DEVELOPMENT OF A RAPID ATP ANALYSIS METHOD: BIOMASS GROWTH ATP METHOD FOR UV DISINFECTION MONITORING IN WASTEWATER TREATMENT

by

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DEDICATION PAGE

献给我的家人和我的故乡。

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ABSTRACT

Adenosine triphosphate (ATP) analysis is gaining popularity as a robust method to quantify microbial content in water supplies and wastewater treatment processes. ATP testing provides cost effective microbial analysis results in a matter of minutes in the laboratory or the field. A challenge with ATP monitoring is that an Ultraviolet (UV) dose sufficient to cause a significant reduction in culturability yields little to no reduction in ATP. To date, few studies have been conducted to investigate the possible techniques that enable the application of ATP assays in assessing the effectiveness of UV disinfection.

The objective of this study was to develop a pre-treatment technique prior to ATP assay that can detect differences in cellular ATP (cATP) between pre and post UV treated wastewater in a single shift (<8hrs) to improve compliance monitoring and disinfection performance. The research approach involved incubating both untreated and irradiated samples in a suitable growth environment to accelerate bacterial growth prior to ATP analysis. The treatment efficacy was determined by the disparity in increase of cATP growth.

A robust Biomass Growth ATP Method (optimum chemical reagent, optimum incubation temperature, 4 hours) has been developed to test UV disinfection efficacy in municipal wastewater treatment. The response of the method indicated a significant direct correlation to the microbial population reduction observed in HPC method (R2A agar, 28℃, 7days) for both pure *E. coli* culture and secondary effluent. The Biomass Growth ATP Method was then tested in field samples from three wastewater treatment plants (WWTPs) to validate its practicality. The result that Biomass Growth ATP Method could detect a 1-2-order of magnitude of microbial inactivation implied a prospective application of this new method in WWTPs with high microbial loading rates to evaluate UV disinfection performance.

LIST OF SYMBOLS AND ABBREVIATIONS USED

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
℃	Degree Celsius
CBU	collimated beam unit
CFU/mL	colony forming units per milliliter
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DBP	disinfection by-product
DNA	deoxyribonucleic acid
DVC	direct viable count
E. coli	*Escherichia coli*
E. coli K12	*Escherichia coli* K12 strain (ATCC MG 1655)
EP	Eastern Passage
ETS	electron transport system
FR	Fall River
H	hour
HPC	heterotrophic plate count
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
MC	Mill Cove
MF	membrane filtration
mJ/cm^2	millijoules per centimeter squared
mL	milliliter (10^{-3} L)
NB	nutrient broth
PBS	phosphate buffered saline
pgATP/mL	pictogram ATP per millilitre

QGA	Quench-Gone-Aqueous
R^2	coefficient of determination
rpm	revolutions per minute
TSA	tryptic soy agar
TSB	tryptic soy broth
USEPA	United States Environmental Protection Agency
UV	ultraviolet
UVT	UV Transmittance
VBNC	viable but non-culturable
WPCC	Water Pollution Control Centre
WPCP	Water Pollution Control Plant
WWTF	Wastewater Treatment Facility
WWTP	Wastewater Treatment Plant
λ	wavelength

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CHAPTER 1 INTRODUCTION

UV disinfection is a valuable and successful alternative in wastewater treatment (Masschelein 2002). It is vital to prevent the receiving water quality from deteriorating and the public health from being jeopardized by the water borne diseases. Monitoring of UV disinfection performance of wastewater treatment is required to ensure that treatment meets the regulatory microbial limits.

Adenosine triphosphate (ATP) analysis is gaining popularity as a robust method to quantify microbial content in water supplies and wastewater treatment processes. ATP assay has the advantages of its capacity of measuring the entire viable microorganisms present in a water sample, which makes it a non-specific indicating tool in the wastewater effluent quality control (Cairns et al. 1979). ATP testing provides cost effective results in a matter of minutes both in the laboratory or the field.

Compared to ATP testing, traditional microbial enumeration techniques such as the heterotrophic plate count (HPC) require at least 24 hours of incubation (Lee & Deininger 2010). These conventional culturing methods are not practical in wastewater treatment process quality control since wastewater treatment plants do not hold secondary effluent while awaiting the results of disinfection efficacy for days. Treated wastewater effluent is

typically discharged into a natural water body before the operation's awareness of whether the effluent is in conformity to pertinent regulatory approval guidelines or not. Also, it is not possible to provide timely results when a contamination event happens and this delay would consequently lead to the failure of protecting the public from the harmful consumption of deteriorated water (Lee & Deininger 2010). Therefore innovative and pragmatic approaches that are able to detect the presence and level of the microbial content in a time-efficient manner are urgently needed in disinfection efficacy monitoring for the wastewater treatment industry. With the assistant of rapid microbial monitoring methods, immediate corrective action can be taken by authorities when facing emergencies like deficient disinfection to mitigate the potential water contamination caused by excessive pathogenic microorganisms being carried with wastewater discharges (Lee & Deininger 2010).

The application of ATP assay to UV disinfection efficacy assessment has not been extensively studied and is not well understood. In this study, ATP technology as a possible candidate for rapid monitoring of UV disinfection performance was evaluated.

1.1 Research Objectives

The overall aim of this research was to develop a pre-treatment technique prior to ATP

assay that can detect differences in cellular ATP (cATP) between pre and post UV disinfected municipal wastewater in a single shift (less than 8 hours) to help improve treatment performance and provide faster options for regulatory compliance monitoring. The pre-treatment method in combination with ATP testing investigated in this study was applied in both controlled laboratory conditions and at field scale.

During the research, five sub-tasks were carried out to achieve the main research objective as elucidated below:

i. Assess the current rapid disinfection monitoring methods: ATP technology and electron transport system (ETS) activity.

ii. Develop a pre-treatment procedure for ATP test, which includes: 1) evaluation and development of the reagent formulation for microorganism cultivation ((i.e., enrichment); 2) assess and optimize the culturing temperature.

iii. Ensure the practicality of the pre-treatment technique, which involves: 1) test of robustness for field sampling; 2) test of repeatability.

iv. Compare the pre-treatment technique: Biomass Growth ATP Method to accepted culturing method.

v. Apply the pre-treatment technique: Biomass Growth ATP Method to secondary wastewater effluent obtained from different wastewater treatment plants.

1.2 Thesis Organization

Each chapter was written based on the five research tasks above. Listed below is a brief description of the content of each chapter.

- Chapter 2 provides a literature review of the application and monitoring of UV disinfection in wastewater treatment. ATP technology and its application as a biomass indicator are introduced as well.
- Chapter 3 describes the materials and methods used in this study. The experimental design and procedures of each sub-task are also provided.
- Chapter 4 presents findings from the five research tasks.
- Chapter 5 presents the interpretations, speculations and implications of the findings. It also discusses the importance and the limitations of the method, how the method fits in with previous work.
- Chapter 6 Provides conclusions from the findings of this research project. It also recommends future research opportunities in the field.

CHAPTER 2 LITERATURE REVIEW

2.1 UV Disinfection in Wastewater Treatment

2.1.1 Mechanism

The inactivation of microorganisms after UV irradiation is likely due to the absorption of UV light between 200 – 300 nm by DNA. DNA is a nucleic acid polymer in a double-stranded helix held together by hydrogen bonds in an orderly sequence from four constituent bases: adenine, guanine, thymine and cytosine. Of the four essential nucleotides, thymine could be linked together to form thymine dimer after UV exposure when they are located adjacent to each other. This disrupts the base pairs (adenine with thymine and cytosine with guanine) structure, as illustrated in Figure 2-1 and Figure 2-2. If a critical number of thymine dimers are formed, the DNA cannot replicate in cell mitosis, resulting in disruption of the multiplication systems of the microorganisms (Zhou & Smith 2002; Bolton & Cotton 2008). Thus the cells cannot infect a host (Zhou & Smith 2002; Bolton & Cotton 2008).

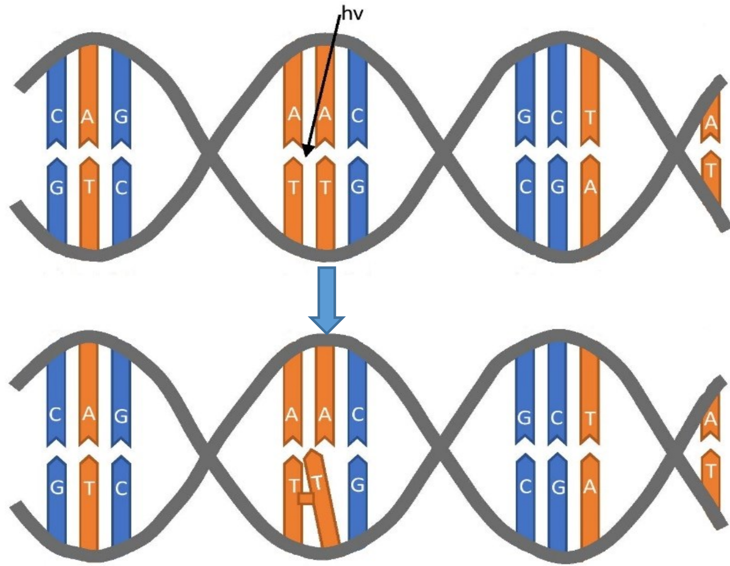


Figure 2-1 Double-stranded DNA chain showing the formation of thymine dimers disrupts the structure of the chain (A = adenine; G = guanine; T = thymine; C= cytosine) (Bolton & Cotton 2008)

