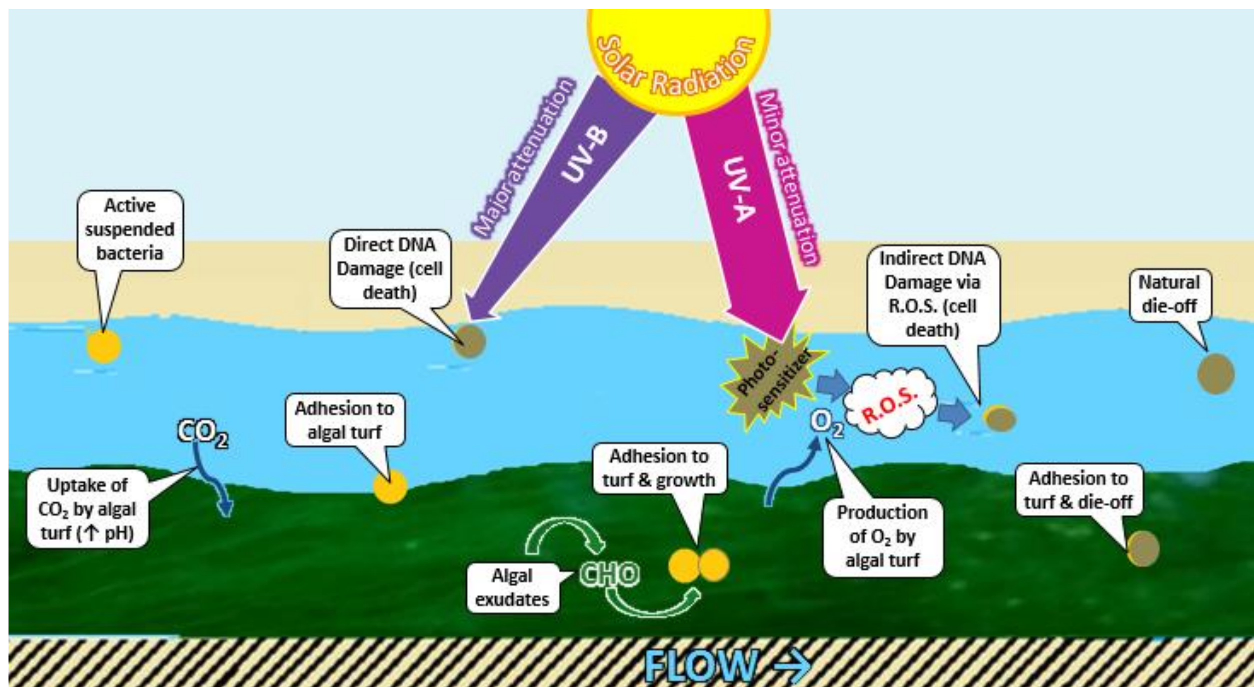

Figure 5.1. Graphic illustration of the pathogen removal processes occurring in the ATS.

The removal of *F. columnare* from the water column appears to be due mainly from the adsorption of *F. columnare* on to the algal turf since no significant difference between treatments

with and without UV were observed (Figure 4.3, 4.4, 4.5 and 4.6). The longer cell length of *F. columnare* compared to *E. coli* may be responsible for the faster removal rate by the algal turf compared to *E. coli* although the ability of *F. columnare* to readily attach and form biofilm must also be considered (Cai et al., 2013). Since it is possible that the surface charge of some green algae and bacteria species may be the same, the most likely candidate for mediating the adsorption of bacteria to algal filaments is the presence of extracellular polysaccharides (EPS) on the surfaces of both algal filaments and bacterial cells (Daniels, 1980). It is also hypothetically possible that the difference in surface charge may indeed play a role in adsorption.

The insignificance of the UV effect of removal on *F. columnare* is most likely due to the combination of adaptation to solar UV radiation along with the low output of UV wavelengths from the Heliovite lamps. The plots of experimental data for *E. coli* removal show that both the presence of an algal turf and UV light do increase the rate of removal; however the effect of UV light was not statistically significant for both treatments with and without an algal turf. However, it can be speculated that for a longer exposure time and/or higher UV intensity that the effect of UV light on removal would be significant.

The operating parameters of the ATS tested in this study appear to have no significant effect on the pathogen removal from the water column. There was no observed difference in removal between a harvested and mature turf suggesting that the physical removal of bacteria by the algal turf appears to be a function of turf surface area rather than volume, turf thickness, or total biomass. The use of a periodic wave pulse or surge also appeared to have no effect on removal compared to the set of experiments without surge; although this experiment was not repeated for the smaller size cells of *E. coli.* The smaller cell size of *E. coli* may have been more susceptible to the effects of the surge than the longer cell size of *F. columnare*. The use of a base

flow may have also affected adsorption by dampening the agitation caused by surge. It is possible that these results do not reflect the behavior on larger flow through systems and may be an artifact of the small recirculating lab scale ATS used in this study. It may be possible that larger ATS would see a greater algal turf concentration of bacteria near the upstream due to surge agitation of algal filaments.

Conductivity and pH varied during the *F. columnare* experiments mainly due to the presence of the algae. As reviewed by Groff and LaPatra (2000) the pH of the water being tested does not significantly affect the rate of die-off *F. columnare* above pH 6. This was confirmed in a preliminary experiment carried out for this research in which the pH values of 7.1, 8.1 and 9.1 saw the same level of die-off after 24 hours (Figure 5.2). The work of Chowdhury and Wakabayshi (1988) seem to indicate that the rate of die-off is not significantly affected by the conductivity difference between algae and no-algae treatments observed during the above experiments. Chowdhury and Wakabayshi (1988) also discovered that the die-off rate of *F. columnare* in F/2 and tap water is not significantly different.

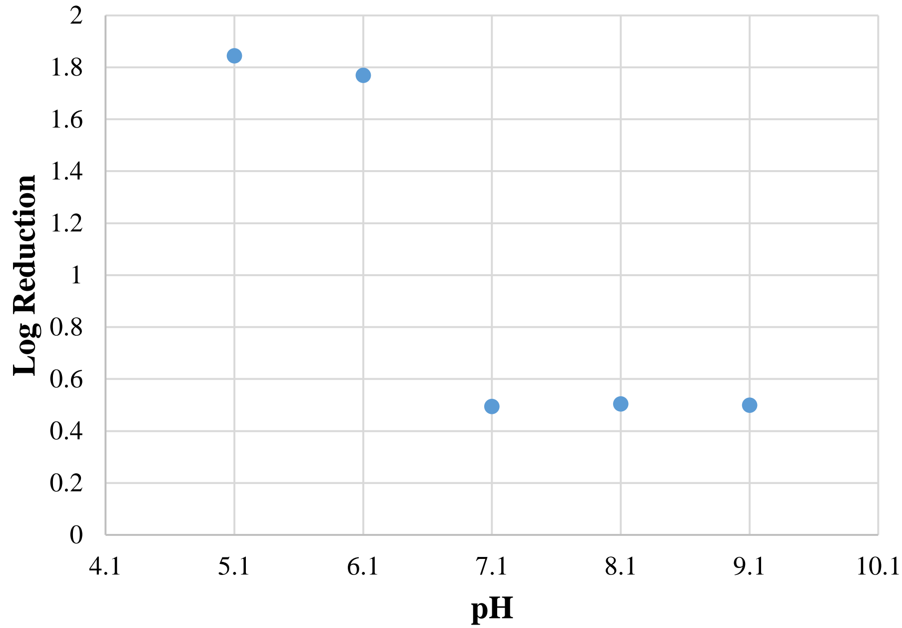

Figure 5.2. Preliminary data for the dark die off rate of F. columnare after 24 hours. The experiment was conducted at 28°C using treatments of similar conductivity and concentration of F/2 algae growth media. Note: data is from one sample (n=1).

For both *F. columnare* and *E. coli* there is very little difference in the shape of the removal curves between a mature and harvested turf suggesting that bacteria are mainly adsorbed to the surface layer of the algal turf and most likely do not readily penetrate into the turf (Figure 4.3, 4.4, 4.7, 4.8). During the course of this study it was observed that when the algal turf was allowed to grow under low nutrient conditions that the resulting turf would be composed of longer, less clumped filaments as opposed to the more matted turf observed under standard F/2 nutrient conditions. This difference in turf structure might have affected the pathogen removal kinetics since the more filamentous turf would likely be more easily agitated by surge than a matted turf allowing for exposure to more adsorption sites on the algae. Likewise, this might have also increased the resuspension or desorption of bacteria into the water column altering the effect of the periodic surge on removal. For the collected removal data, all algal turf was grown

using standard F/2 concentrations. Low nutrient algal growth only occurred during breaks from research when no data was collected.

As experiments with *F. columnare* progressed (experiments with no surge were conducted after the experiments with surge) it was noticed that the overall removal of *F. columnare* from the water column on treatments without algae increased after 24 hours (Figure 4.5 and 4.6). This is seen more prominently in Figure 4.5 since disinfection had not occurred for nine days as opposed to Figure 4.6 which was one day after disinfection, i.e., biofilm formation. This might be due to the buildup of cellular organic matter on the surfaces of the reactor plumbing lending itself to easier biofilm formation. The disinfection protocol used for this study was probably not effective at removing biofilm buildup to completely control for *F. columnare* surface attachment. It was also observed during the experiments with *F. columnare* that the conductivity of the reactors without algae would increase more than the reactors with algae. Again, this may have been due to the release of salts built up on a biofilm on the surfaces of the reactor. After flushing the reactors absent of algae with DI water it was noticed that the conductivity of the DI water increased over a 24 hour period indicating a buildup of salts in the system. The increase in conductivity of the reactors without algae was small and likely had no effect on removal.

The models used for predicting the removal of pathogens from the water column had varying degrees of fitness. For *F. columnare* both the pseudo-first order and pseudo-second order models tended to fall outside of the error bars of the removal curve, and more so for the pseudo-second order model (Figure 4.12). The Daniels (1980) model for adsorption predicted a removal curve that fit the observed data more closely having the lowest RMSD and highest Chi square (Table 4.7). Since die-off is occurring for both bacteria in the water column and adsorbed onto

the algae there is no way of knowing the relative rate of die off between the two using collected data. For modeling purposes it was assumed that all removal of was due to adsorption to the algae and that any die-off that occurred was from bacteria adsorbed to the algae (Figure 4.13). Modeling for UV removal was not included for *F. columnare* since there was no noticeable variation in the data between treatments with UV and without UV.

The adsorption models did not explain the removal of *E. coli* well (Figure 4.14). This may be a result of insufficient experiment time since Figures 4.7 and 4.8 suggest that an equilibrium of *E. coli* adsorption was not reached. After this observation a simple first order reaction model was used which fit more closely to the observed data compared to the other adsorption models (Figure 4.14). The fully developed model for *E. coli*, including the effects of adsorption and UV, fit the observed data fairly well although at 24 hours the predicted data falls outside of the error bars of the observed data (Figure 4.15, Table 4.8). It is possible that after 24 hours the first order model may lose fit with observed data. The first order kinetic coefficient developed by Schumacher and Sekoulov (2003) for removal of *E. coli* using an algal biofilm in sunlight is almost three times greater (k = 0.9009) than the overall coefficient developed in this study. This further substantiates that the removal rate of *E. coli* by the lab scale ATS as a function of UV radiation was conservative.

The adsorption models that were evaluated all have shortcomings in application to the lab scale ATS. The pseudo-first order and pseudo-second order models, which were developed for the adsorption of metal ions, had the least best fit. The Daniels (1980) model, while fitting *F. columnare* reasonably well had poor fit with *E. coli* although the removal curve for E. coli did not appear to reach an equilibrium. This suggests that the adsorption of bacteria to the algal turf is species specific and any adsorption model may need to account for this discrepancy. Also,

since the objective of this study was to establish a context for modeling, the effect of desorption of bacteria from the algal turf was not explored. The development of isotherms for *F. columnare* and *E. coli* adsorption was attempted but not completed due to time limitations on the study.

5.2 Limitations of study

One of the main limitations of this study was the available time for data collection. Each experimental run required a nine-day period of algal turf growth due to the disinfection protocols after each experiment. This limited the sample size of collected data due to the timeframe of the scope of work likely affecting the statistical power of the results. Also, while bringing the experiment into the lab gave more advantages than disadvantages, the ability for the results of the study to be translated to real world applications is still somewhat limited due to the idealized conditions of the lab. However, unlike larger ATS installations that primarily function as a plug flow reactors (PFR) the lab scale ATS is basically a batch reactor. This suggests that the length of channel needed to achieve significant removal in a flow through ATS would be great when considering the rate of removal of removal of the lab scale ATS. The results of this study likely represent a conservative value for the removal rate possible in real world ATS installations.

The initial concentration of bacteria used in the experiment to inoculate the reactors is an exaggerated value for concentrations seen in stormwater (5 CFU/100mL to 1×10^6 CFU/100mL) and surface waters (~1×10^7 CFU/100mL); however, the concentration values used in this study do approach the upper maximum values used in research and measured in wastewater (Clary et al., 2008; Gannon and Busse, 1989; Craig et al., 2004; Shoemaker and Lafrentz, 2014; Kadir and Nelson, 2014).

Although the MS agar used for *F. columnare* was supplemented with 1 µg/mL of Tobramycin, enumeration of *F. columnare* was difficult at the 12 hour and 24 hour sample point

due to the level of background bacteria on the incubated plate. At 24 hours the concentration of *F. columnare* was so low that the background level of bacteria would almost prevent detection of any colonies of *F. columnare* at a dilution level of 10^0. This was always the case for algae samples plated for *F. columnare* enumeration in which under 30 colonies had to be counted at a higher dilution level due to the amount of background bacteria on the plate at the lower dilution level.