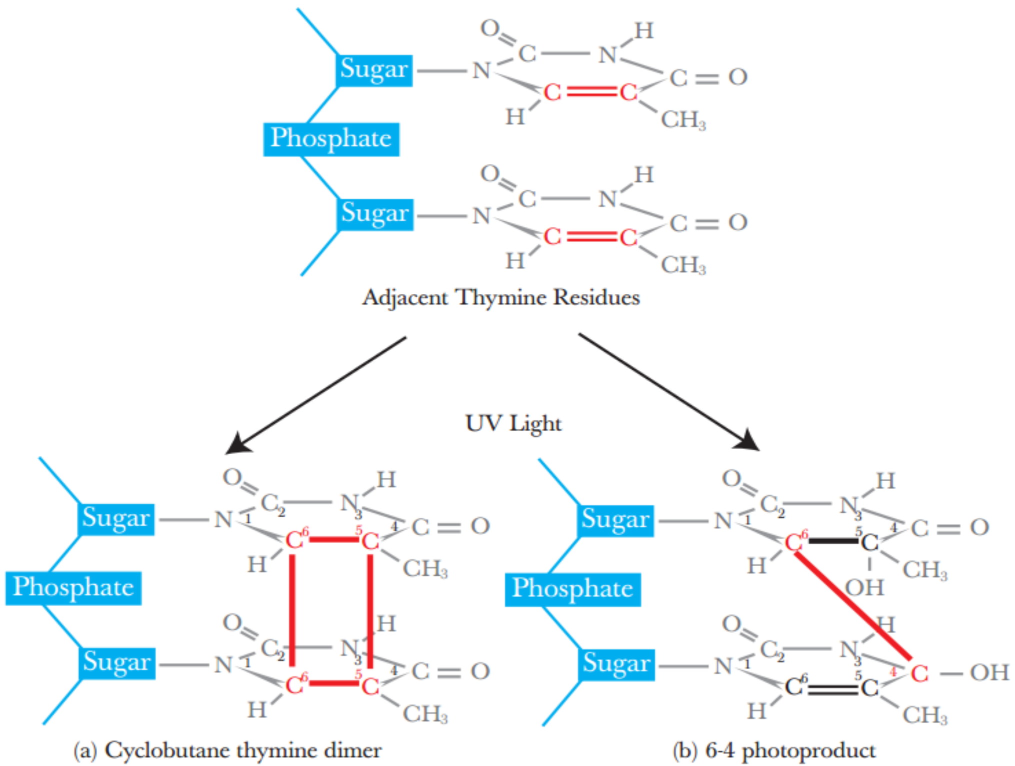


Figure 2-2 Photochemical dimerization of two thymine bases (Matthews & van Holde 1990)

2.1.2 Benefits and Limitations

UV disinfection is a very effective disinfection technology for the inactivation of chlorine-resistant parasites such as *Cryptosporidium* (Bolton & Cotton 2008). UV disinfection does not consume chemicals, form harmful byproducts, or significantly alter the water quality; that is, no change in total organics carbon (TOC), pH, corrosivity, DBP formation potential, or turbidity (Droste 1997; Bolton & Cotton 2008). Moreover, UV disinfection is very fast. The exposure can be completed in the range of a few seconds (Bolton & Cotton 2008).

The major limitation of UV disinfection is that many organisms have developed multiple pathways to compensate and reconstitute the altered DNA. Photoreactivation and dark repair are two main removal pathways used to correct UV-induced DNA damage (Zimmer 2002). This is not a universal behavior as some organisms are more subject to repair than others (Masschelein 2002). Photoreactivation is catalysed by photolyase enzyme in presence of visible light (λ = 370 nm) to dissociate the thymine dimers formed from UV exposure and restoring the original monomer (Basu et al. 2007; Masschelein 2002). Dark repair is much slower than photoreactivation. Nucleotide excision repair is the dominant form of dark repair mechanism, where repair is done by physical incision (cutting out) of the dimer from damaged DNA strand by an enzyme

complex. Thus it is removed and the DNA is repaired (Basu et al. 2007). The fact that UV treatment does not provide a disinfectant residual leads to the concern of the possible consequences the repair mechanisms might bring. Several studies were directed to determine the relationship between the repair and UV dose which suggested that repair mechanisms can be inhibited at a higher UV dose (Zhou & Smith 2002).

Another limitation with UV disinfection is that particle-associated coliform bacteria present in secondary effluents could be shielded from UV irradiation and result in ineffectiveness of disinfection treatment (Parker & Darby 1995). The research conducted by Loge et al. (1999) and Emerick et al. (1999) implied that the number of particle-associated coliform is directly correlated to residual coliform bacteria concentration after high UV doses, thus the particles containing completely blocked target organisms is likely to be a fundamental factor influencing the UV disinfection performance. Other constituents such as organic compounds and ferric ions in water may also affect the transmission and absorption of UV rays (Droste 1997).

The disinfection feature that no residual being left after UV treatment is huge benefit in wastewater treatment, but a big disadvantage in drinking water treatment. The beneficial perspective is that there is no chemical left in the disinfected water to interfere with water quality and lead to potential harmful DBP formation after being released to natural

watershed (Droste 1997). The disadvantage is that especially in drinking water treatment, there is no residual disinfecting power left to prevent the water from possible contamination in the distribution system.

2.1.3 Monitoring Approaches

UV Transmittance

UV transmittance (UVT) is the measurement of the amount of UV light at a particular wavelength (usually at 254 nm) transmitted through water from UV lamp, usually expressed in percentage (%) (Bolton & Cotton 2008). UVT is a key operational parameter in UV disinfection process monitoring, since the UV light absorbed by the water itself would render it unavailable to reach microorganisms (Qiang et al. 2013; Bolton and Cotton 2008). The higher the UVT, the more light is able to penetrate the water and therefore the more efficient the inactivation of pathogens. In an empirical UV disinfection model developed by Loge et al. (1996), UVT was adopted as one of the input parameters to the model to predict the inactivation of coliforms in the tailing region of UV dose - response curve where log (N/N_0) is less than minus three. Other parameters in the model include the suspended solids concentration, coliform densities and the applied UV dose.

UVT can indicate if the influent wastewater contains substances that could interfere with and even impair the UV disinfection performance. However, UVT is not a direct indicator of the microbial level of the influent. Clearly, the effectiveness of UV irradiation is subject to the clarity of the water being treated. However, small colloids and particle attachment suggest that UVT cannot be guarantee that a sufficient inactivation of the pathogenic microorganisms has been be achieved and thus complimentary tools are required.

Membrane Filter (MF) Technique & Colilert®

The membrane filter technique has been approved and fully accepted as a standard procedure for isolating, enumerating and identifying the coliform indicator group in water and wastewater quality assessment (Hsu & Williams 1982; APHA 2005; USEPA 2000). The MF Technique is an effective method that can provide presence or absence information within 24 hours and involves less preparation and cost than many traditional culturing methods. The usage of MF method is limited when the testing waters contain high turbidity level or high levels of toxic metals or toxic organic compounds.

Colilert® (Idexx Laboratories, Westbrook, ME) is a commercially available test kit that uses the defined substrates technology (DST) to allow quick and simple microbial

analysis process in the laboratory compared to MF technique. It is an easy and straightforward testing procedure with only 24 hours of incubation time to obtain the results. The application of Colilert as a viable method for determining the presence and number of total coliforms and *E. coli* in wastewater effluent was favored by the evidence found in studies conducted by Elmund et al. (1999), Kramer and Liu (2002) and Eccles et al. (2004). Its usage has been certified by the USEPA (2003). An over 3-year of study conducted by Buckalew et al. (2006) further validated that Colilert DST is a reliable method that showed a statistically significant positive correlation with MF technique.

Heterotrophic Plate Count

Heterotrophic Plate Count (HPC) is a universal and versatile biological analysis method for examination of waterborne bacteria, the origin and development of which can be traced to 120 years ago (Reasoner 2004). Basically, HPC involves colony counts of bacteria on or in solid media containing organic compounds of carbon and nitrogen as sources of energy and nourishment, which is able to approximate the concentration of culturable heterotrophic bacteria in water and wastewater under investigation (Bartram et al. 2003). As a traditional standard culture-based technique, HPC plays an important role in estimating general microbial population in water and wastewater effluent quality and typically requires an incubation time of 7 days before obtaining the results. HPC does not, however, give an indication of the types of organisms and a full extent of pathogens.

It underestimates the total microbiological population by enumerating only 0.1% to 1% of the living organisms (Zengler 2008). This could be possibly explained by the presence of the bacteria in viable but non-culturable state and the fact that HPC media are relatively deficient in nutrients and not able to accommodate the complex nutritional substances required for the growth of all heterotrophs.

2.2 ATP Technology

2.2.1 ATP as a Biomass Indicator

ATP, a universal molecule providing primary energy source for cellular metabolism, is present as the driving force in bioenergetics reactions in all living cells (First & Drake 2013; Tifft et al. 1976). ATP content is rarely found in nonbiological systems and the ATP that is released by moribund microorganisms is rapidly decomposed and the half-time after cell death are negligible (Tifft et al. 1976; Neethling et al. 1985). Therefore, ATP assay measures all and only the living organisms present in water samples (Tifft et al. 1976).

ATP concentrations can be quantified by measuring the light produced through its reaction with naturally-occurring firefly enzyme, luciferase, in the presence of

magnesium ions and oxygen, using a luminometer (Neethling et al. 1985; Lee & Deininger 2010). Each ATP molecule reacts to produce one quantum of light. The principal of the light reaction between the ATP and the luciferin/luciferase are showed in the following chemical equations (Neethling et al. 1985; Lee & Deininger 2010; Tifft et al. 1976).

Step 1: LH2 (luciferin) + E (luciferase) + ATP $\overset{Mg++}{\Longleftrightarrow}$ E-LH-AMP (luciferyl adenylate complex) + PP (pyrophosphate);

Step 2: E-LH-AMP + O_2 → E-L-AMP (dehydroluciferyl adenylate) + light.

With decades of research and development, ATP testing can be performed onsite and requires no advanced technical skills of the personnel who runs the tests or complicated and labor-intensive preparation of samples by using ATP test kit. It can produce results within a few minutes. However, wastewater samples include various organic and inorganic compounds, suspended particles, and ions, as well as residual chemicals added during treatment processes which may interfere with the luminescent reaction of the ATP assay. Filtration is necessary to rule out the factors that may hinder the accuracy of the ATP assay when measuring cellular ATP. Another disadvantage with ATP assay is that it is a non-specific quantification tool which is not able to differentiate between species or identify the contamination of a particular source.

2.2.2 Application in Water and Wastewater Treatment

The rapid ATP assay has shown a strong correlation with the conventional heterotrophic plate count (HPC) method and the direct viable count (DVC) method when tested on a considerable quantities of drinking water samples from the United States and worldwide (Dininger & Lee 2011). This result profoundly indicated that the estimation of the bacterial population in drinking water could be achieved in a practical and timely manner, thus it could help prevent waterborne disease (Dininger & Lee 2011). Linklater and Ormeci (2014) collected samples throughout a drinking water treatment plant and observed a step-wise decrease with increasing level of treatment in both HPC colony counts and ATP contents. This suggested that ATP could be potentially used in microbial quality and treatment performance monitoring of each treatment process in a water treatment plant. ATP technology also showed promising applicability in the microbiological quality control of a drinking water distribution network (Delahaye et al. 2003) and the drinking water contaminant warning system (Ghazali 2010; Linklater and Ormeci 2014).

ATP technology has shown promise as a potential rapid indicator to control wastewater disinfection process using chlorine as the disinfectant in studies conducted by Tifft and Spiegel (1976), and Cairns et al. (1979). Linker and Ormeci (2014) observed a good

correlation between the culture-based methods and the ATP assay results from the before and after chlorinated effluent collected at a WWTP, which confirmed the aforementioned conclusion that ATP assay could be potentially used as a process and effluent microbiological quality monitoring tool. ATP technology has also been applied in activated sludge control in wastewater treatment. ATP served as an indicator of the metabolic activity of the sludge (Levin 1975) and early warning signs of bulking in an activated sludge reactor (Brault 2011).

ATP technology is capable of developing into an alternative monitoring tool and being more widely employed in rapid assessment of disinfection performance. However, very few research studies have been conducted to investigate the influence of UV light on ATP concentration or the applications of ATP technology in UV disinfection application in wastewater treatment. Thus a study focusing on the development and application of a rapid ATP analysis method is very necessary and valuable for UV disinfection monitoring in wastewater treatment.

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental Timeline and Design

The experiments were conducted at the Water Quality Laboratory in the Centre for Water Resources Studies, Dalhousie University. The experiments involved testing the pre-treatment technique: Biomass Growth ATP Method applied to UV disinfection and the application of the Biomass Growth ATP Method to three wastewater treatment facilities in Halifax Regional Municipality (HRM), NS, Canada. The development of Biomass Growth ATP Method was conducted from September of 2012 to September of 2013, and the application of Biomass Growth ATP Method was conducted from May of 2014 to June of 2014.

For the Biomass Growth ATP Method, the following water samples were tested: pure *E. coli* cultures irradiated using UV collimated beam unit in the laboratory, wastewater samples from Mill Cove Water Pollution Control Centre (WPCC) treated by the UV collimated beam unit in the laboratory and wastewater samples from Mill Cove WPCC disinfected by the UV system in the plant.

Different pre-treatment conditions were performed on the method with different water samples. Different temperatures were tested in order to optimize the methods. All the

experiments were conducted in laboratory batches. HPC and ATP assay were employed as the microbial population quantification methods.

3.2 Experimental Materials and Analytical Procedures

Wastewater Source

The Mill Cove Water Pollution Control Centre (Mill Cove WPCC) in Bedford (Nova Scotia, Canada) is the largest municipal secondary treatment facility in Atlantic Canada (Halifax Water 2014). The plant has a daily flow capacity of 5.0 million imperial gallons per day (MImpGa/Day), serving a population of 52,500 in the communities of Bedford and Sackville (Hu & Gagnon 2006; Comeau et al. 2008; Halifax Water 2014). Mill Cove WPCC was originally constructed in 1969, operated in 1970, expanded in 1981 and upgraded in 1997 by Halifax Water, with a new process design consisting of flow equalization, primary clarification, pure oxygen activated sludge biological treatment, secondary clarification and ultra violet light disinfection (Halifax Water 2014). The plant is located in a residential area as is showed in Figure 3-1 and Figure 3-2. The effluent is discharged to the Bedford Basin (in the south coast of Nova Scotia) through an underwater pipe which is a few kilometers offshore (Crouse et al. 2012). A process schematic of the plant is presented in Figure 3-3. For this research, secondary wastewater

effluent (both prior to and post UV disinfection) was sampled from the plant. The wastewater samples were transported in a cooler to the laboratory immediately after collection and the experiments were performed within 5 hours after the collection.

Figure 3-1 Satellite map of Mill Cove WPCC (Google map 2014)

Figure 3-2 Top view of Mill Cove WPCC (Halifax Water 2014)

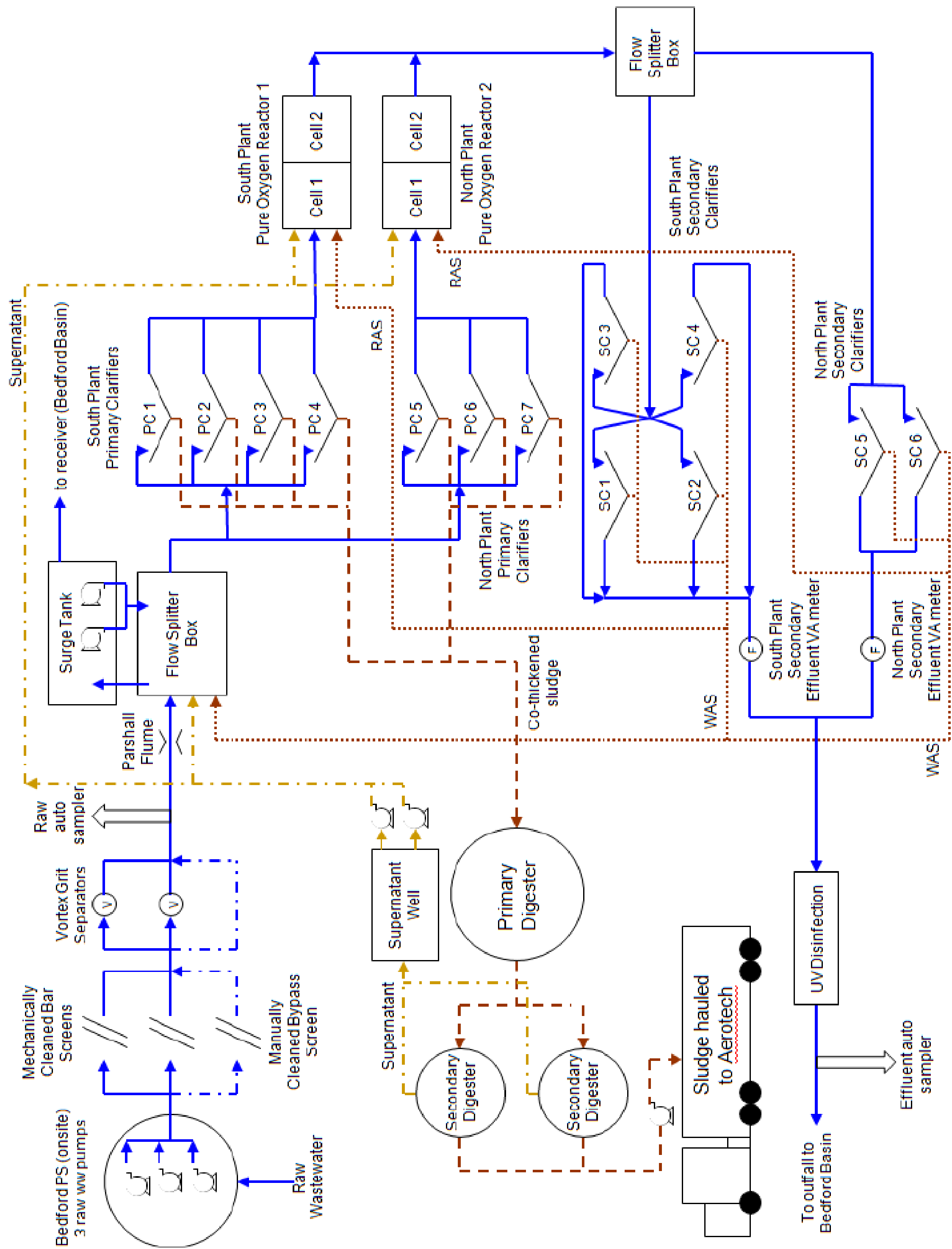


Figure 3-3 Mill Cove WPCC process flow diagram (Halifax Water 2014)

UV Light Source and Exposures

The UV collimated beam unit (CBU) system (Calgon Carbon Cooperation, PA, USA) with a 40W low-pressure (LP) Mercury UV lamp tube emitting light at a wavelength of 254 nm was used as the UV light source to irradiate the samples, illustrated in Figure 3-4 and Figure 3-5. The CBU consists of a lamp chamber, an electrical cabinet complete with control indicators, and a UV collimator with a shutter for instant directed ultraviolet light (Calgon Carbon Cooperation 2013). The CBU is designed to expose liquid or solid samples under uniform UV light with a consistent intensity on a batch basis, which has an irradiation area of 6 cm diameter (Calgon Carbon Cooperation 2013). The operational steps instructed in the Collimated beam unit operation and maintenance manual (Calgon Carbon Cooperation 2013) were followed to perform the tests. First, turning on the UV lamp to warm it up. When the full power was attained, UV irradiance was measured at the centre of the beam using an ILT-1400 radiometer photometer (International Light Technologies, MA, USA). Then the water sample in a petri dish on a magnetic stir plate was placed under the UV collimator for a fixed period of exposure time while being stirred, which was controlled by opening and closing the shutter to achieve a desired UV dose. The UV Lamp was turned off after all the required samples were treated. The protocol for collimated beam test employed were the procedures that outlined by Bolton and Linden (2003) and Kuo et al. (2003). Average irradiance (E_{avg}) was calculated by

using the UV irradiance reading from the radiometer ($E_{reading}$), the thickness of the water sample in petri dish, the absorbance of the water per centimeter thickness (A_{1cm}), adsorption factor, petri factor, and integration factor, expressed by Equation 3.1 (Bolton & Linden 2003; Calgon Carbon Cooperation 2013). Desired UV dose divided by average irradiance is exposure time, expressed by Equation 3.2 (Calgon Carbon Cooperation 2013).

$$E_{avg} = E_{reading} \times \text{integration factor} \times \text{Petri factor} \times \frac{1-10^{-A_{1cm}\times l}}{2.303\times A_{1cm}\times l} \quad (3.1)$$

$$\text{UV dose (mJ/cm}^2\text{)} = E_{avg}\text{(mW/cm}^2\text{)} \times \text{exposure time (s)} \quad (3.2)$$