The MacConkey agar used for the enumeration of *E. coli* most likely did not allow for a total count of bacteria in sampled water (0.63 orders of magnitude fewer bacteria recovered compared to LB-Luria agar). This was likely due to the fact that the strain DH10B was unable to utilize lactose, the primary carbon source in MacConkey agar, relying on less abundant carbon sources in the media. This does not necessarily affect the results of the experiment since this error was evenly distributed among treatments preserving the overall rate of removal.

The variation in conductivity between the algae and no algae treatments using *E. coli* is significant but well under the limit that can accelerate die-off of *E. coli* (He et al., 2007). It has been observed that at conductivity values less than 2 mS/cm there is no significant effect on die-off in ponded water which is well above the range of conductivity values observed during the experiments (He et al., 2007).

The adsorption models used to predict the behavior do not always fit the data. Also, since bacteria are able to die and avoid enumeration when plated, it is difficult to predict adsorption kinetics compared to non-living particles although adesorption term in the equation could satisfy this deficiency. A possible solution would be to use an inactive solution of bacteria (non-destructive method) and observe the optical density over time. This would prevent any cell

reproduction allowing for the measurement of adsorption through change in absorbance of sampled reservoir water over time.

This study also does not consider other variables outside of adsorption and UV disinfection such as the influence of temperature, pH, DO and potentially toxic algal exudates as well as their interaction with UV radiation. The introduction of these other variables may or may not enhance the removal rate of active bacteria from the water column but treatment pond research would seem to indicate an increase in removal (Davies-Colley et al., 1999; Craggs et al., 2004; Curtis et al., 1992; Cole, 1982).

5.3 Translation of study results to real world application

It can be seen in Figure 5.3 that the spectral output of the lamp configuration used for experimentation was much less than full sunlight at noon on a spring day. Referring to Figure 3.6, the UV lamp configuration provides a little more irradiance than a cloudy day. This may explain why the effect of direct and indirect cellular damage by UV light was not clearly seen in collected data. Overall the Sunsource Heliovite lamps provided only 36% of UV-B and 13% of UV-A available in noon sunlight in the data collected in Figure 3.6. In particular the lack of UV-A irradiance most likely contributed to the inability of ROS formation via photosensiters. Past research studying disinfection with ROS used natural sunlight or solar simulators that more closely approached the spectral output of incident solar radiation (Kohn and Nelson, 2007; Liltved and Landfald, 2000).

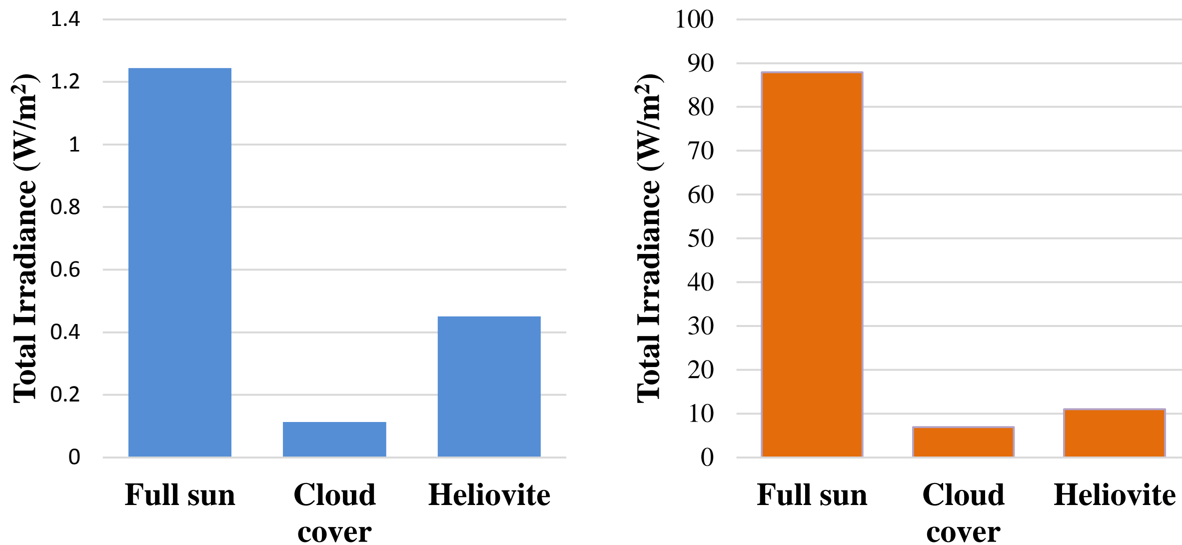

Figure 5.3. Comparison of the observed irradiance for the UV-B (Left) and UV-A (right)band between natural sunlight and the Heliovite lamp measured at 5 cm. The UV irradiance data was extracted from the spectral irradiance data in Figure 3.6.

In the experiments of Beckinghausen et al. (2014) a Reptisun 10.0 UV-B (Zoo Med, San Luis Obispo, CA) lamp was used to simulate solar UV proving output similar to the observed output of the Heliovite bulb. However, Beckinghausen et al. (2014) used a greater distance between the lamp and the receiving surface (12 cm) compared to the distance used in this study (5 cm). This suggests that the Reptisun 10.0 UV-B lamp probably provides more UV radiation and would have been a better lamp to use for simulating solar UV. The Heliovite lamp was selected since it provided high PAR for algal photosynthesis although it was decided after the fact that the Heliovite lamps would not be used to grow the algal turf since they were expensive and might require replacement to maintain consistent output.

As stated above it can be speculated that larger ATS installations exposed to solar radiation likely experience enhanced removal rates of *E. coli* in full sunlight. The removal model developed for *E. coli* can be adjusted to predict the removal in this environment. Figure 5.4 shows model output using the measured solar irradiance for full sun (approximately 1208 W/m^2)

in Figure 3.6. However, the model uses the same value of k_s associated with the lab scale ATS which may or may not be an underestimation of the k_s associated with larger ATS installations. Figure 5.4 projects an increase in the removal rate of *E. coli* in full sun and that a low concentration of *E. coli* at 100 CFU/mL, which may be considered as a typical concentration in stormwater, can be reduced to 10 CFU/100mL in three hours (Clary et al., 2008; Gannon and Busse, 1989). At a velocity of 25.4 cm/s an ATS flowway would need to be ~2700m in length to achieve this level of reduction in stormwater. For a typical 100 m long flow through ATS at the same velocity a reduction to 92 CFU/mL from the initial 100 CFU/mL would be realized.

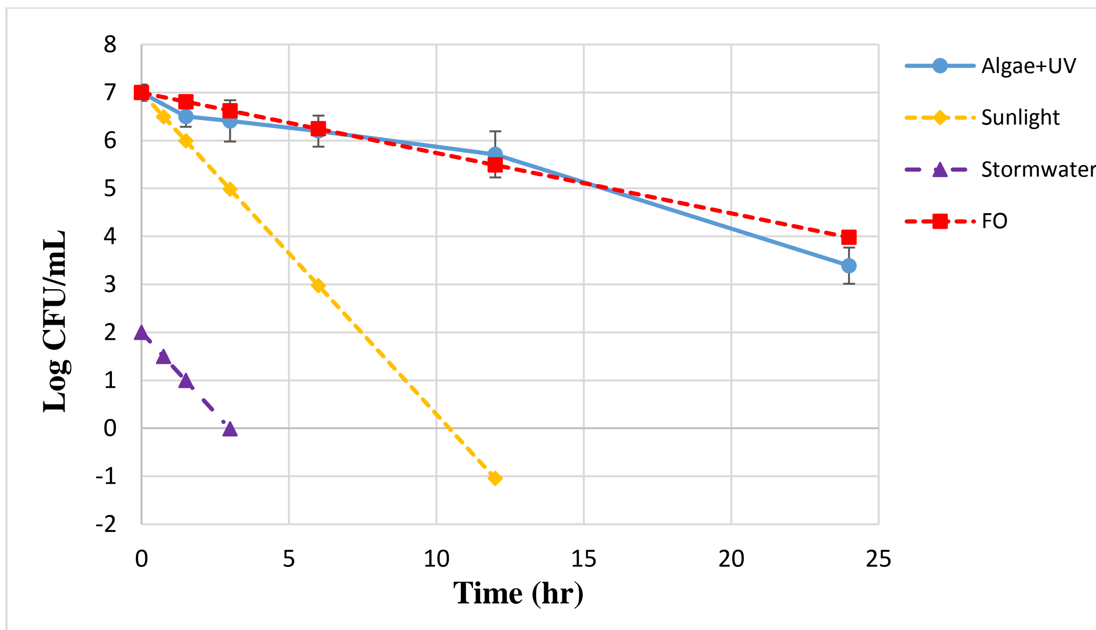

Figure 5.4. Model output for removal of E. coli with surge where Algae+UV is the removal from the lab scale ATS as a function of lamp irradiance, Sunlight is the removal of the lab scale ATS adjusted for solar irradiance and Stormwater is the removal of an estimated concentration of E.coli in stormwater adjusted for solar irradiance.

5.5 Conclusions

The research presented in this study indicates that algal turf scrubber technology has the potential for the treatment of pathogens in polluted waters. The combination of the effects of bacterial sorption to algal filaments and ultraviolet radiation promote the removal and die-off of

pathogens from the water column. Several conclusions can be made regarding the results of this study:

1. An algal turf is able to remove bacteria from a column of water over time in which the rate of removal is a function of the species of bacteria. This is seen when comparing the graphs of log removal from the water column between *F. columnare* and *E. coli* in which the longer and biofilm prone *F. columnare* had a faster rate of removal compared to the shorter and less biofilm prone *E. coli*. On average, the lab scale algal turf scrubber was able to achieve a 3.3 and 2.6 order of magnitude greater removal than control treatments over a 24 hour period 0for *F. columnare* and *E. coli*, respectively.
2. The removal of bacteria from a column of water by an algal turf is most likely a function of turf surface area and not dependent on the mass of the algal turf. This suggests that the rate of removal by the algal turf is not dependent on the growth stage of the turf.
3. While not statistically significant, UV radiation did affect the removal rate of *E. coli* from water column. Under greater UV exposure or recirculation time it would be expected that the effect would be statistically significant.
4. The first order model for *E. coli* removal developed from observed lab scale ATS data suggests that the length of channel required for significant removal of *E. coli* in outdoor flow through pilot and landscape installations would most likely not be feasible. However, if these systems are operated in a batch/recirculation mode then the ATS shows promise for remediating water of microbial pollutants.

5.4 Recommendations for future work

Continued research on this lab scale reactor system could include validating and utilizing a UV lamp that produce spectral output similar to sunlight in the UV-B and UV-A range. This would probably allow for a more accurate comparison of what could occur on larger outdoor systems. The supplementation of humic acids could also be integrated into future research to validate their importance in exogenous disinfection mechanisms. Experimentation at lower concentrations of pathogen would also benefit this study by simulating concentrations found in surface and storm waters.

The interaction effect of high pH and sunlight enhances solar mediated disinfection for enteric pathogens (Davies-Colley et al., 1999). This effect was not tested for in this study although it probably warrants further investigation since the ATS typically increases the pH of treated water over the length of the reactor due to algal photosynthesis.

The adsorption process of bacteria to algae is probably more complex than presented in this study and deserves additional research into mechanisms of adsorption. This area of future research would greatly aid in the development of models that can more accurately predict the behavior of bacterial adhesion in algal turf environments.

More importantly, validation of the removal observations seen in this research should be conducted on pilot and landscape scale systems that are exposed to the variability of weather and season. The model could also be expanded to include other variables that can affect pathogen die-off such as pH and temperature. The observation of removal in outdoor settings over a one year period would give a good indication of the effectiveness of these systems for removing pathogenic bacteria from treated water. Eventually a calibrated model could be used for the

design of outdoor systems if observations of real world systems prove effective pathogen removal.

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Appendix A: Lab scale ATS design and validation

Introduction

Algal turf scrubbers (ATS) are a promising technology for the remediation of natural and waste waters using the energy from the sun to drive the bulk of the remediation work. Prior research has shown that these systems are able to remove inorganic nutrients from treated water as well as metals and organic compounds (1, 2, 5, 6 and 7). Although these systems have been under study for decades now there are still many aspects of this system that have not been sufficiently studied such as the effect of wave pulse on biomass productivity. Since these systems are usually large in size and are located in outdoor settings, ATS research is limited due to the constraints of replication and environmental variability. In order to test treatments and control variables in algal turf scrubbers it is necessary to scale these systems down and bring them into the lab where the environmental variables are more easily controlled and multiple identical systems can be used for increased replication.

Currently, there are no published studies for lab scale algal turf scrubbers that follow the same design criteria as pilot scale and landscape flow way systems such as the characteristically high length to width ratio and period wave pulse (3). This publication presents a pilot growth study on a low cost and off the shelf recirculating algal turf scrubber design for laboratory use that more closely resembles the flow way design of larger systems in research and industry. It is the intent of this paper to initiate a standardized lab scale ATS reactor that will fulfill the need for a more consistent method of collecting and reporting data and results from ATS experiments as concluded by Berner et al. (3). The main design goal was therefore to draft a lab scale ATS design that utilizes low cost and off the shelf parts allowing for researchers to construct multiple reactors for greater replication.