Figure 3-4 Bench-scale Calgon Carbon collimated beam unit

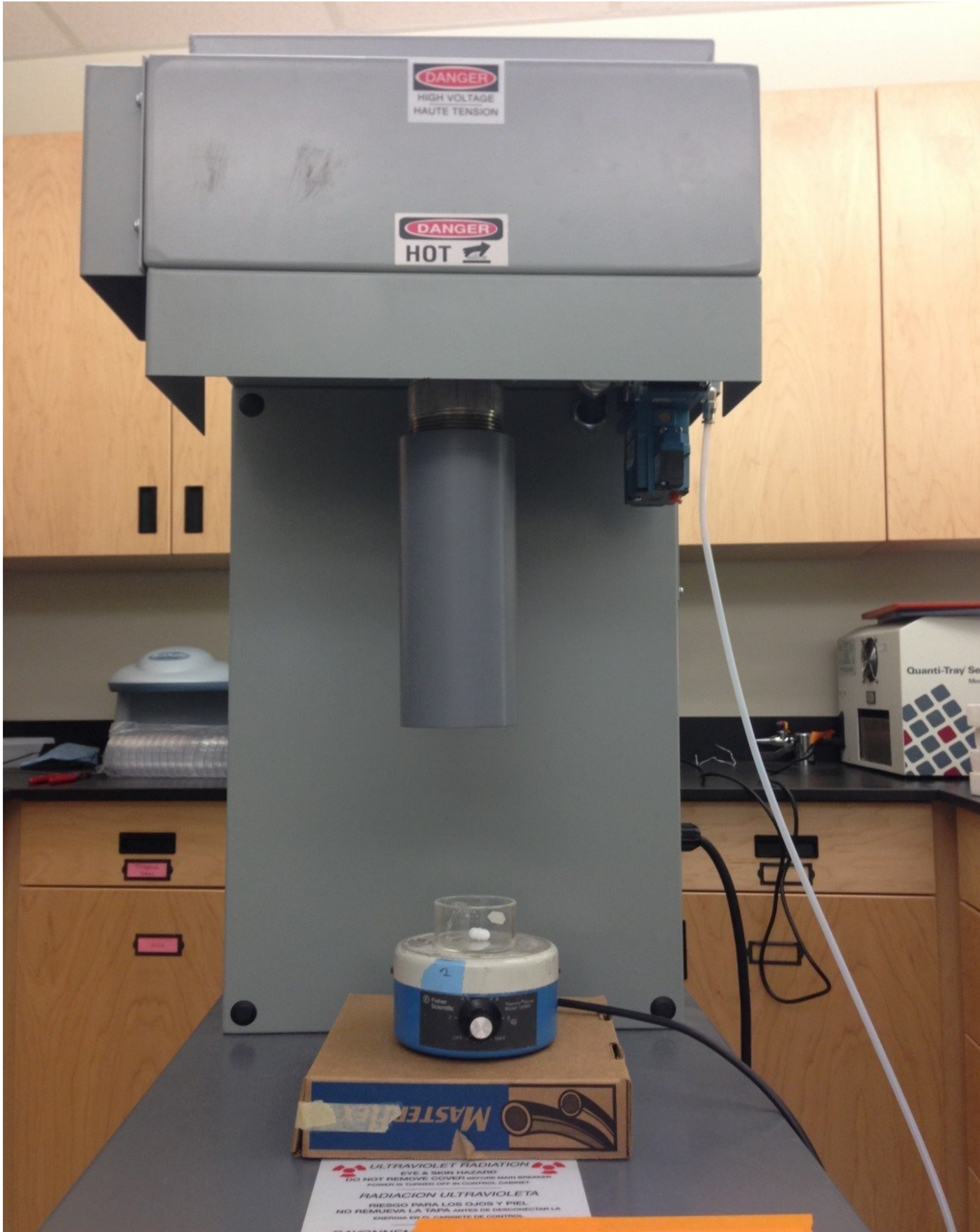


Figure 3-5 Bench-scale collimated beam unit experimental setup

E. coli Inoculation, Cultivation and Dilution Procedure

Pure *E. coli* K12 culture (ATCC#47076 Strain Designations: MG1655) purchased from

the American Type Culture Collection (ATCC) was aseptically streaked using a sterile inoculating loop onto Tryptic Soy agar (TSA) (Becton Dickinson and Co., MD, USA) and incubated at 37.5℃ for 24 hours. Colonies were aseptically removed from the plates using a sterile inoculating loop and placed into a sterile disposable 15 mL polypropylene centrifuge tube (Fisherbrand, Fisherscientific Canada) containing about 14 mL Tryptic Soy broth (TSB) (Becton Dickinson and Co., MD, USA). The *E. coli* inoculated TSB tubes were incubated for 24 hours at 35℃. Then 1.37 mL of *E. coli* in TSB was added into 0.23 mL of 70% glycerol and preserved in a 2 mL sterile Nalgene cryogenic polypropylene vial (Sigma-Aldrich, MO, USA) for later usage. Twenty four vials were made by repeating this procedure, then stocked and frozen at -80℃.

For each batch of experiment, one of the vials was taken out of the freezer and thawed while it reached the room temperature. 0.4 mL stock *E. coli* were pipetted into ~15 mL TSB in a sterile disposable 15 mL polypropylene centrifuge tube and incubated overnight at 35℃. After incubation, the tube was centrifuged at 3000 rpm for 15 minutes, the supernatant was decanted. The pellet and the tube were rinsed once using sterile PBS to ensure that there was less TSB remaining in the solution. Then the pellet was re-suspended by vortexing in ~15 mL of sterile PBS, which was diluted further in 500 mL PBS.

The buffered pure *E. coli* samples was utilized as monobacterial solutions to provide uniform, consistent and controlled experimental conditions. This allowed for the assessment of performance and limitations of the ATP bioluminescence assay and the reproduction of the experiments.

Glassware Preparation and Stock Preparation

An ultrapure water purification system (Millipore Corporation, MA, USA) with typical resistance of ≈18,2 MΩ. cm at 25℃ was used throughout the experiments to provide deionized (DI) water which is as known as Milli-Q water. All glassware were thoroughly cleaned by washing and triply rinsing with Milli-Q prior to experiments. Phosphate buffered saline (PBS) solution was prepared freshly in accordance to Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 22nd edition), and autoclaved before usage.

Heterotrophic Plate Count

Spread Plate Method was employed to determine the total microbial population present in the samples according to the Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 22nd edition). Desired volumes (0.1mL

– 0.5mL) of sample or diluted sample were pipetted onto a suitable predried and absorbent R2A agar (Becton Dickinson and Co., MD, USA) plate or nutrient agar (Becton Dickinson and Co., MD, USA) plate using autoclaved tip of an 10–100 μL microlitre pipet (VWR International, PA, USA). Then the spot was distributed over the agar surface with a sterile bent glass rod. The R2A agar plates were incubated at 28℃ for 7 days, and the nutrient agar plates were incubated at 35℃ for 48 hours. After desired incubation time was achieved, the colonies were counted with the aid of a Quebec colony counter (American Optical Company, NY, USA).

ATP Assay

The ATP assay was performed by employing the Quench-Gone-Aqueous (QGA) Test Kits provided by LuminUltra Technologies Ltd., Fredericton, NB, Canada and the Kikkoman Lumitester C-110 (HACH Co., CO, USA), illustrated by Figure 3-6 and 3-7 respectively.

Cellular ATP (cATP) is quantified by measuring the light produced through its reaction with the naturally-occurring firefly enzyme Luciferase using a Lumitester. The QGA kit measures down to 0.1 pg/mL using the standard protocol claimed by LuminUltra Technologies Ltd.. The standard procedures include filtering the water sample through a

syringe, then lysing the microorganisms left on the filter to release the ATP. After dilution, the amount of light can be measured using the Lumitester, which was directly proportional to the amount of cATP present in the sample. The reading in relative light unit (RLU) from the Lumintester can be transformed into the cATP concentration using the Equation 3.3 (LuminUltra Technologies Ltd. 2014).

$$cATP\left(\frac{pgATP}{mL}\right) = \frac{RLU_{cATP}}{RLU_{UC1}} \times \frac{10{,}000(pgATP)}{V_{sample}(mL)} \qquad (3.3)$$

An instruction provided by LuminUltra Technologies Ltd. is attached as Figure 3-8 to show how to conduct the ATP test step by step.

Figure 3-6 Quench-Gone-Aqueous (QGA) Test Kit (LuminUltra Technologies Ltd. 2014).

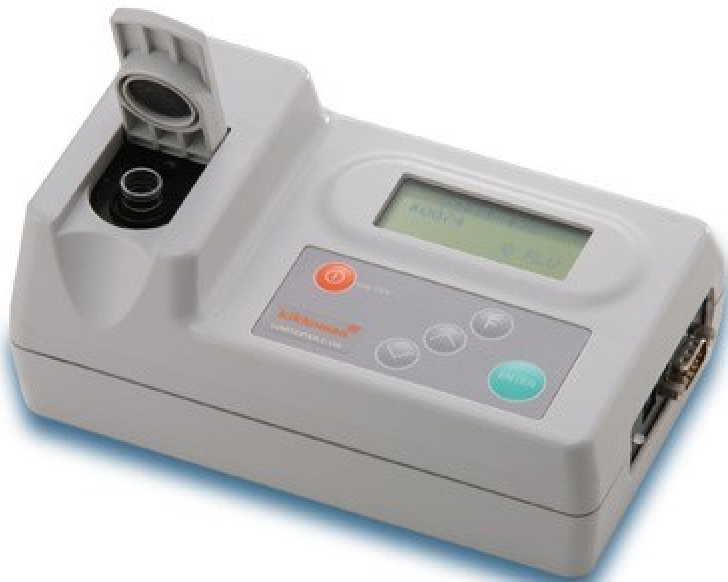


Figure 3-7 Kikkoman Lumitester C-110 (LuminUltra Technologies Ltd. 2014).

Quick Reference Guide

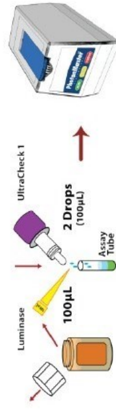
Quench-Gone™ Aqueous Test Kit

Product #: QGA-25 / QGA-100

NOTE: Please refer to Test Kit Instructions during first product use and for additional details including legal statements.

Step 1 - UltraCheck™ 1 Calibration

Perform one UltraCheck 1 calibration per day or per each set of samples analyzed.

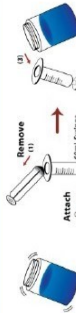


NOTE: If $RLU_{ATP1} \le 5{,}000$ using a PhotonMaster or Lumitester C-110, rehydrate a new bottle of Luminase for maximum sensitivity.

Step 2 - Cellular ATP (cATP™) Analysis

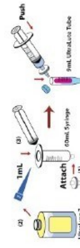
2.1 – MEASURE SAMPLE VOLUME
Determine volume and filter sample.

Sample Type	Volume (mL)
Cooling or Process Water	10 to 25
Fresh Brackish & Salt Water	25 to 50
Reclaimed Water, Effluents	25 to 50
Drinking and Sanitary Water	50 to 100
High Purity Water	> 100

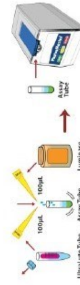


2.2 – FILTRATION
Filter sample.

2.3 – EXTRACTION
Extract ATP from filter & dilute.



2.4 – ASSAY
Measure ATP concentration.



NOTE: If $RLU_{cATP} \le 10$ using a PhotonMaster or Lumitester C-110, you are below the low- detection limit

NOTE: If $RLU_{cATP} \le 50$ using a PhotonMaster or Lumitester C-110, consider accounting for background (RLU_{bg}). See Test Kit Instructions for guidance.

Calculations

Cellular ATP (cATP) Calculation):

$$cATP\ (pg\ ATP/mL) = \frac{RLU_{cATP}}{RLU_{ATP1}} \times \frac{10{,}000\ (pg\ ATP)}{V_{Sample}\ (mL)}$$

Microbial Equivalent (ME/mL):

$$cATP\ (ME/mL) = cATP\ (pg\ ATP/mL) \times \frac{1\ ME}{0.001\ pg\ ATP}$$

NOTE: 1 ME (Microbial Equivalent) assumes 0.001 pg (1fg) ATP per cell.