Methods

Construction

All of the parts used to assemble the lab scale ATS reactors were purchased from builder supply stores excluding the magnetic drive pumps (Danner Supreme Classic MD3, Danner Manufacturing, Inc.) and plastic bat house netting (Industrial Netting, Minneapolis, MN) used as a substratum. The reactor frame of the system was constructed from dimension lumber and plywood and assembled using off the shelf hardware (Figure 1). The frame itself is composed of several sub-assemblies that can be broken down in order to more easily relocate the system if needed. A residential rain gutter with a symmetrical cross section is used as the channel for the reactor. Steel brackets are used to constrain the rain gutter to an adjustable sub-frame on the reactor that allows for slope adjustment from 0 to 3% (Figure 2).The reactor plumbing from the reservoir is routed through the middle of the frame assembly using 305 cm of schedule 40 PVC. Vinyl hose was used for a flexible plumbing connection from the reservoir line to a ½” tee connected to two ball valves that controlled flow into the gutter (base flow) and to the surge device (Figure 3).

The surge device is modified from an original design by Eric Borneman in *Aquarium corals: Selection, Husbandry, and Natural History* to allow for adjustment of surge volume (amplitude) and period (frequency) (4). The Borneman surge uses a toilet flush valve to produce a surge once the height of water in a tank has reached a preset level determined by the length of chain connecting a float to the valve. The modification was accomplished using foam inserts that can be added or removed from a plastic container to alter the volume of the surge. A Coroplast stop to prevent the valve from completely opening can also be installed inside the device to allow for additional control of surge amplitude. The period of the surge is controlled by a ball valve that allows for flow rate into the surge tank to be adjusted. The surge device requires bi-weekly cleaning to remove biofilm on the flush valve in order to maintain consistent surge amplitude. Tipping buckets that were initially manufactured and tested for use in the reactor proved to be unreliable at the scale required for the reactor and would require more costly materials for proper function. The Borneman surge device was used as a less costly alternative to the standard tipping bucket that also eliminated splashing which can lead to significant water loss in reservoirs of small volume. It is also a low tech solution and comparable in cost to a solenoid valve operated surge which was also explored as an alternative to the tipping bucket in this project.

The total volume of the water in the reactor used for the pilot study was 45.4 liters yielding a reservoir residence time of 1.5 minutes for the combined flowrate of the two 22 LPM pumps. The volume of the reservoir has no upper bounds but lower volumes are limited by flowrate or retention time in the channel, reservoir geometry, and physical properties of the pump. The smallest volume utilized during testing of the reactor was 4.7 L. The substrate used for attachment of benthic algae was 1/8" UV stabilized polyethylene bat house netting which was installed in each gutter. The netting was cut to fit the gutter and measured 305 cm in length by 7.6 cm (2318 cm^2); however only a 244 cm by 7.6 cm (1854 cm^2) area was fully illuminated to allow for significant algal growth.

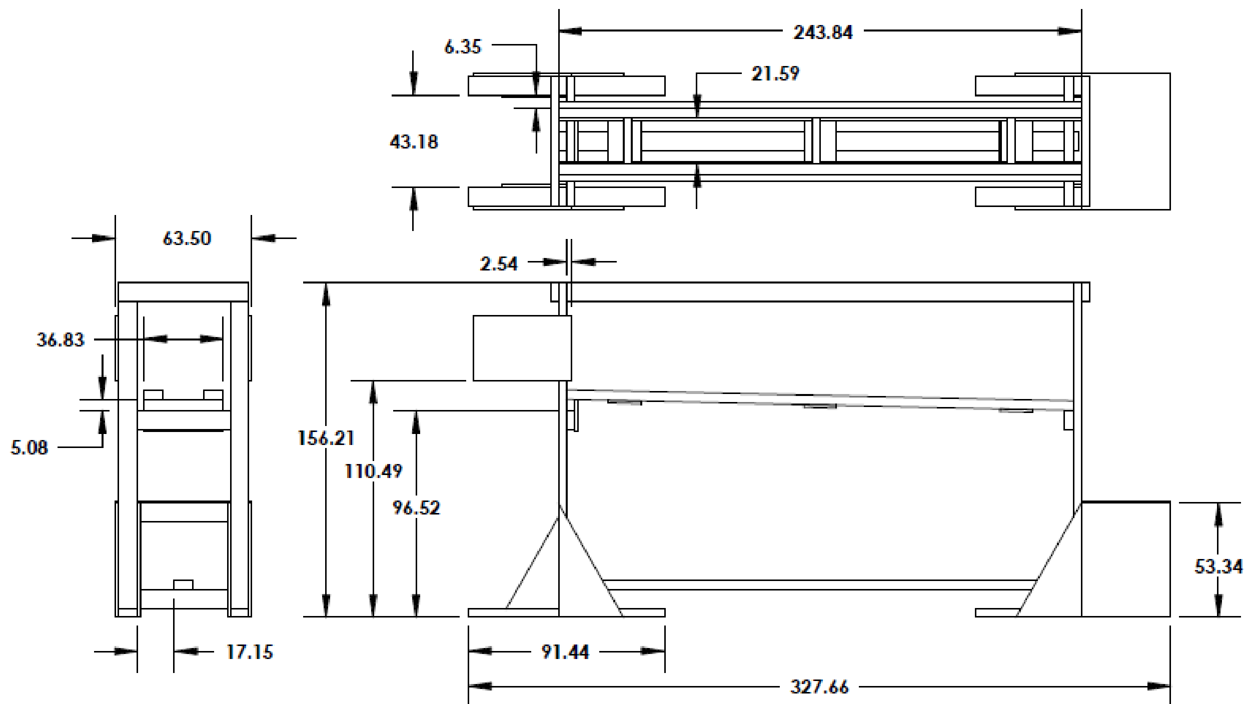

Figure 1. Overall dimensions for the lumber and plywood components of the reactor frame assembly. All dimensions are in centimeters.

1. Valve and surge mounting board
2. Surge device
3. 305cm vinyl rain gutter
4. Mounted power strips
5. 122cm T8 fluorescent fixture
6. Gutter mounting bracket
7. Reservoir/pump

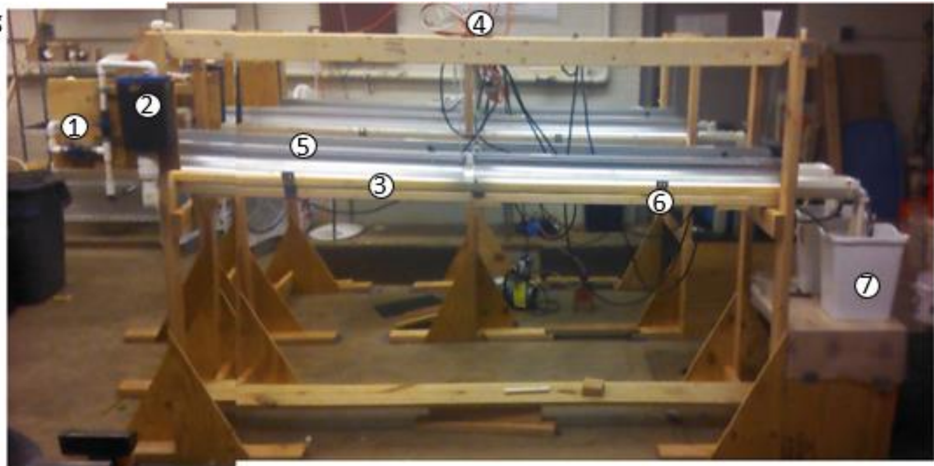

Figure 2. Composite side view image of the reactor with callouts for major components in the complete reactor assembly.

1. Vinyl hose connecting pump to valves
2. Base flow valve
3. Surge device valve
4. Mounting board
5. Surge device tank
6. Foam inserts
7. Flush valve
8. Surge to gutter outlet
9. Base flow to gutter outlet

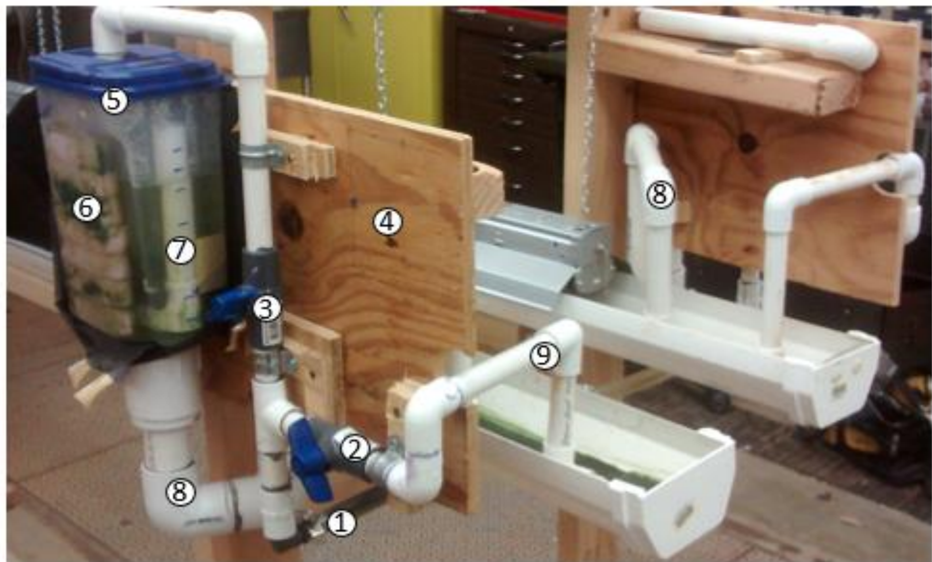

Figure 3. View of reactor plumbing components showing adjustable ball valves distributing flow to the surge device and gutter. Also shown are the components of the surge device: tank, foam inserts and flush valve.

Table 1. Parts list used for constructing the system used for the pilot growth experiment. All cost values are in US dollars and were priced on 1/13/2016 from vendor websites using local in stock items.

Sub-system	Item	Source	Cost	Qty	Total
Frame	2 in. x 4 in. x 96 in. Premium Kiln-Dried Whitewood Stud	Home Depot	$ 2.82	12	$ 33.84
	15/32 in. x 4 ft. x 8 ft. 3-Ply RTD Sheathing	Home Depot	$ 17.95	1	$ 17.95
	Grip-Rite #8 x 2-1/2 in. Philips Head Coarse Thread Drywall Screws (1 lb.)	Home Depot	$ 6.47	1	$ 6.47
	Campbell Commercial 15-ft Weldless Zinc Plated Steel Chain	Lowes	$ 8.98	1	$ 8.98
	Blue Hawk 12-Pack Steel Screw Hooks	Lowes	$ 2.93	1	$ 2.93
	USP 2-in x 4-in Triple Zinc Slant Nail Joist Hanger	Lowes	$ 0.75	4	$ 3.00
	1/4 in.-20 tpi x 5/8 in. Nylon Hex Bolt (2-Piece per Bag)	Home Depot	$ 0.65	3	$ 1.95
	Everbilt 1/4 in. x 5/8 in. Black Neoprene Washer (4-Piece)	Home Depot	$ 0.86	2	$ 1.72
	1/4 in.-20 tpi x 1-1/2 in. Nylon Hex Bolts (2-Piece)	Home Depot	$ 0.71	1	$ 0.71
	USP 5-in x 1-13/16-in Nail Plate	Lowes	$ 0.58	8	$ 4.64
	The Hillman Group 20-Count #6 x 0.5-in Flat-Head Zinc-Plated Wood Screws	Lowes	$ 1.24	2	$ 2.48
Electrical and Power	Utilitech 6-Outlet Power Strip with Built-in Circuit Breaker	Lowes	$ 3.97	2	$ 7.94
	4 ft. T8 32-Watt Cool White Alto Linear Fluorescent Light Bulb (2-Pack)	Home Depot	$ 8.97	4	$ 35.88
	Lithonia Lighting 2-Light Fluorescent White Cold Weather Shop Light	Home Depot	$ 19.89	4	$ 79.56
	Danner magnetic drive pump 350 gph	Pentaires.com	$ 69.99	2	$ 139.98
Surge Device	Charlotte Pipe 2-in dia PVC Fitting Adapter Fitting	Lowes	$ 1.81	2	$ 3.62
	LASCO 2-in Dia x 1-1/4-in Dia PVC Sch 40 Bushing	Lowes	$ 1.35	2	$ 2.70
	LASCO 1-1/4-in Dia x 1-in Dia PVC Sch 40 Bushing	Lowes	$ 1.26	2	$ 2.52
	LASCO 1-in Dia x 3/4-in Dia PVC Sch 40 Bushing	Lowes	$ 0.77	2	$ 1.54
	LASCO 3/4-in Dia 90-Degree PVC Sch 40 Slip Elbow	Lowes	$ 0.53	6	$ 3.18
	LASCO 3/4-in Dia 90-Degree PVC Sch 40 Slip Elbow	Lowes	$ 2.67	1	$ 2.67
	LASCO 1-1/4-in Dia 90-Degree PVC Sch 40 Slip Elbow	Lowes	$ 1.26	2	$ 2.52
	Mainstays 14 Cup Storage Dispenser	Walmart	$ 5.86	2	$ 11.72
	Plumb Pak Universal Flush Valve	Lowes	$ 10.39	4	$ 41.56
	2 ft. x 2 ft. Foamular insulation project panel	Home Depot	$ 5.48	1	$ 5.48
	Nalgene® Polypropylene Economy Bottles, Wide Mouth, Thermo Scientific	VWR	$ 3.16	2	$ 6.32
Plumbing	Samar 5/8-in x 10-ft PVC Clear Vinyl Tubing	Lowes	$ 4.59	1	$ 4.59
	1/2 in. x 10 ft. PVC Sch. 40 Plain-End Pipe	Home Depot	$ 2.10	3	$ 6.30
	LASCO 1/2-in Dia 90-Degree PVC Sch 40 Slip Elbow	Lowes	$ 0.19	12	$ 2.28
	NDS 1/2 in. PVC Sch. 40 Slip Ball Valve	Home Depot	$ 2.65	4	$ 10.60
	1/2 in. Schedule 40 PVC Female Adapter	Home Depot	$ 0.64	6	$ 3.84
	Genova 1/2-in Dia Insert Adapter Plastic Coil Fittings	Lowes	$ 1.43	2	$ 2.86
	Genova 1/2-in Dia 90 Elbow Plastic Coil Fittings	Lowes	$ 0.83	6	$ 4.98
	DURA 1/2 in. Schedule 40 PVC Tee	Home Depot	$ 0.73	2	$ 1.46
	AMERICAN VALVE 10-Pack 1/2-in-1/2-in dia Galvanized 2-Hole Pipe Straps	Lowes	$ 1.97	2	$ 3.94
	Murray 10-Pack 3/4-in - 1-1/2-in Dia Stainless Steel Adjustable Clamps	Lowes	$ 8.22	1	$ 8.22
	Genova 4.5-in x 120-in Half Round Gutter	Lowes	$ 6.98	2	$ 13.96
	Raingo Vinyl Half Round Gutter End Cap	Lowes	$ 3.28	2	$ 6.56
					$ 467.31

Pilot Growth Experiment

To observe and analyze algal growth of the design, a pilot growth experiment was conducted between a reactor with a wave pulse (surge treatment) and a reactor without a wave pulse (no surge control). During the experiment the reactor was housed in the basement of the Auburn University Biosystems Engineering department and subject to the temperature variation of the lab. The reactors were seeded using a mixed culture of algae collected from a freshwater aquaculture production facility. This algae was introduced into the reactor and allowed to colonize for several weeks before beginning the growth experiment. During the experiment, the mixed culture of algae was grown using Proline F/2 algae food (Pentaire Aquatic Ecosystems) at half of the recommended concentration using dechlorinated tap water at a volume of 45.4 liters in the reactor reservoir.

An average channel velocity for the surge and no surge reactors was set at 22 and 23 cm/s respectively using a 2% channel slope. The modified Borneman surge device was configured to provide a periodic surge averaging 700 mL (±30 mL) every 15 seconds (±0.23 sec.) for the surge treatment resulting in 54% of overall system flow through the surge and 46% to the base flow outlet. For the no surge treatment all of the system flow went to the base flow outlet. Flow rate measured at each gutter outfall at the reservoir averaged 5.0 LPM (±.2 LPM) for the surge reactor and 5.2 LPM for the no surge reactor. The water depth above the substratum averaged approximately 1.3 cm.

Each reactor was illuminated with four 48 inch Phillips Alto II fluorescent lightbulbs that covered 80% of the reactor channel and averaged 306 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at the water surface. The light fixtures were suspended at a height of 6.4 cm from the bottom of the gutter to the edge of the fluorescent lamps using steel chain that allowed for height adjustment of the fixtures. For the duration of the growth experiment the diurnal cycle was maintained at 16 hours of light to 8 hours of dark using a wall outlet timer. To control for water quality variation between the treatment and control both reactors utilized the same reservoir in which the water was exchanged daily to maintain a nutrient concentration and conductivity within a determined range. Water quality measurements including temperature, pH and conductivity were taken daily using a Hannah HI 98130 meter (Hanna Instruments). A submerged data logger was also used to record water temperature at 15-minute intervals. Nitrate was measured periodically using a RQflex plus 10 reflectometer (EMD Millipore) to within ±1 mg/L.

Each reactor channel was divided into three zones for sampling separated by 15.2 cm of buffer that would not be sampled. Each zone was divided into a 24 by 3 inch grid (61 by 7.62 cm). Using a random number generator, a list of grid coordinates within each zone was created that would be used for sampling during each 5-day harvest. Each sample was taken within the selected grid coordinate to the nearest centimeter. Sampling was conducted according to *Methods in Stream Ecology* (8). Briefly, a 1 inch section of PVC pipe was used to isolate the random grid location in a zone of the reactor. Algae was loosened using a stiff bristle brush followed by vacuuming into a small vacuum flask. After collecting the sample, the flask was emptied into a sample bottle following a rinsing of the flask with DI water that was transferred to the sample bottle. The brush and 1 inch PVC section were also rinsed off into the sample bottle. Samples were then emptied into pre-weighed and pre-combusted aluminum weigh boats before drying at 105 °C for 24 hours. Following oven drying the samples were weighed for ash free dry mass (AFDM) and then combusted at 575 °C for three hours to yield ash content.

The remaining algae were harvested by manually scraping the netting using a plastic scraping tool made from chloroplast. The loose algae from each reactor was then collected into plastic beakers and set aside. A plastic mesh paint strainer was used at the outfall of each reactor to catch any loose algae entrained in flow once the pumps were turned back on. After five minutes, any loose alga caught in the netting was added to the respective beaker. This algae was then dried in a food dehydrator on pre-dried and pre-weighed plastic trays for 24 hours before weighing for air dry mass (ADM). Before the algae was placed in the food dehydrator a 15mL centrifuge tube was filled with algae from the beaker and preserved with formalin to observe species composition using light microscopy. Each sample was visually observed for composition and abundance but no formal method was used to quantify species abundance or composition thus microscopy results are anecdotal relying only on visual observation.

Results

Data was collected for ten 5-day harvests over the course of the pilot growth experiment. Water temperature averaged at 23.7 °C ± 1.6 and although there were spikes and drops in temperature data (27.4 °C max, 18.6 °C min), linear regression with harvest productivity data found no significant correlation

between average weekly temperature and harvested biomass (P<0.004). Over the course of the experiment nitrate, pH and conductivity of the reservoir averaged 26 mg/L ± 8, 8.68 ± 0.76 and 0.45 µS/cm ± 0.04 respectively. The mixed culture of algae appeared to be dominated by *Sirogonium sp.* but also included other filamentous species including *Oedogonium,* and *Spirogyra sp.* Suspended colonial algae were also present in the culture and appeared to be predominately *Scenedesmus sp. The* suspended algae growth might have lowered the productivity of benthic algae during the experiment and its abundancem which caused 'greening' of the reservoir water, might be explained by the use of the F/2 medium which is formulated more towards suspended algae species than benthic. Visually, there was no observed shift in species composition or abundance during the course of the pilot growth experiment.

The total biomass harvested from the reactors (ADM) does not appear to show any significant difference between the surge treatment and no surge control (Figure 4). Statistical analysis using a two independent T-test (SAS 9.3, SAS Institute Inc.) agreed with this observation that there is no significant difference in ADM between the two reactors (P=0.2033). AFDM productivity between the surge and no surge reactors show that the surge effect does produce an increase in harvested biomass (Figure 5). The surge effect also appears to increase the percent ash content of harvested biomass as indicated in Figure 6. A two independent T-test revealed that the increase in AFDM and percent ash by the surge treatment was indeed significant with P=0.0409 for AFDM and P=0.0183 for percent ash.

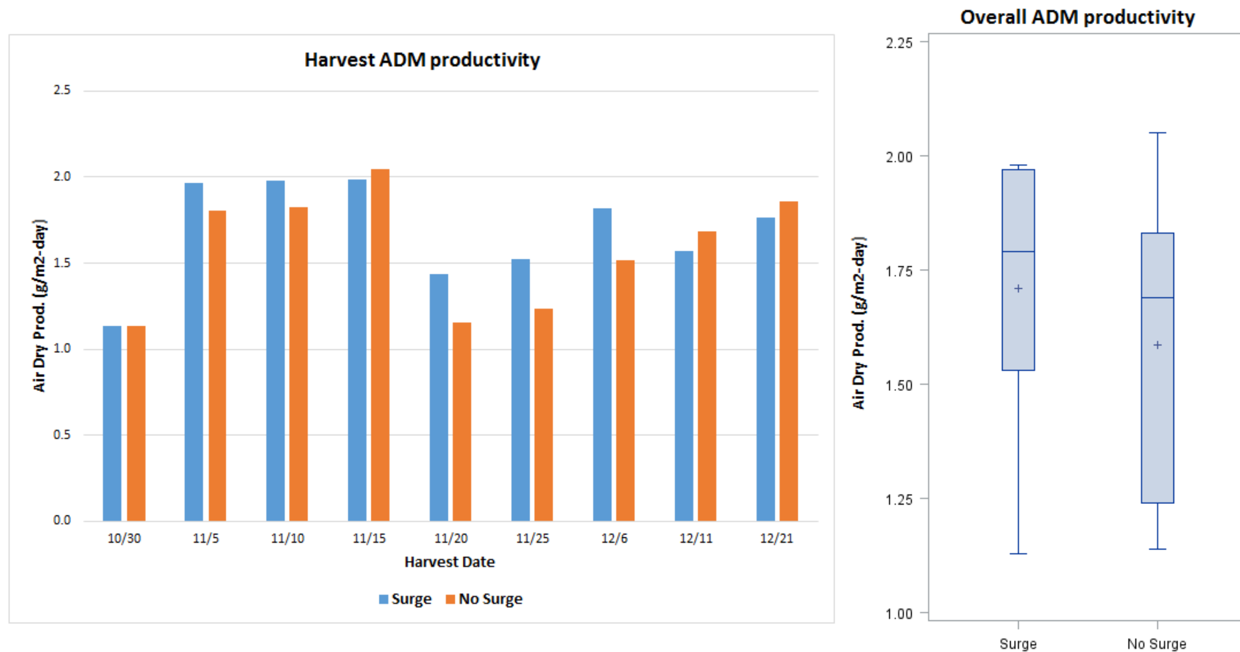

Figure 4. Total harvested air dry mass (ADM) from each 5 day harvest. Note: a portion of the algae collected on 12/6 from the 'no surge' reactor was lost during the harvesting and was omitted from statistical analysis.

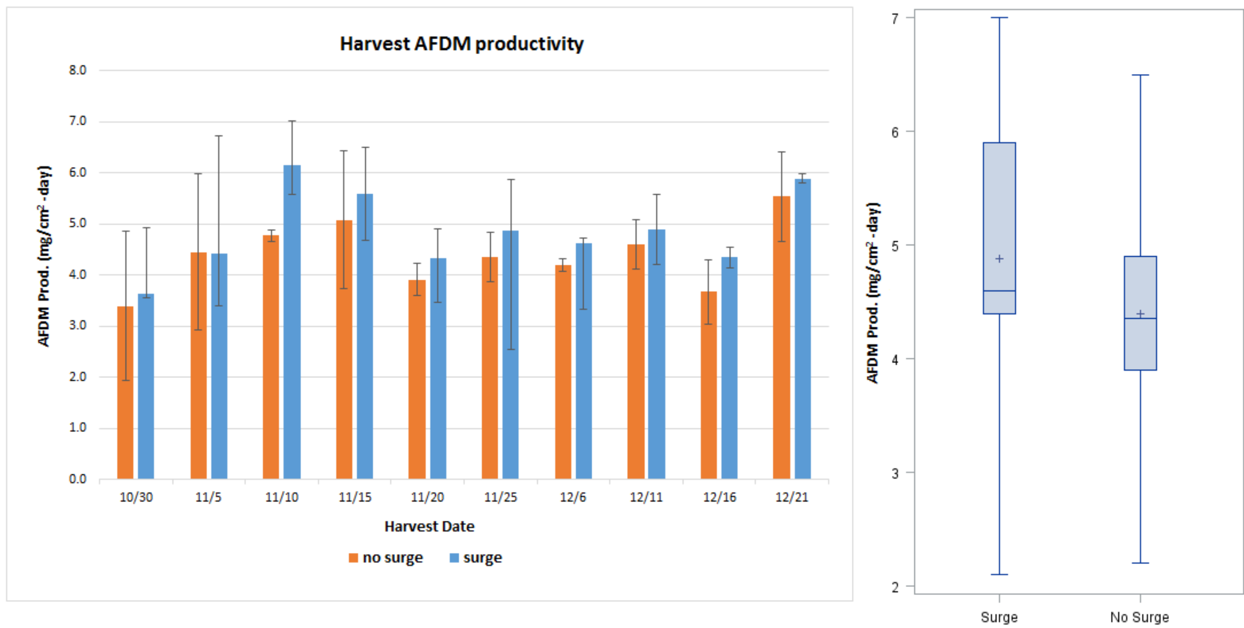

Figure 5. Average AFDM productivity and standard deviation of the sub samples collected from the three zones for each five day harvest (Left) and box plot of the overall sub sample average AFDM productivity for all harvests during the pilot growth experiment (right).