Interpretations Guidelines

Application	Good Control (pg cATP/mL)	Preventative Action (pg cATP/mL)	Corrective Action (pg cATP/mL)
High Purity Water	< 0.1	0.1 to 1.0	> 1.0
Water for Consumption (Potable, Sanitary)	< 0.5	0.5 to 10	> 10
Raw Make-up Water (Fresh Brackish, Salt, Reclaimed)	< 10	10 to 100	> 100
Cooling & Process Water (Oxidizing Biocides)	< 10	10 to 100	> 100
Cooling & Process Water (Non-Oxidizing Biocides or Non-Chemical Treatment)	< 100	100 to 1,000	> 1,000

NOTE: Interpretation Guidelines provided for general guidance. For best results, establish your own baseline and control levels.

Date Modified: 5-Apr-13

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Figure 3-8 ATP assay procedures instruction (LuminUltra Technologies Ltd. 2014)

Statistical Analysis

Experimental data was recorded, processed and plotted by using Microsoft Excel 2013 (Seattle, WA, USA) for further analysis and discussion to compare the effectiveness of each method. Basic statistical values were reported as means and coefficient of variance. One-way and two-way ANOVA, Pearson correlation coefficient and regression analysis (with a confidence interval set at 95%) were performed using Minitab 16 Statistical Software (State College, PA, USA)

3.3 Technical Approach

3.3.1 Assessment of Current Rapid Monitoring Technologies

ATP Technology

Four experiments of ATP assay were conducted on before and after UV treated secondary wastewater effluent samples by employing the Quench-Gone-Aqueous (QGA) Test Kits provided by LuminUltra Technologies Ltd., Fredericton, NB, Canada and the Kikkoman Lumitester C-110. Duplicate or triplicate tests were performed for each experiment. Wastewater samples were taken from Mill Cove WPCC, Halifax, NS, Canada. Experiments of inactivation of microorganism according to different UV doses applied to pure *E. coli* culture samples was also performed in laboratory. Pure K12 *E. coli* culture

was prepared as described in the section of "*E. coli* Inoculation, Cultivation and Dilution Procedure".

Electron Transport System (ETS) Activity Assay

The phenomenon described above had been further proved by measuring the electron transport system (ETS) activity.

Cellular respiration is the process that releases energy by breaking down three main foodstuffs—carbohydrates, fats, and proteins in the presence of oxygen. By converting food energy into ATP energy, cells are provided with the energy they need to carry out the activities of life. This requires three distinct but interconnected pathways: Glycolysis, the Krebs cycle and the electron transport system, as is illustrated in Figure 3-9. Most oxidative phosphorylation of ADP to form ATP takes place in the electron transport system (ETS). The ETS, which is also called electron transport chain or the respiratory chain, is located in a cell's mitochondria. It consists of a complex chain of cytochromes, flavoproteins, and metallic ions that transport electrons from catabolized foodstuff to oxygen (Packard 1971).

The Electron transport system activity assay, which is also as known as INT-

dehydrogenase assay, is a rapid measurement to demonstrate the presence of the respiratory processes in cells indicating the microbial activity in water samples utilizing the redox dye, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (Lee et al. 1988). INT is a relatively colorless chemical. However, when it is reduced by the ETS of actively metabolizing cells, it becomes a purple-red compound iodonitrotetrazolium formazan (INTF) that precipitates within the cells. This INTF can be extracted from microbial cells by dimethyl sulfoxide (DMSO) and quantified by colorimetric measurement. The optical density of the INTF extraction directly corresponds to the intensity of ETS activity.

The ETS assay employed in this research was a modified version of Dufour and Colon's work (1992). To prepare the reagents, 200 mg 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was dissolved in 50ml of autoclaved DI water in a 100 mL beaker. To facilitate solubilisation, the solution was stirred vigorously on a hot plate set at low heat. The solution should be colorless or faint yellow. Then the INT concentration was stored at 4℃ refrigerator in a sterile container.

The tests were performed on buffered DI water sample that has been inoculated with pure *E. coli* culture. The treatment of each UV dose was duplicated so that it was able to duplicate ETS assay for each dose. After irradiating 30 mL water sample with UV

collimated beam unit, and removing a subsample for HPC analyses, 2 ml of INT concentrate was added into the remaining volume (28 mL), followed with an incubation of 1 hour at 35°C. The same steps were performed for 28 mL non-UV-treated sample. The before UV sample had an easily visible purple-red color after incubation. After incubation, analysis was completed immediately by extracting and measuring the formazan produced by ETS activity. The detailed steps of this method are elaborated in the following paragraph.

Each sample was centrifuged at 3000 rpm for 15 minutes so that the bacteria were pelleted into the bottom of the centrifuge tube. The supernatant was decanted and the pellet was suspended in 5 ml dimethyl sulfoxide (DMSO). Incubate in the dark for 20 minutes. Then the absorbance which is also as known as optical density was measured using a DR5000 spectrophotometer (HACH Co., CO, USA) at a wavelength of 465 nm. DMSO was used as the blank sample.

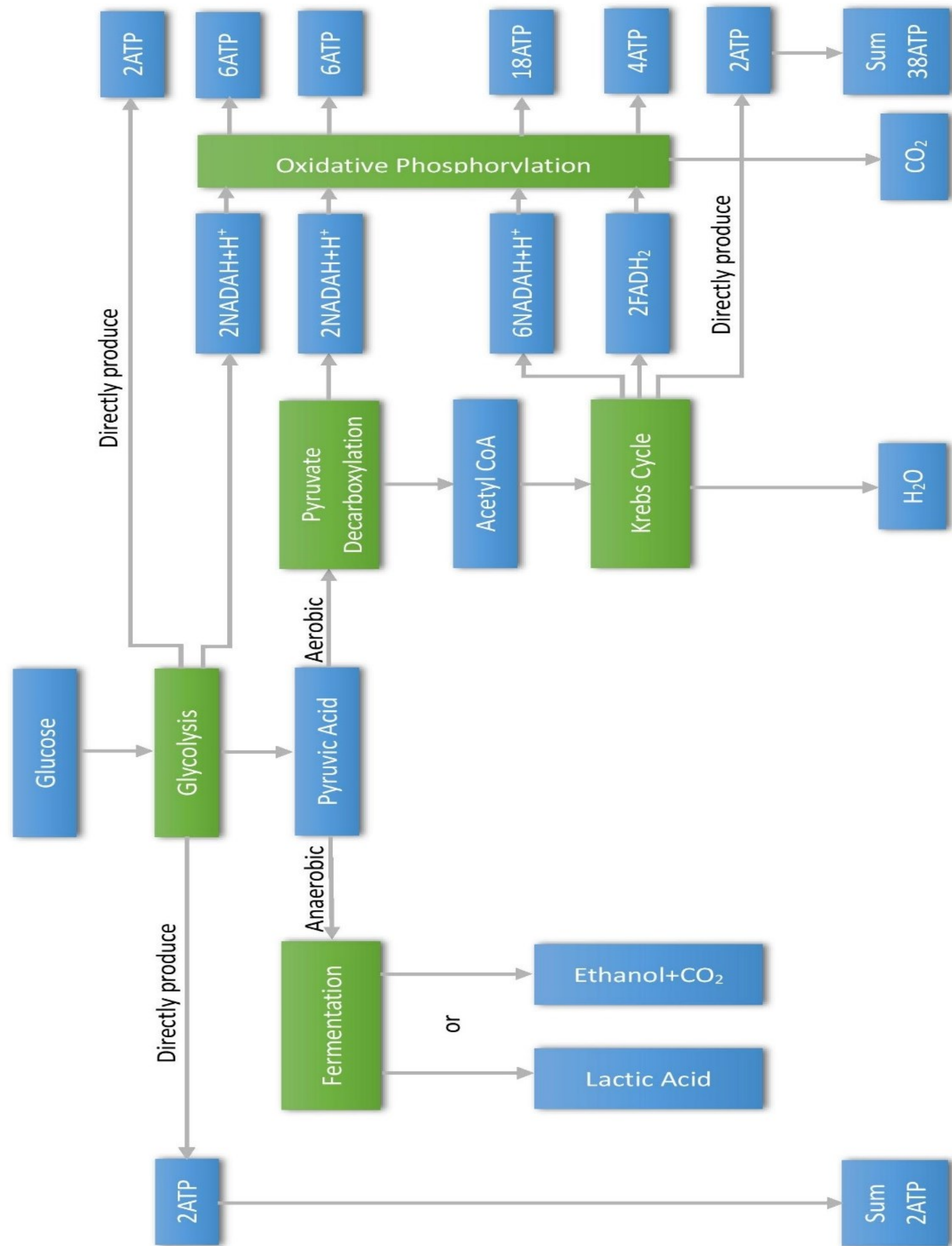


Figure 3-9 Flow chart of cellular respiratory process (Rich 2003; Lodish et al. 2000; Nelson & Cox 2000)

3.3.2 Development of a Pre-treatment Technique for ATP Test

Optimization of Biomass Growth Reagents

In this experiment, different chemical reagents developed in partnership with LuminUltra Technologies Ltd. were tested to select the most suitable reagent for bacterial cultivation.

Five millilitres of before UV treatment secondary wastewater effluent from Mill Cove WPCC was added into 45 mL of the chemical reagent in a 50 mL sterile centrifuge tube, which were incubated for 4 hours at a range of temperatures (20 - 40℃). Then a standard ATP assay was performed by using the Quench-Gone-Aqueous (QGA) test kits to assess the microbial growth after the incubation.

Optimization of Incubation Temperature

In the experiment of optimizing the incubation temperature, temperatures in the range of 20 to 40℃ were tested to determine the optimum temperature for microbial cultivation. The incubation condition was optimized by tweaking the combination of temperature and reagent to further decrease the incubation time. 5 mL of before or after UV treated wastewater samples from Mill Cove WPCC were subjected in 45 mL chemical reagent in sterile 50 mL Falcon centrifuge tubes and incubated at different temperatures for 4 or 6

hours. The incubation was followed by the standard ATP assay was followed using the QGA test kits.

3.3.3 Ensuring the Practicality of the Method

Test of Robustness for Field Sampling

In the experiment of testing the stability of the Biomass Growth ATP Method, before and after UV treatment wastewater secondary effluent samples were taken from Mill Cove WPCC. After the wastewater being transported to the laboratory, both straight ATP assay and ATP assay with Biomass Growth ATP Method were performed on the fresh wastewater sample. Data was collected for later comparison with stored sample.