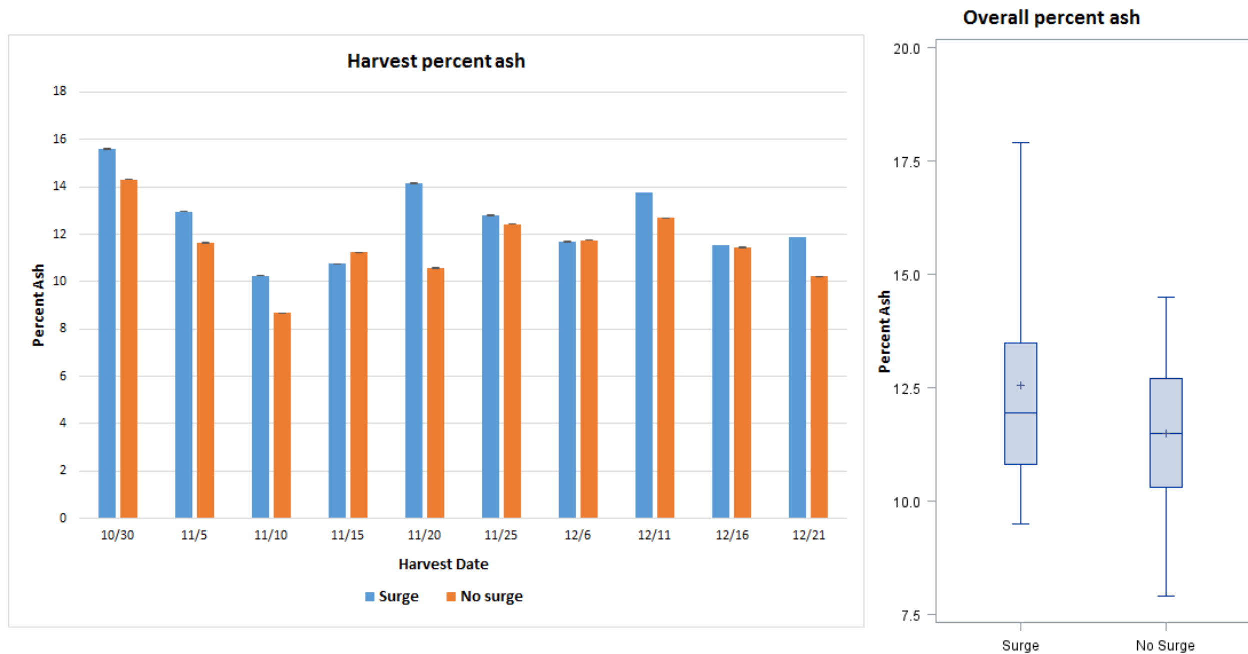

Figure 6.Average percent ash and standard deviation of the sub samples collected from the three zones for each five day harvest (Left) and box plot of the overall sub sample average percent ash content for all harvests during the pilot growth experiment (right).

Since only one treatment and one control reactor were used for data collection, replication was reliant on multiple 5 day harvest which should be treated as time series data. In an attempt to remove the week to week variation in harvest data resulting from variable environmental factors and the prior harvest, a

percent subsidy was used to treat the no surge condition as a baseline value of zero in which any increase in biomass due to the surge treatment could more easily be detected (Equation 1).

$$\% \, Subsidy = \frac{S - NS}{NS} \times 100 \qquad (1)$$

For AFDM the subsidy was calculated using the average biomass productivity of the three zones in each reactor since the sub samples were not distinguished by zone number during harvesting. Using a one sample T Test with ho=0 ($\alpha = 0.05$)the percent subsidy of the surge was tested for a significant increase over the no surge base line of zero. In all cases the percent subsidy of the surge treatment was found to be significantly greater than the no surge baseline (ADM, P=0.0497; AFDM, P=0.0009; %ASH, P=0.0035).

Discussion

The ATS community tends to agree that a period wave pulse increases biomass production of benthic algal communities testifying to the use of surge generating mechanisms such as tipping buckets in larger ATS systems. In the case of this pilot growth study the increase in biomass production was significant with the surge treatment in subsamples taken for AFDM but not significant with the overall harvest for ADM. However, once the data was normalized to remove week-to-week variation in data ADM was found significant. The variability in ADM productivity can in part be explained by the method of harvesting. After several harvest it was noticed that scraping the algae off of the netting during harvest did not always remove all of the algae on the netting leaving small random patches. A more careful approach to harvesting by scraping or vacuum harvesting could reduce this variability in future experiments. Also, algae colonized the sloped sides of the gutter exposed to water but was not harvested; however, some of this algae might have unintentionally ended up in the final harvested amount used to determine ADM further increasing variability. Since sub samples from each zone collected during the harvests were not labeled according to zone, it cannot be determined if some of the variability in AFDM can be explained by a decrease in productivity along the length of the reactor from zone 1 to 3. D'Auito et al. have reported higher productivity in the upper portion of a larger ATS system but it cannot be determined in this experiment whether or not this effect is seen with the lab scale ATS (6).

The percent ash content in Figure 6 shows a week-to-week variation that appears more prominent in the first three harvests and has an inverse relationship with AFDM in figure 5. Ten days prior to the start of the pilot growth experiment a new flow regime was started in both reactors to equalize flow between the surge and no surge treatment. This period of time prior to the start of the experiment might have been insufficient for allowing the algae to adapt to the new flow conditions resulting in harvest-to-harvest variability in the first three harvests. The remaining variability might be explained by water quality variables, which were kept constant but only within a certain range. To reduce this variability, future designs could include dosing pumps to maintain a constant nutrient concentration and the reactor could be housed in a more controlled environment.

The objective of this project was to develop an inexpensive design for a lab scale ATS using off the shelf parts that would function similar to large ATS systems and allow for increased experimental replication. The Borneman surge device provided a low tech and inexpensive means for replacing a tipping bucket to provide a wave pulse in the system. This was the best alternative to a more expensive but less variable solenoid valve and tank system which would have required more skill and funds for construction. Although collected data showed variability among harvest events, some of this variability can be explained by the low tolerance of water quality parameters and the initial harvesting methodology. The pilot growth experiment presented in this research is meant to serve as a preliminary study with no solid

conclusion on the effect of surge on biomass productivity since the statistical analysis suffers from a low sample size and therefore low statistical power.

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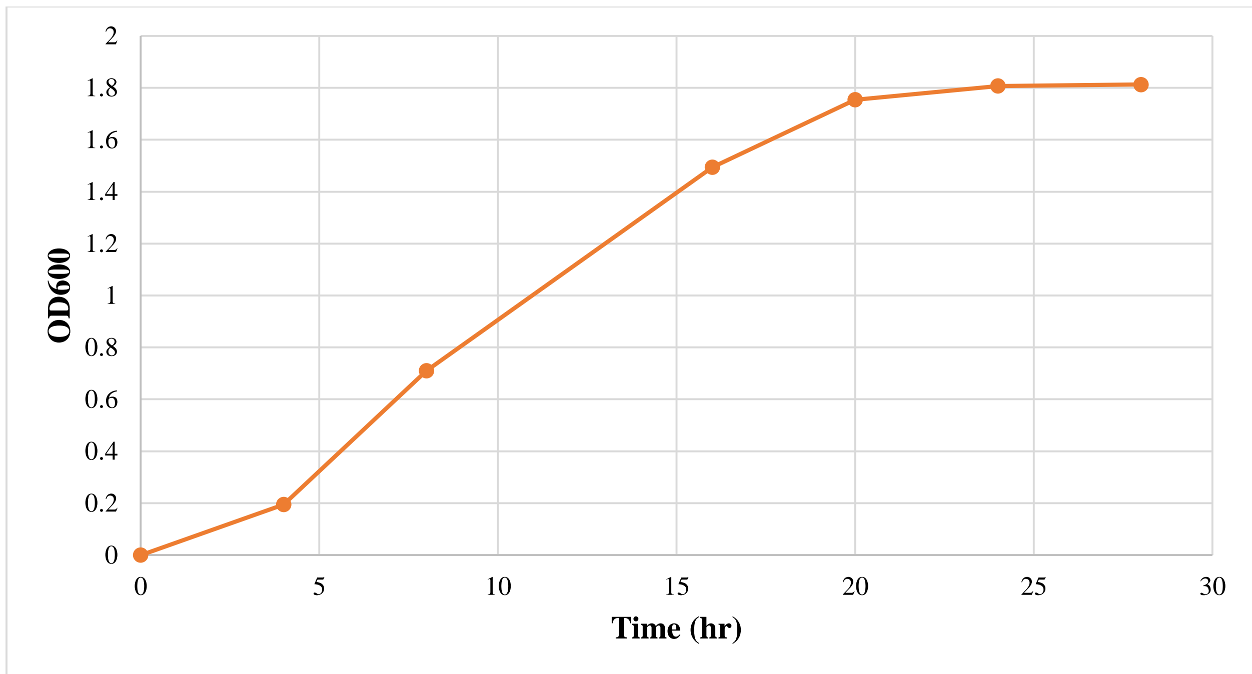
Appendix B: Growth curves for *F. columnare* and *E. coli*

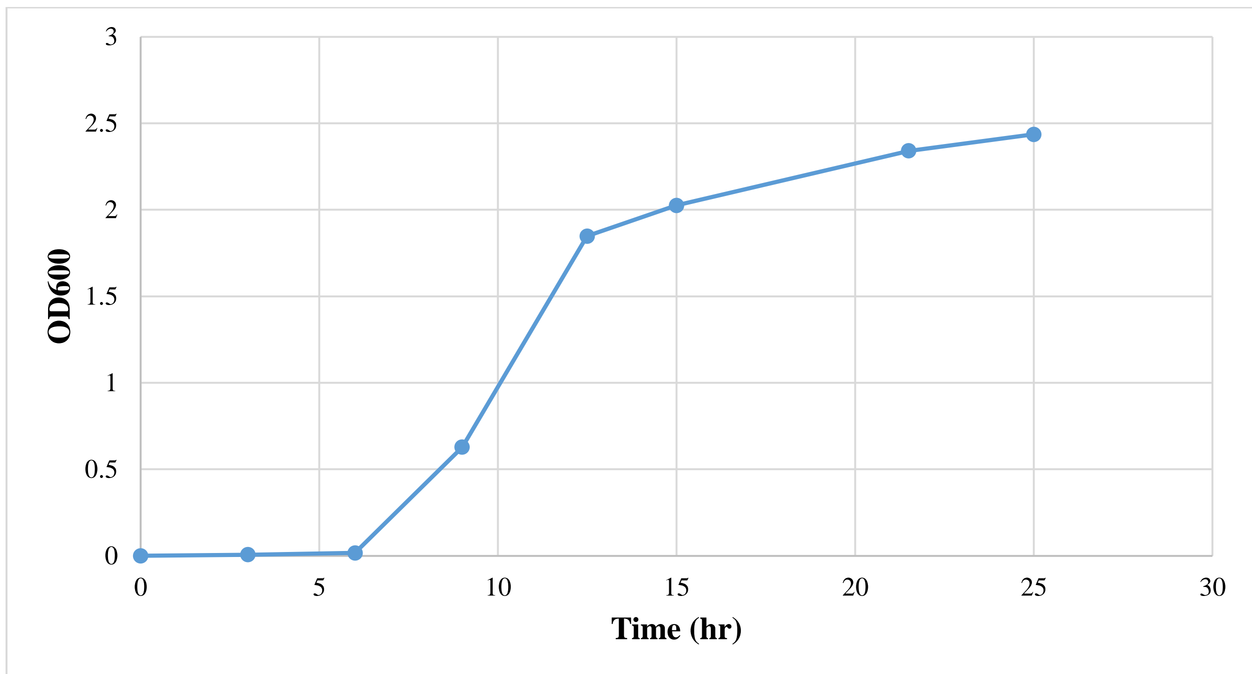
The figures below are growth curves of *F. columnare* and *E. coli* measured using the optical density at 600 nm of a sample from an incubated flask culture using a UV-VIS spectrometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer, Inc.). *F. columnare* was grown at 28 °C and 150 RPM on 200 mL (including 10mL inoculum) of MS broth in a 1 L flask. *E. coli* was grown at 37 °C and 150 RPM on 200 mL of LB-Luria broth from a single isolated colony using a 1 L flask. The charts show that the cultures were grown up to the stationary phase of growth for use during the pathogen removal experiments.

***Flavobacterium columnare* growth curve**



Growth curve of F. columnare grown at 28 °C and 150 RPM on 200 mL (including 10mL inoculum) of MS broth in a 1 L flask for 28 hours. The curve shows that at 24 hours of incubation the log phase of growth has ended and the stationary phase has commenced.

***Escherichia coli* growth curve**



Growth curve of E. coli grown at 37 °C and 150 RPM on 200 mL of LB-Luria broth in a 1 L flask for 28 hours. The curve shows that at 24 hours of incubation the log phase of growth has ended and the stationary phase has commenced.

Appendix C: SAS output

Appendix C.1: Subsidized AFDM difference between UV and No UV algal productivity

AFDM Percent Subsidy

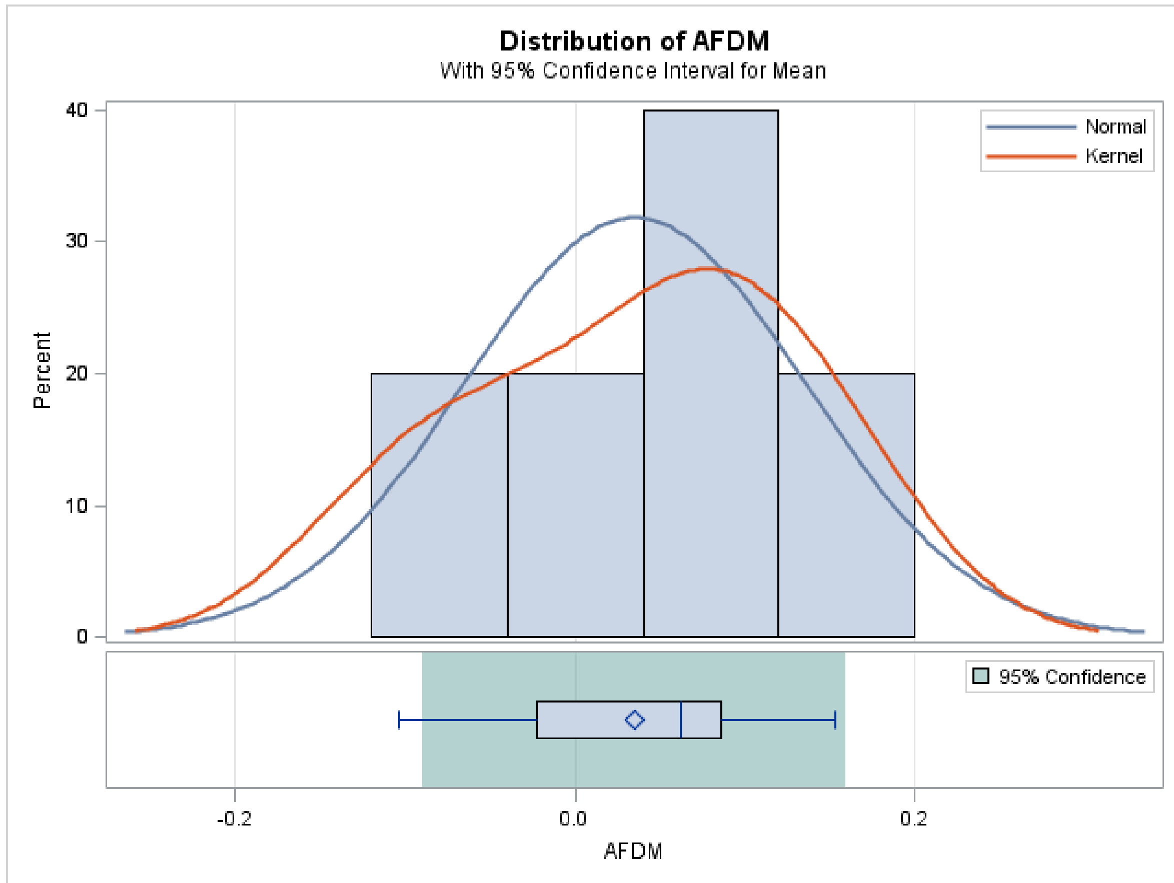
The TTEST Procedure

Variable: AFDM

N	Mean	Std Dev	Std Err	Minimum	Maximum
5	0.0349	0.1002	0.0448	-0.1044	0.1534

Mean	95% CL Mean		Std Dev	95% CL Std Dev	
0.0349	-0.0895	0.1594	0.1002	0.0600	0.2879

DF	t Value	Pr > |t|
4	0.78	0.4790



Appendix C.2: Water quality variation for *F. columnare* w/ surge and mature turf

Among treatment ANOVA for pH *(Mature Turf)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	8.63546250	2.87848750	9.06	<.0001
Error	116	36.84331667	0.31761480		
Corrected Total	119	45.47877917			

R-Square	Coeff Var	Root MSE	pH Mean
0.189879	6.824642	0.563573	8.257917

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	8.63546250	2.87848750	9.06	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.317615
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.3793

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	8.5577	30	2
A			
A	8.4907	30	1
B	8.0237	30	3
B			
B	7.9597	30	4

Among treatment ANOVA for EC (Mature Turf)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.07462250	0.02487417	11.02	<.0001
Error	116	0.26177000	0.00225664		
Corrected Total	119	0.33639250			

R-Square	Coeff Var	Root MSE	ec Mean
0.221832	10.91421	0.047504	0.435250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	0.07462250	0.02487417	11.02	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.002257
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.032

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	0.47167	30	3
A			
A	0.44500	30	4
B	0.41267	30	1
B			
B	0.41167	30	2

Appendix C.3: Water quality variation for *F. columnare* w/ surge and harvest turf

Among treatment ANOVA for pH *(Harvest Turf)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	9.50523667	3.16841222	19.20	<.0001
Error	116	19.13861333	0.16498805		
Corrected Total	119	28.64385000			

R-Square	Coeff Var	Root MSE	pH Mean
0.331842	4.865974	0.406187	8.347500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	9.50523667	3.16841222	19.20	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.164988
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.2734

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	8.6740	30	2
A			
A	8.5793	30	1
B	8.0860	30	4
B			
B	8.0507	30	3

Among treatment ANOVA for EC (Harvest Turf)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.04820250	0.01606750	3.12	0.0288
Error	116	0.59775667	0.00515307		
Corrected Total	119	0.64595917			

R-Square	Coeff Var	Root MSE	ec Mean
0.074622	15.37425	0.071785	0.466917

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	0.04820250	0.01606750	3.12	0.0288

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.005153
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.0483

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	Trt
	A	0.49900	30	4
	A			
B	A	0.46867	30	3
B	A			
B	A	0.45100	30	2
B				
B		0.44900	30	1

Appendix C.4: Water quality variation for *F. columnare* no surge and mature turf

Among treatment ANOVA for pH *(mature turf)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	10.71667583	3.57222528	30.10	<.0001
Error	116	13.76820333	0.11869141		
Corrected Total	119	24.48487917			

R-Square	Coeff Var	Root MSE	pH Mean
0.437685	4.297276	0.344516	8.017083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	10.71667583	3.57222528	30.10	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.118691
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.2319

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	8.32900	30	1
A			
A	8.30200	30	2
B	7.73667	30	4
B			
B	7.70067	30	3

Among treatment ANOVA for EC (mature turf)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.54294333	0.18098111	42.53	<.0001
Error	116	0.49358667	0.00425506		
Corrected Total	119	1.03653000			

R-Square	Coeff Var	Root MSE	ec Mean
0.523809	13.27178	0.065231	0.491500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	0.54294333	0.18098111	42.53	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.004255
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.0439

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	0.60633	30	4
B	0.47167	30	3
B			
B	0.44567	30	1
B			
B	0.44233	30	2

Appendix C.5: Water quality variation for *F. columnare* no surge and harvest turf

Among treatment ANOVA for pH *(harvest)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	8.66196917	2.88732306	18.37	<.0001
Error	116	18.23243000	0.15717612		
Corrected Total	119	26.89439917			

R-Square	Coeff Var	Root MSE	pH Mean
0.322073	4.983662	0.396454	7.955083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	8.66196917	2.88732306	18.37	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.157176
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.2668

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	8.2613	30	2
A			
A	8.1827	30	1
B	7.7063	30	4
B			
B	7.6700	30	3

Among treatment ANOVA for EC (Post-harvest)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.21212250	0.07070750	23.47	<.0001
Error	116	0.34947667	0.00301273		
Corrected Total	119	0.56159917			

R-Square	Coeff Var	Root MSE	ec Mean
0.377712	11.31524	0.054888	0.485083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	0.21212250	0.07070750	23.47	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.003013
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.0369

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	0.53967	30	3
A			
A	0.51233	30	4
B	0.44467	30	2
B			
B	0.44367	30	1

Among treatment ANOVA for temp *(harvest)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	6.21700000	2.07233333	3.54	0.0170
Error	116	67.99600000	0.58617241		
Corrected Total	119	74.21300000			

R-Square	Coeff Var	Root MSE	temp Mean
0.083772	2.946388	0.765619	25.98500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	6.21700000	2.07233333	3.54	0.0170

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.586172
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.5153

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	Trt
	A	26.2867	30	1
	A			
B	A	26.1000	30	2
B	A			
B	A	25.8667	30	3
B				
B		25.6867	30	4

Appendix C.6: Water quality variation for *E. coli* w/ surge and mature turf

Among treatment ANOVA for temp *(mature turf)*

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	5.60025000	1.86675000	24.61	<.0001
Error	116	8.79900000	0.07585345		
Corrected Total	119	14.39925000			

R-Square	Coeff Var	Root MSE	temp Mean
0.388927	1.070508	0.275415	25.72750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	5.60025000	1.86675000	24.61	<.0001

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.075853
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.1854

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	Trt
A	26.02000	30	2
A			
A	25.85000	30	1
B	25.52000	30	3
B			
B	25.52000	30	4

Among treatment ANOVA for DO (mature turf)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.12690917	0.37563639	2.85	0.0402
Error	116	15.26318333	0.13157917		
Corrected Total	119	16.39009250			

R-Square	Coeff Var	Root MSE	DO Mean
0.068756	4.083857	0.362738	8.882250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	1.12690917	0.37563639	2.85	0.0402

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.131579
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.2441

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	Trt
	A	8.99767	30	2
	A			
B	A	8.93700	30	1
B	A			
B	A	8.85533	30	3
B				
B		8.73900	30	4

Appendix C.7: Water quality variation for *E. coli* w/ surge and harvest turf

Among treatment ANOVA for temp (harvest)

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3.55100000	1.18366667	4.42	0.0056
Error	116	31.09400000	0.26805172		
Corrected Total	119	34.64500000			

R-Square	Coeff Var	Root MSE	temp Mean
0.102497	2.020438	0.517737	25.62500

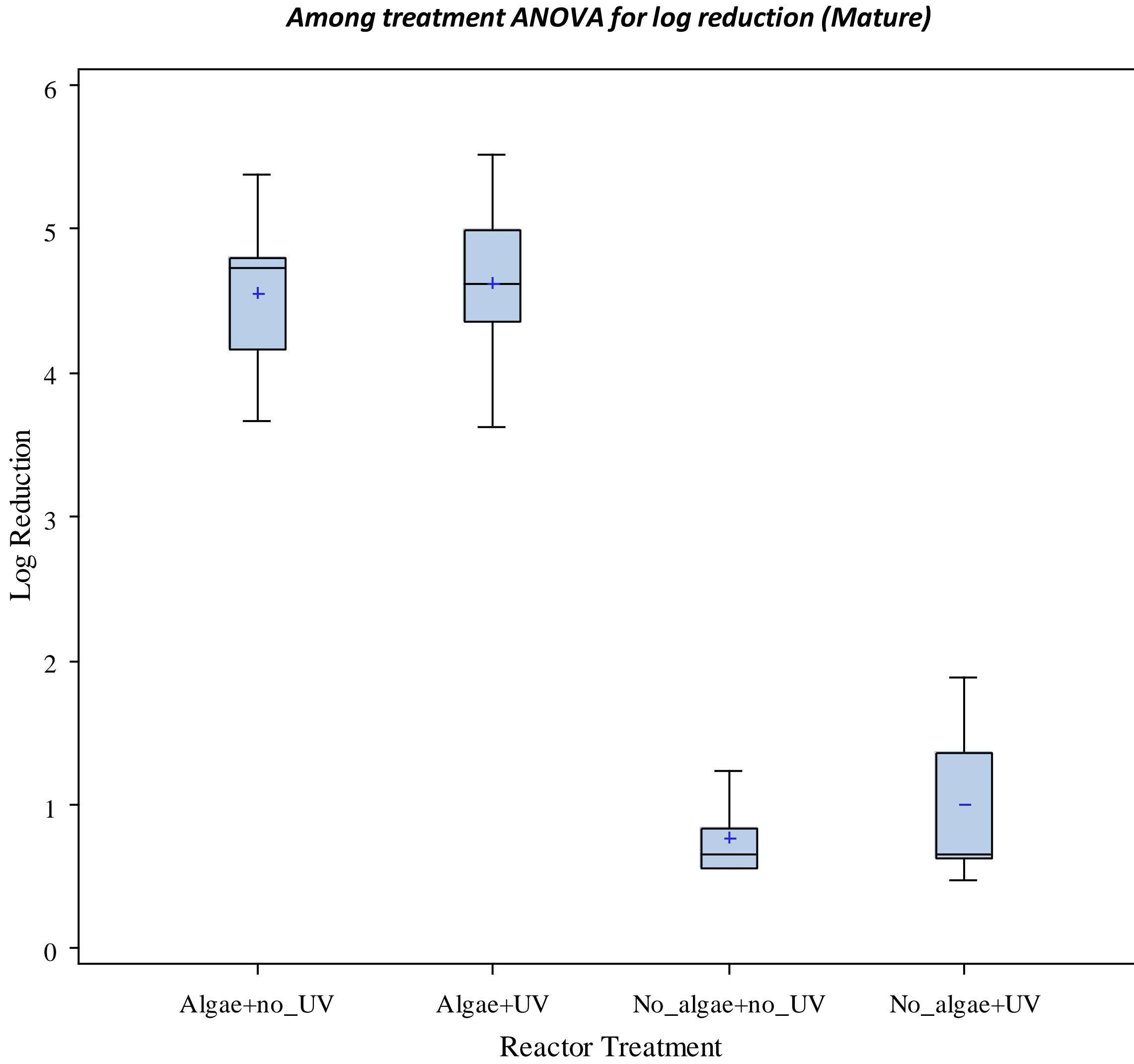
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	3	3.55100000	1.18366667	4.42	0.0056

Alpha	0.05
Error Degrees of Freedom	116
Error Mean Square	0.268052
Critical Value of Studentized Range	3.68638
Minimum Significant Difference	0.3485

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	Trt
	A	25.8067	30	Algae+no_UV
	A			
	A	25.7767	30	Algae+UV
	A			
B	A	25.5167	30	No_algae+no_UV
B				
B		25.4000	30	No_algae+UV

Appendix C.8: Log reduction of *F. columnare* with mature turf and surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	68.56277500	22.85425833	66.76	<.0001
Error	16	5.47708000	0.34231750		
Corrected Total	19	74.03985500			

R-Square	Coeff Var	Root MSE	Mature Mean
0.926025	21.40403	0.585079	2.733500

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	68.56277500	22.85425833	66.76	<.0001

Adjustment for multiple comparisons: Tukey

Trt	Mature LSMEAN	LSMEAN Number
Algae+UV	4.62200000	1
Algae+no_UV	4.54400000	2
No_algae+UV	1.00000000	3
No_algae+no_UV	0.76800000	4