The rest of the fresh sample was stored at 4℃ in a refrigerator overnight. Then a portion of sub-sample was taken from the overnight 4℃wastewater sample and preheated to room temperature. Straight ATP assay without pretreating the water samples was carried out to test both refrigerated wastewater sample (4℃) and the room temperature wastewater sample. The chemical reagent was kept at room temperature. Part of the reagent was put into a sterile 500 mL clear glass laboratory bottle to be preheated in the incubator. Then the Biomass Growth ATP Method was performed to pretreat the samples.

5 mL of the refrigerated wastewater sample (4℃) or the room temperature wastewater sample were added into 45 mL of room temperature reagent or pre-warmed reagent in 50 mL centrifuge tubes. These four combination are showed in Table 3-1. After 4 hours' incubation, the ATP assay was followed to measure the total microbial population.

Table 3-1 Combinations of samples and reagents of different temperatures

Groups	Sample (5 mL)	Reagent (45 mL)
①	Refrigerated overnight sample (4℃)	Room temperature reagent
②	Refrigerated overnight sample (4℃)	Pre-warmed reagent
③	Room temperature overnight sample	Room temperature reagent
④	Room temperature overnight sample	Pre-warmed reagent

Test of Repeatability

In the experiment of testing the repeatability of the Biomass Growth ATP Method, three trials of the method were tested with an increment of UV dosages. Fresh *E. coli* K12 pure culture which was spiked into sterile phosphate buffered water was prepared daily to be used as synthetic water samples. Details of *E. coli* inoculation is described in Chapter 3. 30 mL of the sample was pipetted into a glass Petri dish. The Petri dish had a diameter of 5.9 cm and a depth of 3.5 cm. Samples were placed under the UV light to be treated using the UV collimated beam unit (CBU) system (Calgon Carbon Cooperation, PA, USA).

Figure 3-10 showed the experimental set-up for UV treatment in laboratory scale. A timer was used to keep track of the exposure time to obtain a designed UV dose. For the first trial, a wide UV dose range from 0 to 150 mJ/cm^2 was tested to get a general idea of the trend. The UV doses applied to the first trial were 10 mJ/cm^2, 50 mJ/cm^2, 100 mJ/cm^2, 150 mJ/cm^2. Then the UV dose range was reduced to 100 mJ/cm^2 to test more specific doses within this interval. The UV doses employed in the second batch of the test were 5 mJ/cm^2, 8 mJ/cm^2, 12 mJ/cm^2, 20 mJ/cm^2, 50 mJ/cm^2, 80 mJ/cm^2 and 100 mJ/cm^2. Then in the third trial, the practical UV dose range (0 mJ/cm^2 – 20 mJ/cm^2) was tested again. Then the Biomass Growth ATP Method was performed to pretreat the samples. Either before UV or after UV treated 5 mL of the *E. coli* sample was added into 45 mL of the new formulation reagent in 50 mL centrifuge tubes, and incubated at a certain temperature for 4 hours. The ATP assay was performed after the incubation. Then the quantification of total *E. coli* population was performed by carrying out the ATP assay.

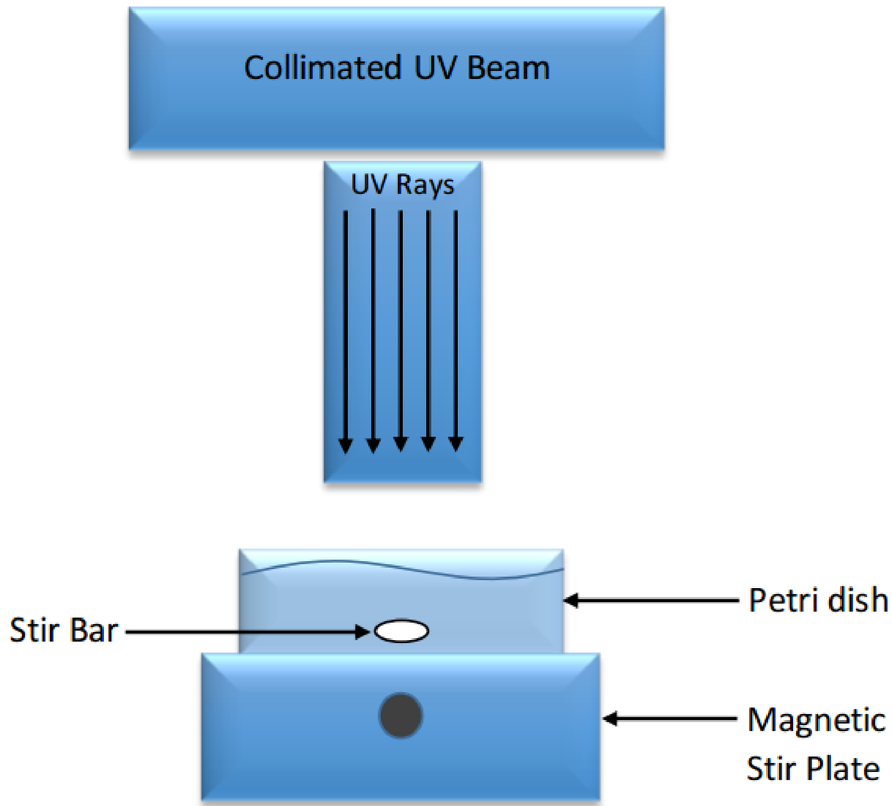


Figure 3-10 Experimental set-up for UV treatment in laboratory scale

3.3.4 Comparison to Accepted Culturing Method

The pure *E. coli* cultures inoculated in buffered water and before UV treated secondary wastewater effluent taken from Mill Cove WPCC were used as water samples.

Thirty millilitres of sample was pipetted into a glass Petri dish. The Petri dish had a diameter of 5.9 cm and a depth of 3.5 cm. Samples were placed under the UV light to be treated using the UV collimated beam unit (CBU) system (Calgon Carbon Cooperation,

PA, USA). Figure 3-10 showed the experimental set-up for UV treatment in laboratory scale. A timer was used to keep track of the exposure time to obtain a designed UV dose. The *E. coli* cultures were tested to prove that Biomass Growth ATP Method works for controlled and laboratory scale experiment. The UV doses applied to treat pure *E. coli* cultures were 5 mJ/cm^2, 8 mJ/cm^2, 12 mJ/cm^2, 20 mJ/cm^2, 50 mJ/cm^2, 80 mJ/cm^2 and 100 mJ/cm^2. Then the UV dose range was narrowed down to a more practical dose range to test this optimum pre-treatment technique on secondary wastewater effluent. The UV doses employed in irradiating wastewater samples were 5 mJ/cm^2, 8 mJ/cm^2, 12 mJ/cm^2, 20 mJ/cm^2 and 50 mJ/cm^2.

A small volume of the sample was taken from the UV-treated solution in the petri dish to be proceeded with HPC method. Microbial reduction of pure *E. coli* solution and wastewater samples was monitored using HPC method (nutrient agar, 35℃, 48 hours) and HPC method (R2A agar, 28℃, 7 days) respectively.

Meanwhile, the Biomass Growth ATP Method was performed to pretreat the samples after irradiation. 5 mL sample taken from the rest of UV-treated solution (28 mL) in the petri dish was added into 45 mL of the optimal reagent in 50 mL centrifuge tubes. After 4 hours' incubation at the optimum temperature, the ATP assay was followed immediately to measure the total microbial population.

3.3.5 Field Scale Evaluation of Biomass Growth ATP Method

Three wastewater treatment facilities within HRM were chosen as sampling sites in this research, which are Eastern Passage Wastewater Treatment Facility (Eastern Passage WWTF), Mill Cove Water Pollution Control Centre (Mill Cove WPCC) and Fall River Water Pollution Control Plant (Fall River WPCP). Both before and after UV treated wastewater were taken weekly at these three wastewater treatment plants for four weeks. Flow rate and UV Transmittance % of the UV chamber at each facility was recorded. After the fresh samples were transported to laboratory, Biomass Growth ATP Method was performed to pre-treat the samples immediately. 5 mL of the sample was added into 45 mL of the optimal reagent in 50 mL centrifuge tubes. After 4 hours of incubation at the optimum temperature, the ATP assay was followed to measure the total microbial population. The microbial reduction was also monitored by using HPC method (R2A agar, 28℃, 7days).

The information of Mill Cove WPCC is elaborated in section 3.2. General summaries of Eastern Passage WWTF and Fall River WPCP are described in the following paragraphs.

Eastern Passage WWTF

The Eastern Passage WWTF in Dartmouth (Nova Scotia, Canada) has a daily flow capacity of 25,000 m^3, serving a population of 50,000 in the communities of Cole Harbour, Eastern Passage and Shearwater. Eastern Passage WWTF was originally constructed in 1974, expanded in 1987 and further expanded and upgraded in 2013 by Halifax Water. The new process design flow diagram is showed in Figure 3-12. The effluent is discharged to the Halifax harbour (Halifax Water 2014).

The UV disinfection process is incorporated with an open channel UV disinfection system. It includes two UV banks which are equipped with low pressure high output amalgam lamps with flow-proportional lamp dimming capability. The UV system is followed by common serpentine weir. An average UV Dose of 32 mJ/cm^2 was applied to be complied with the disinfection criteria of no grab sample to exceed 400 MPN/100 mL fecal coliform (Halifax Water 2014).

Figure 3-11 Top view of Eastern Passage WWTF (Halifax Water 2014)