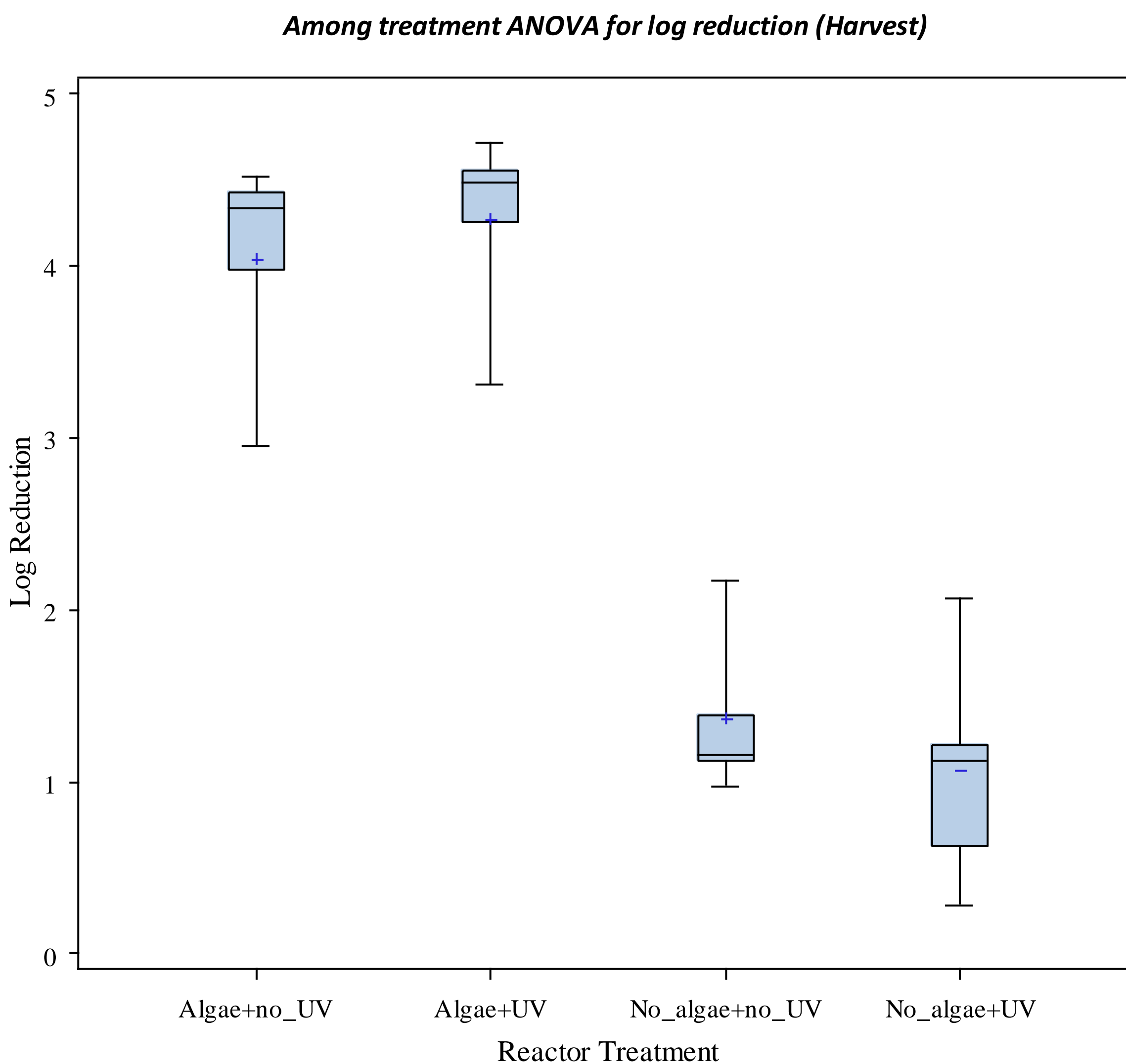
Least Squares Means for effect Trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Mature

i/j	1	2	3	4
1		0.9965	<.0001	<.0001
2	0.9965		<.0001	<.0001
3	<.0001	<.0001		0.9219
4	<.0001	<.0001	0.9219	

Appendix C.9: Log reduction of *F. columnare* with harvested turf and surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	43.50370000	14.50123333	41.21	<.0001
Error	16	5.62968000	0.35185500		
Corrected Total	19	49.13338000			

R-Square	Coeff Var	Root MSE	Harvest Mean
0.885420	22.14161	0.593174	2.679000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	43.50370000	14.50123333	41.21	<.0001

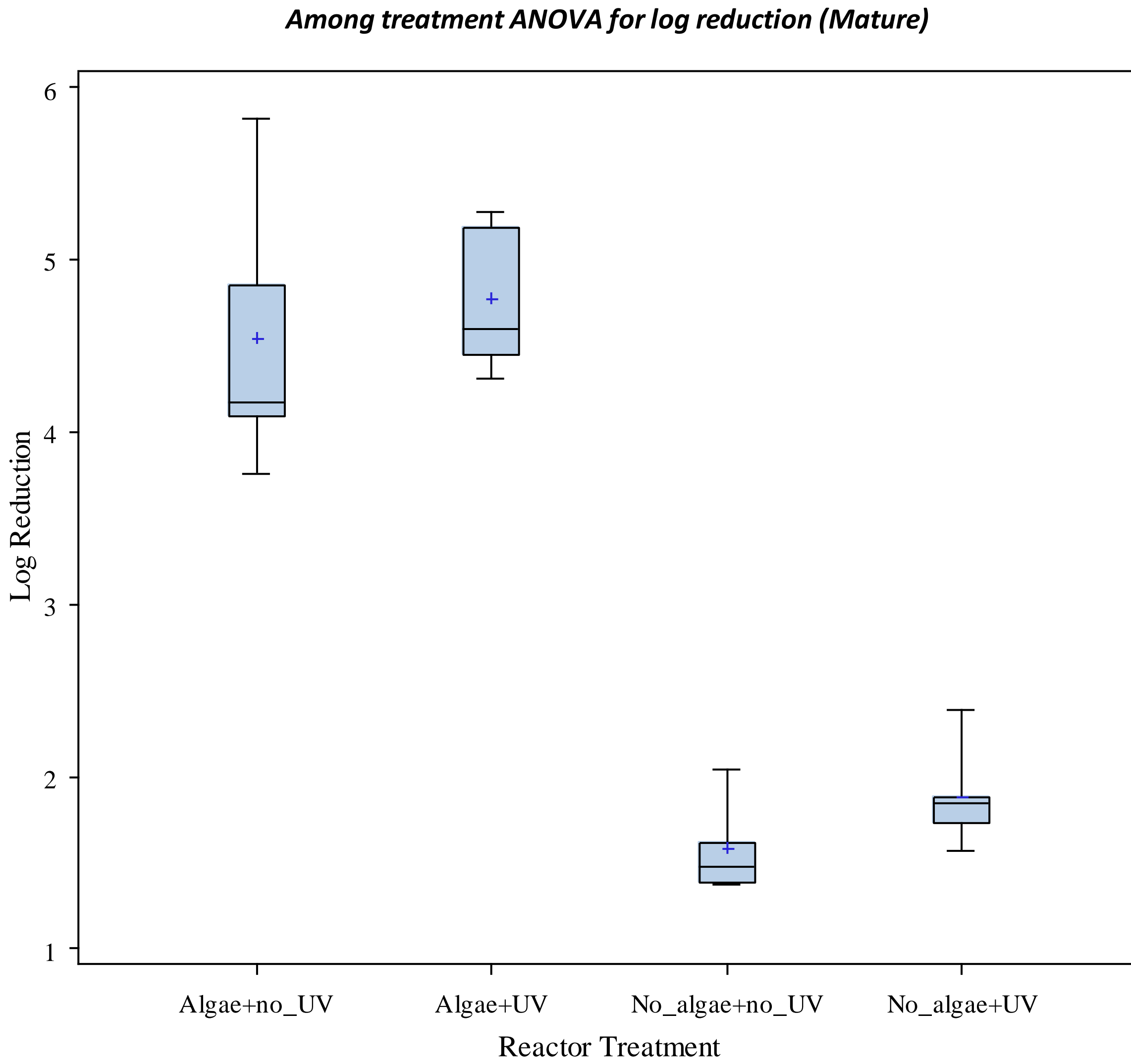
Adjustment for multiple comparisons: Tukey

Trt	Harvest LSMEAN	LSMEAN Number
Algae+UV	4.26000000	1
Algae+no_UV	4.03600000	2
No_algae+UV	1.06200000	3
No_algae+no_UV	1.35800000	4

Least Squares Means for effect Trt Pr > |t| for H0: LSMean(i)=LSMean(j) Dependent Variable: Harvest				
i/j	**1**	**2**	**3**	**4**
1		0.9315	<.0001	<.0001
2	0.9315		<.0001	<.0001
3	<.0001	<.0001		0.8584
4	<.0001	<.0001	0.8584	

Appendix C.10: Log reduction of *F. columnare* with mature turf and no surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	42.96233500	14.32077833	55.48	<.0001
Error	16	4.12976000	0.25811000		
Corrected Total	19	47.09209500			

R-Square	Coeff Var	Root MSE	Mature Mean
0.912305	15.92369	0.508045	3.190500

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	42.96233500	14.32077833	55.48	<.0001

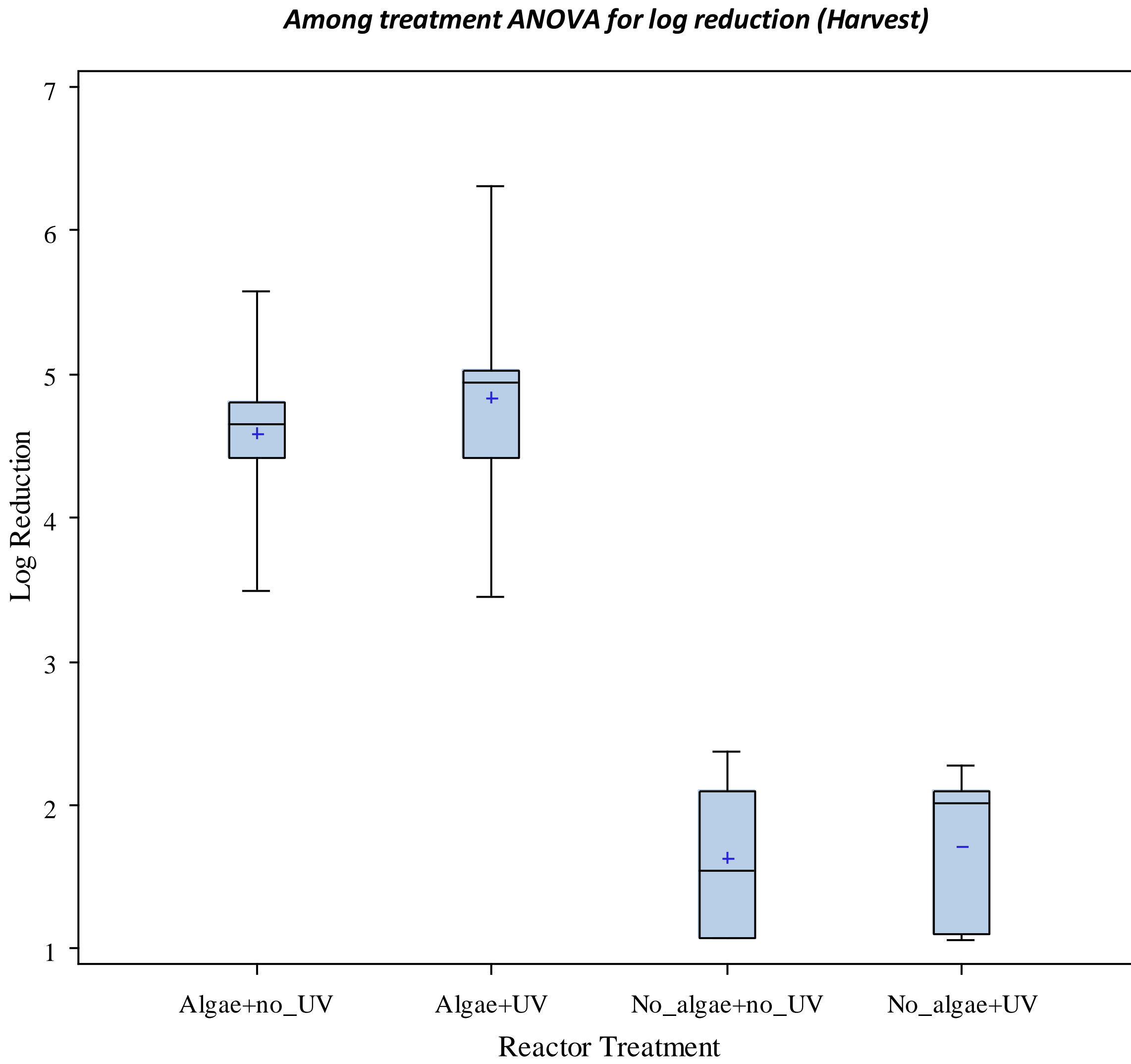
Adjustment for multiple comparisons: Tukey

Trt	Mature LSMEAN	LSMEAN Number
Algae+UV	4.76200000	1
Algae+no_UV	4.53800000	2
No_algae+UV	1.88400000	3
No_algae+no_UV	1.57800000	4

Least Squares Means for effect Trt Pr > |t| for H0: LSMean(i)=LSMean(j) Dependent Variable: Mature				
i/j	**1**	**2**	**3**	**4**
1		0.8967	<.0001	<.0001
2	0.8967		<.0001	<.0001
3	<.0001	<.0001		0.7776
4	<.0001	<.0001	0.7776	

Appendix C.11: Log reduction of *F. columnare* with harvested turf and no surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	46.31220000	15.43740000	26.59	<.0001
Error	16	9.28952000	0.58059500		
Corrected Total	19	55.60172000			

R-Square	Coeff Var	Root MSE	Harvest Mean
0.832927	23.90112	0.761968	3.188000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	46.31220000	15.43740000	26.59	<.0001

Adjustment for multiple comparisons: Tukey

Trt	Harvest LSMEAN	LSMEAN Number
Algae+UV	4.83000000	1
Algae+no_UV	4.58400000	2
No_algae+UV	1.70600000	3
No_algae+no_UV	1.63200000	4