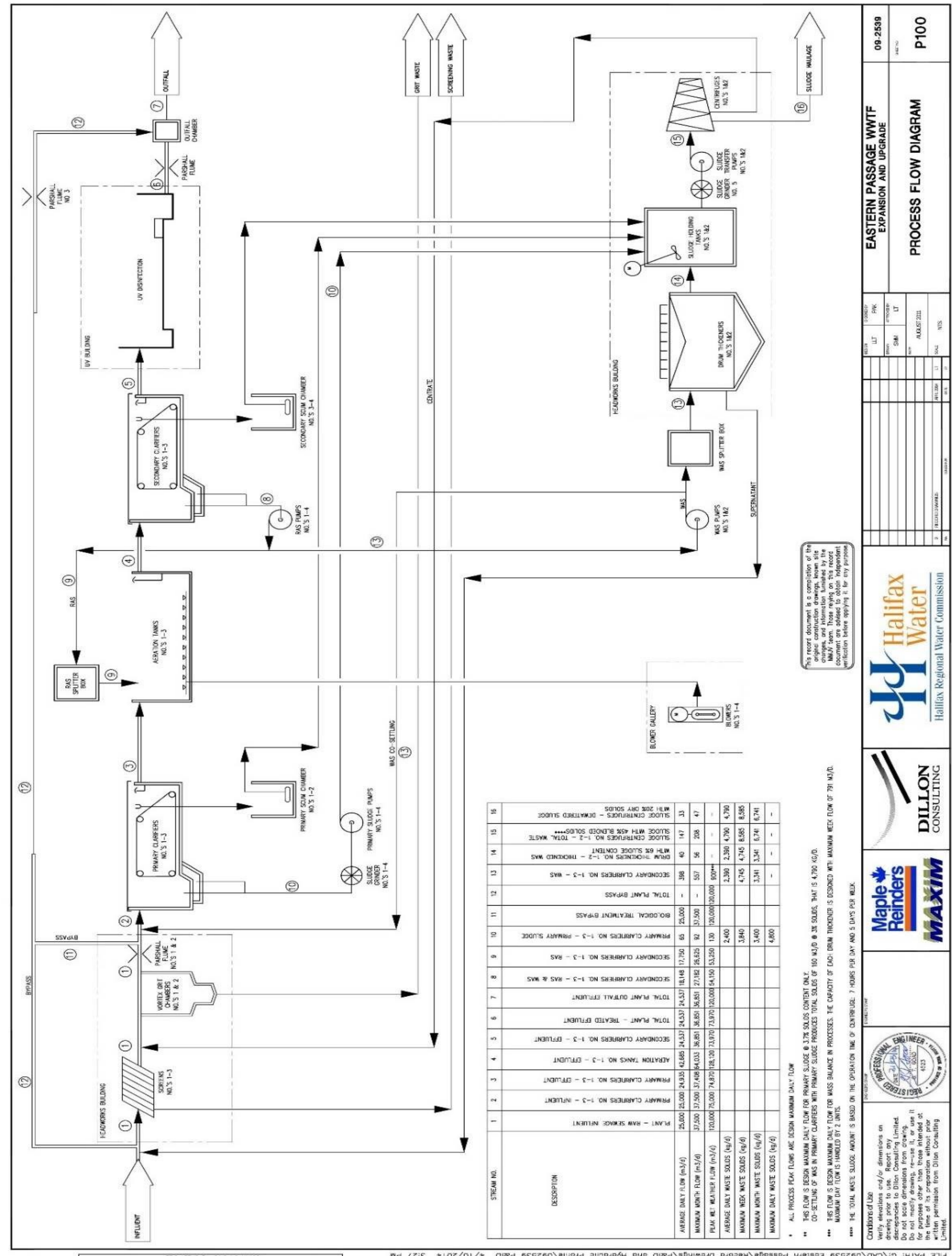


Figure 3-12 Eastern Passage Wastewater Treatment Facility process flow diagram (Halifax Water, 2014)

Fall River WPCP

Fall River WPCP was designed and commissioned in 1994. As is presented in the top view picture of the facility (Figure 3-13) and the process schematic of the plant process schematic of the plant (Figure 3-14), it is a small community plant which is incorporated 100,000 gal/day extended aeration treatment process with tertiary filtration, nutrient (phosphorus) removal, nitrification and ultra violet disinfection. A high quality tertiary effluent is required due to the environmental sensitivity of the Fletcher Lake receiving water (Halifax Water 2014).

Figure 3-13 Top view of Fall River WPCP (Halifax Water 2014)

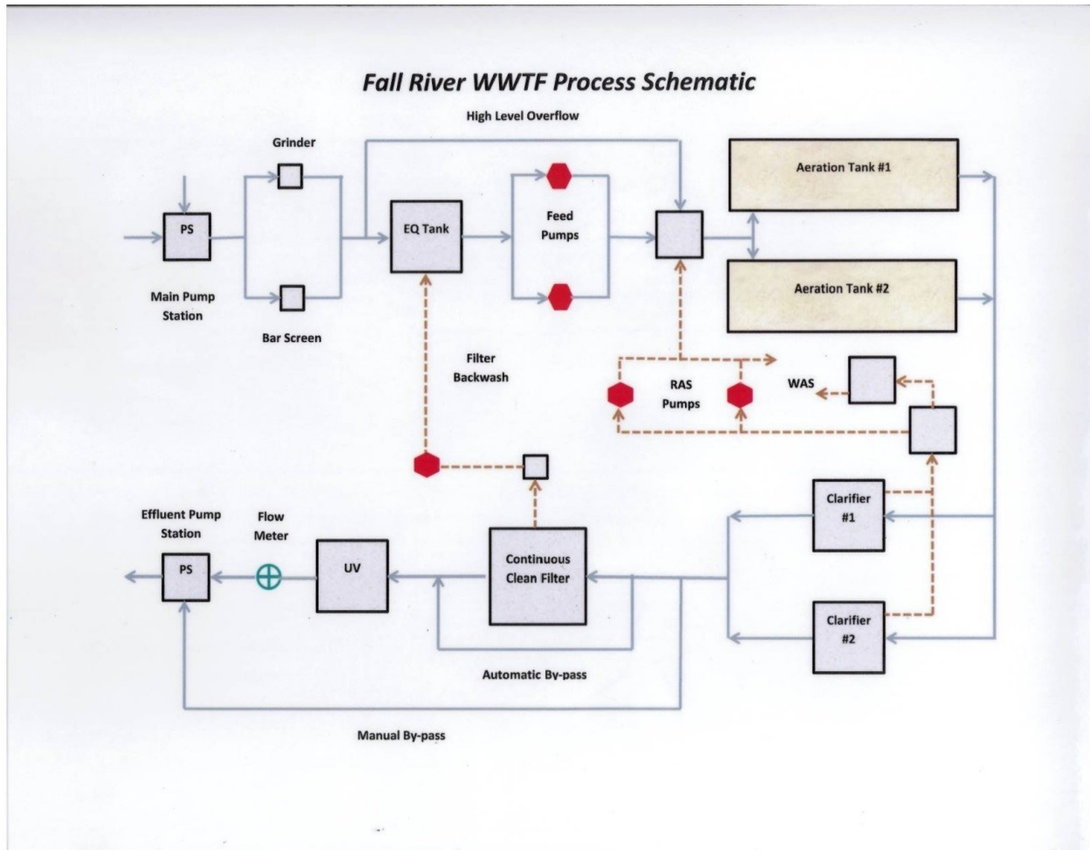


Figure 3-14 Fall River WPCP process flow diagram (Halifax Water 2014)

CHAPTER 4 RESULTS ANALYSIS

4.1 Assessment of Current Rapid Monitoring Technologies

ATP Technology

A challenge with current ATP monitoring technology confronted by industry is that an ultraviolet (UV) dose sufficient to cause orders of magnitude reduction in culturability yields little to no reduction in ATP (Villaverde et al. 1986; First & Drake 2013). As is indicated in Table 4-1, the HPC method can detect an average 1.40 log reduction of the microorganism presented in the secondary wastewater effluent samples, while the ATP test showed almost no disparity of common logarithm value of ATP concentration between before and after UV treated water samples. This was proven by performing an ANOVA test on the data of ATP assay. The statistical analysis showed that there was no detectable difference between the means of before and after UV treatment samples ($p = 0.707$).

The results of the experiment which applied different UV doses to irradiate pure *E. coli* culture solution are presented in Table 4-2. The data obtained from ATP assay fluctuated in the same order of magnitude (about log 4.40), and failed to show a clear decreasing trend with increasing UV irradiation (from 0 to 100 mJ/cm^2). Whereas the HPC method

observed a microbial log reduction of 4.38 at the UV doses of 50 and 100 mJ/cm^2 in microorganism density. This confirmed the results obtained with wastewater sample that the ATP approach did not perceive a noticeable variation of microbial response to different UV treatments compared to culture-based enumeration technique. This likely occurs because UV irradiation causes minimal immediate damage to cell respiration, as well as ATP testing detects viable but non-culturable (VBNC) microorganisms (Shimomura 2006; Basu et al. 2007; Bolton & Cotton 2008).

Table 4-1 Comparison of effectiveness of current ATP assay and HPC method tested on secondary wastewater effluent samples from Mill Cove WPCC

Sampling date	Log [ATP (pgATP/mL)]		Log [HPC (CFU/mL)]	
	Before UV	After UV	Before UV	After UV
Nov. 2nd, 2012	4.04	4.15	6.11	4.76
Nov. 28th, 2012	4.17	4.02	6.14	4.48
Dec. 13th, 2012	3.95	3.91	5.73	4.02
Dec. 19th, 2012	4.32	4.26	6.17	5.26

[1] Data was reported in common logarithm value

Table 4-2 Comparison of effectiveness of current ATP assay and HPC method tested on pure *E. coli* culture samples

UV Dose (mJ/cm^2)	Log [ATP (pgATP/mL)]	Log [HPC (CFU/mL)]
0	4.40	6.97
6	4.36	6.90
8	4.26	6.79
12	4.48	5.72
20	4.40	2.76
50	4.63	2.59
80	4.55	2.82
100	4.49	2.59

Electron Transport System (ETS) Activity Assay

The results displayed in Table 4-3 showed that the difference of absorbance between before and after UV treatment. Statistical analyzing tools like ANOVA test, Tukey test and Dunnett's test were employed to determine if the UV irradiation alters the cellular respiration. The results of ANOVA test showed that UV irradiation did have a significantly adverse effect on the ETS activity ($p = 0.002$). However, it can be observed from the absorbance data in Table 4-3 that the INTF precipitate produced through the ETS activity still was maintained at a relatively high level (92.2%) at the UV dose of 50 mJ/cm^2 when compared to the straight sample which was not treated by UV light. This indicated that the intensity of ETS activity did not dramatically decrease or even cease

even when the UV dose is sufficiently high to inactivate almost 99.999% of the microbial population presented in the water sample. This is consistent with the research outcome found by Kelner (1953) that UV light has no immediate effect on aerobic cellular respiration. Thus it allowed us to conclude that the majority of rapid monitoring methods based on the detection of cellular respiration and ATP content probably would fail for UV disinfection. More data points would be required to generate an ETS activity – UV response curve that can show the trend of the change of ETS activity intensity clearly.

Table 4-3 Comparison of effectiveness of ETS assay and HPC method

UV dose (mJ/cm^2)	ETS (Absorbance @465 nm)	Log [HPC(CFU/mL)]
0	0.0965	6.9
10	0.0915	5.9
50	0.0890	2.3

4.2 Development of a Pre-treatment Technique for ATP Test

Optimization of Biomass Growth Reagents

The results of experiment of optimization of biomass growth reagents are showed in Table 4-4. In general, the growth condition of B.1.1.2 achieved the maximum biomass growth after 4 hours incubation.