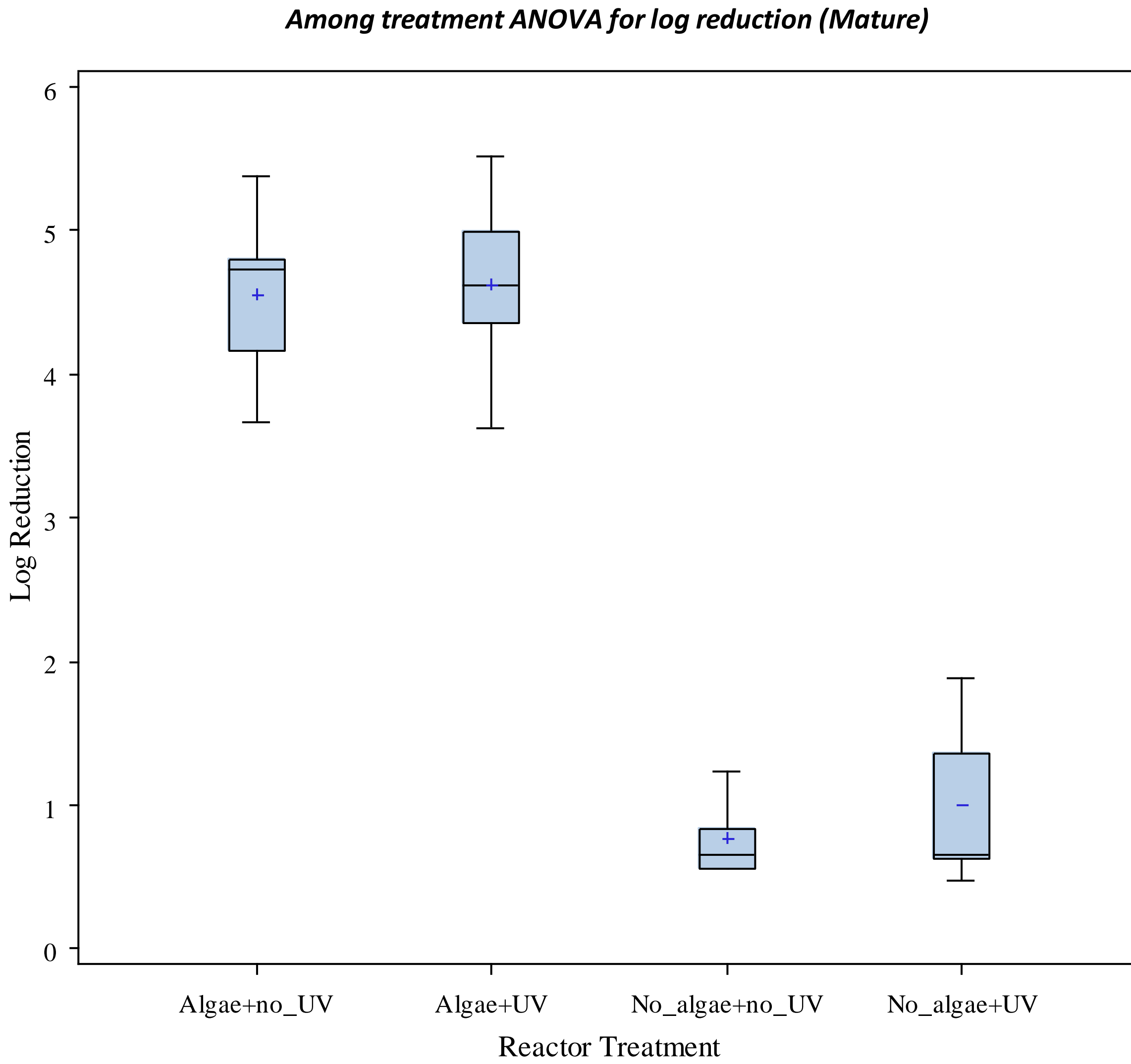
Least Squares Means for effect Trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Harvest

i/j	1	2	3	4
1		0.9554	<.0001	<.0001
2	0.9554		0.0001	<.0001
3	<.0001	0.0001		0.9987
4	<.0001	<.0001	0.9987	

Appendix C.12: Log reduction of *E.coli* with mature turf and surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	31.70101327	10.56700442	32.26	<.0001
Error	16	5.24012820	0.32750801		
Corrected Total	19	36.94114147			

R-Square	Coeff Var	Root MSE	Mature Mean
0.858149	28.25356	0.572283	2.025526

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	31.70101327	10.56700442	32.26	<.0001

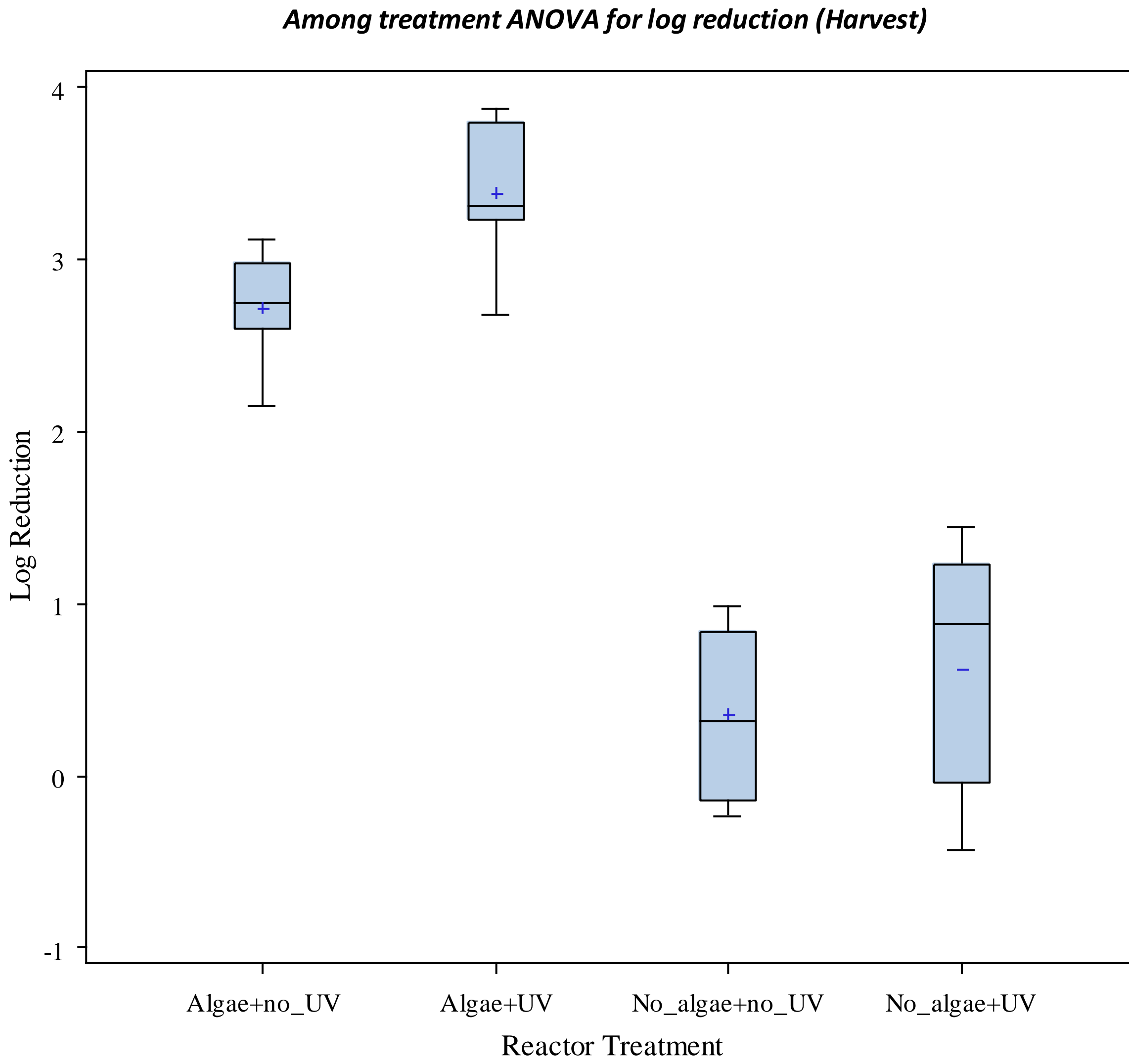
Adjustment for multiple comparisons: Tukey

Trt	Mature LSMEAN	LSMEAN Number
Algae+UV	3.64927800	1
Algae+no_UV	2.77114800	2
No_algae+UV	1.25357800	3
No_algae+no_UV	0.42809800	4

Least Squares Means for effect Trt Pr > |t| for H0: LSMean(i)=LSMean(j) Dependent Variable: Mature				
i/j	**1**	**2**	**3**	**4**
1		0.1119	<.0001	<.0001
2	0.1119		0.0035	<.0001
3	<.0001	0.0035		0.1443
4	<.0001	<.0001	0.1443	

Appendix C.13: Log reduction of *E.coli* with harvested turf and surge

Boxplot



ANOVA table

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	34.11563732	11.37187911	33.86	<.0001
Error	16	5.37397634	0.33587352		
Corrected Total	19	39.48961367			

R-Square	Coeff Var	Root MSE	Harvest Mean
0.863914	32.87654	0.579546	1.762795

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Trt	3	34.11563732	11.37187911	33.86	<.0001

Adjustment for multiple comparisons: Tukey

Trt	Harvest LSMEAN	LSMEAN Number
Algae+UV	3.37547000	1
Algae+no_UV	2.71315200	2
No_algae+UV	0.61292600	3
No_algae+no_UV	0.34963200	4

Least Squares Means for effect Trt
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Harvest

i/j	1	2	3	4
1		0.3061	<.0001	<.0001
2	0.3061		0.0002	<.0001
3	<.0001	0.0002		0.8884
4	<.0001	<.0001	0.8884	

Appendix D: Media and buffer formulations

Appendix D.1: Modified Sheih

Modified Shieh

1. First measure out below in 200mL of water

- 200 ml, 4.1 mM $FeSO_4 \cdot 7H_2O$ (FW 278.01): 0.2 g in 200 ml water
- 200 ml, 45.6 mM $CaCl_2 \cdot 2H_2O$ (FW 147): 1.34 g in 200 ml water
- 200 ml, 0.108 M KH_2PO_4 (monobasic) (FW 136.09): 2.94 g in 200 ml water
- 200 ml, 0.12 M $MgSO_4 \cdot 7\ H_2O$ (FW 246.47): 5.91 g in 200 ml water

2. Then use the below amounts of the above mixed compounds

	500 ml	**1 liter**
4.1 µM $FeSO_4 \cdot 7H_2O$ (1000 X)	500 µl	1 ml
45.6 µM $CaCl_2 \cdot 2H_2O$ (1000 X)	500 µl	1 ml
1.08 mM KH_2PO_4 (monobasic) (100 X)	5 ml	10 ml
1.2 mM $MgSO_4 \cdot 7\ H_2O$ (100 X)	5 ml	10 ml
Tryptone	2.5 g	5 g
Yeast extract	1 g	2 g
Water		
ph	7.2-7.4	7.2-7.4
Agar	4-6 g	8-12 g

3. Add NaOH to adjust pH to required range

4. Supplemented with tobramycine at a concentration of 1 µg ml^{-1}

Source: Decostere, A., Haesebrouck, F. and Devriese, L. A. (1997). Sheih medium supplemented with Tobramycin for selective isolation of *Flavobacterium columnare (Flexibacter columnaris)* from diseased fish. *Journal of Clinical Microbiology*, 332-324.

Appendix D.2: Buffer recipe

1. Salt solution
    a. Add 34 g Seachem Marine Salt (Seachem Laboratories, Madison, GA) to 1L of DI water.
    b. Stir solution for 2-12 hours and store refrigerated
2. Dry buffer mix
    a. 6g $CaCO_3$
    b. 14g $NaHCO_3$
    c. 1.7g NH_4Cl
    d. Mix thoroughly
3. Reservoir water
    a. Add 2.6 mL/L salt solution
    b. Add 2.6 mg/L dry buffer mix
    c. Ensure adequate mixing

Appendix D.3: Gulliard's F/2 recipe

F/2 medium

Add components below with trace element and vitamin solutions and bring to a final volume of 1L. Autoclave and refrigerate.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82×10^{-4} M
$NaH_2PO_4\ H_2O$	5 g/L dH_2O	1 mL	3.62×10^{-5} M
$Na_2SiO_3\ 9H_2O$	30 g/L dH_2O	1 mL	1.06×10^{-4} M
trace metal solution	(see recipe below)	1 mL	---
vitamin solution	(see recipe below)	0.5 mL	---

Trace Metal Solution

Add all stock solutions to DI water for a final volume of 1L, autoclave.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3$ $6H_2O$	---	3.15 g	1.17×10^{-5} M
Na_2EDTA $2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4$ $5H_2O$	9.8 g/L dH_2O	1 mL	3.93×10^{-8} M
Na_2MoO_4 $2H_2O$	6.3 g/L dH_2O	1 mL	2.60×10^{-8} M
ZnSO4 $7H_2O$	22.0 g/L dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2$ $6H_2O$	10.0 g/L dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2$ $4H_2O$	180.0 g/L dH_2O	1 mL	9.10×10^{-7} M

Vitamin solution

Dissolve thiamine in DI water, add stock solutions and bring to a final volume of 1L. Filter sterilize and refrigerate.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	1.0 g/L dH_2O	1 mL	2.05×10^{-9} M
cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	3.69×10^{-10} M

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