Table 4-4 Comparison of reagent formulations for microorganism cultivation (4 hours) tested on before UV wastewater secondary effluent samples

Reagent	Log cATP
Straight wastewater sample without reagent (0 hour)	3.41
A.0.0.2	3.21
A.0.2.2	3.13
A.1.0.2	3.40
A.1.1.2	3.49
B.0.0.2	3.46
B.0.2.2	3.45
B.1.0.2	3.58
B.1.1.2	5.14

Optimization of Incubation Temperature

The optimum biomass growth reagent was tested at different temperatures to achieve the maximum log difference between pre and post UV treated wastewater samples. As is shown in Table 4-5, by subjecting the before and after UV wastewater samples to the optimum biomass growth reagent, and incubating at a certain temperature, a maximum difference of 1.53 log value between before and after UV treatment was achieved. The incubation time was successfully reduced to 4 hours. This is the Biomass growth ATP method, which requires 4 hours of incubation at the growth condition of B.1.1.3.

Table 4-5 Comparison of incubation temperatures for microorganism cultivation

Reagent	Maximum log (cATP) difference between before and after UV treatment	Incubation time (hours)
B.1.1.2	1.53	6
B.1.1.3	1.50	4

In summary, a pre-treatment technique of the water samples prior to the ATP assay named "Biomass Growth ATP Method" was developed. The Biomass Growth ATP Method, which is incubating 5 mL of water sample in 45 mL of the optimum chemical reagent at the optimum temperature for 4 hour, is expected to capture the maximum discrepancy between before and after UV treated wastewater.

4.3 Ensuring the Practicality of the Method

Test of Robustness for Field Sampling

Table 4-6 and Table 4-7 present the results of experiments of determining the effect of sample storage in 4℃ refrigerator overnight on microbial population response when applying the Biomass Growth ATP Method prior to ATP assay, before UV treated wastewater secondary effluent sample showed a 24% decrease of the cellular ATP content compared to the result obtained from testing the fresh wastewater sample. The after UV treated wastewater sample showed a 29% increase of the cATP concentration. Further analysis was performed on the raw data by calculating the common log value of the cATP

value. From Table 4-8, it can be seen that the log difference between before and after UV treated sample of overnight sample (log difference = 1.27) was close to the result obtained from testing fresh sample (log difference = 1.50). Thus it can be concluded that for overnight storage of the sample, the result of only before or after UV sample which varied by around 25% may not be that accurate to reflect the real microbial pollution level of the wastewater. However, paired results of before and after UV treated sample and the log difference between those two sets of data could be used as an indicator of UV disinfection efficacy.

Therefore, it is not recommended to use stored samples, which is to store the wastewater samples in 4℃ for around 24 hours. But if one can't do the tests on the same day of sampling, it won't affect too much of the accuracy of the results. It will still show the trend of treatment efficacy after pretreating the sample using the Biomass Growth ATP Method.

Table 4-9 and Table 4-10 display the raw data obtained from the test of effect of temperature shock on microbial population response. Before UV treated samples, the percentage remaining of the cATP content compared to fresh sample was varied from 40% (refrigerated overnight sample + pre-warmed reagent) to 76% (room temperature overnight sample + room temperature reagent). After UV treated samples, the cATP

content compared to fresh sample was increased from 18% (room temperature overnight sample + pre-warmed reagent) to 46% (refrigerated overnight sample + room temperature reagent). The results obtained from calculating the log difference between before and after UV treated samples in Table 4-11 showed that the closest result compared to fresh sample (log difference = 1.50) occurred in the condition of subjecting the room temperature overnight sample into room temperature reagent (log difference = 1.27). The results of refrigerated overnight sample (4℃) + pre-warmed reagent indicated that the larger the temperature difference between the storage sample and the reagent, the more significant the temperature shock. It can be concluded that the impact of temperatures of both sample and reagent have on microbial population response cannot be neglected. The least variation was obtained from testing the room temperature overnight sample in room temperature reagent.

In summary, in the experiment of evaluation of microbial response on sample storage and temperature shock, fresh sample showed a better log discrepancy in differentiating between before and after UV treated wastewater samples. A room temperature sample added into room temperature reagent (i.e., the minimum temperature difference) was the optimal incubation condition.

Table 4-6 Effect of sample storage in 4℃ refrigerator overnight on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay - before UV treated wastewater secondary effluent sample.

Sample + Reagent	cATP (pgATP/mL)	% Remaining
Fresh sample + Room temperature reagent	11,116	100%
Room temperature overnight sample + Room temperature reagent	8,425	76%

Table 4-7 Effect of sample storage in 4℃ refrigerator overnight on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay - after UV treated wastewater secondary effluent sample.

Sample + Reagent	cATP (pgATP/mL)	% Remaining
Fresh sample + Room temperature reagent	353	100%
Room temperature overnight sample + Room temperature reagent	455	129%

Table 4-8 Effect of sample storage in 4℃ refrigerator overnight on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay - log value and log difference between before and after UV treated wastewater secondary effluent sample.

Sample + Reagent	Before UV Log cATP	After UV Log cATP	Log difference
Fresh sample + Room temperature reagent	4.05	2.55	1.50
Room temperature overnight sample + Room temperature reagent	3.93	2.66	1.27

Table 4-9 Effect of temperature shock on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay - before UV treated wastewater secondary effluent sample.

Sample + Reagent	cATP (pgATP/mL)	% Remaining
Fresh sample + Room temperature reagent	11,116	100%
Refrigerated overnight sample (4°C) + Room temperature reagent	6,295	57%
Refrigerated overnight sample (4°C) + Pre-warmed reagent	4,493	40%
Room temperature overnight sample + Room temperature reagent	8,425	76%
Room temperature overnight sample + Pre-warmed reagent	7,176	65%

Table 4-10 Effect of temperature shock on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay - after UV treated wastewater secondary effluent sample.

Sample + Reagent	cATP (pgATP/mL)	% Remaining
Fresh sample + Room temperature reagent	353	100%
Refrigerated overnight sample (4°C) + Room temperature reagent	515	146%
Refrigerated overnight sample (4°C) + Pre-warmed reagent	450	128%
Room temperature overnight sample + Room temperature reagent	455	129%
Room temperature overnight sample + Pre-warmed reagent	416	118%

Table 4-11 Effect of temperature shock on microbial population response in the application of the Biomass Growth ATP Method prior to ATP assay – common log value and log difference between before and after UV treated wastewater secondary effluent sample.

Sample + Reagent	Before UV Log cATP	After UV Log cATP	Log difference
Fresh sample + Room temperature reagent	4.05	2.55	1.50
Refrigerated overnight sample (4℃) + Room temperature reagent	3.80	2.71	1.09
Refrigerated overnight sample (4℃) + Pre-warmed reagent	3.65	2.65	1.00
Room temperature overnight sample + Room temperature reagent	3.93	2.66	1.27
Room temperature overnight sample + Pre-warmed reagent	3.86	2.62	1.26

Test of Repeatability

In the test of repeatability, *E. coli* K12 culture was employed. *E. coli* is an indicator of fecal contamination and completely measurable by culturing methods using a general purpose agar. As is displayed in Figure 4-1, in general, those three trials showed consistent results from visual observation of the graph. It can be concluded that the Biomass Growth ATP Method pretreatment technique was repeatable using pure *E. coli* culture at laboratory scale. More ATP data at the same UV doses would be required to further provide statistical information on the repeatability of the Biomass Growth ATP Method.

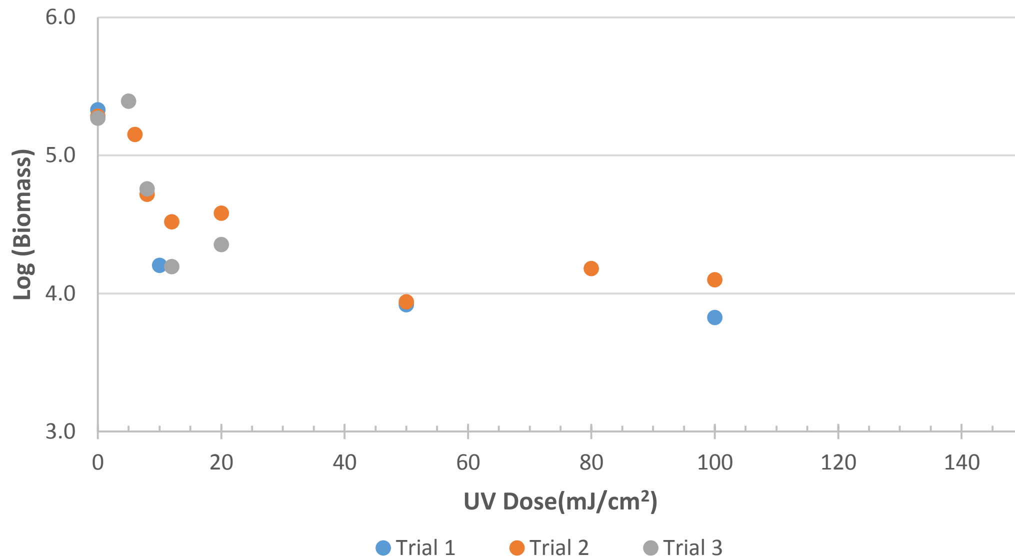


Figure 4-1 Comparison between three trials of repeatability test with pure *E. coli* culture using Biomass Growth ATP Method. Plots display the biomass concentration in common logarithm value. Unit of Biomass Growth ATP Method: pgATP/mL.

4.4 Comparison to Accepted Culturing Method

UV Dose Response Curves

The microbial inactivation response curves obtained are displayed in Figure 4-2 where the logarithm of the number of surviving microorganisms (y-axis), measured in either colony forming units per milliliter or pictogram ATP per millilitre, is plotted against corresponding UV dose (x-axis), measured in millijoules per centimeter squared. As expected, the wastewater samples showed similar trend with controlled samples, which is the inactivation rate of microorganism decreases with increased UV irradiation. For both Biomass Growth ATP Method and HPC method, there was no further significant

reduction of microbial population when the UV doses were higher than 20 mJ/cm^2. It can be observed from the orange lines that the Biomass Growth ATP Method was effective to detect the difference within 1 log between before and after UV treatment. It was also noticeable that for Instant ATP test, the ATP concentration was not affected even when the UV dose was sufficiently high to cause 4 orders of magnitude reduction in HPC.

Figure 4-2 can be analyzed by dividing the UV doses applied into three phases. In Figure 4-2, from 0 mJ/cm^2 to 5 mJ/cm^2 UV dose, Biomass Growth ATP Method showed paralleled change with HPC method. The second phase was from 5 mJ/cm^2 to 20 mJ/cm^2. During this phase, the results of microorganism inactivation with HPC method showed dramatic change. While the Biomass Growth ATP Method didn't show a same degree of decrease, but it showed a relatively comparable trend of change. With even greater doses applied ($>$ 20 mJ/cm^2), the inactivation became more gradual and reached a plateau where additional UV dose didn't inactive additional microbial population. This type of non-linear inactivation kinetics is commonly observed. This plateau was probably due to the residual bacteria concentration or resistance to the UV treatment. Another reason to cause this would be the parallel culturing technique used to quantify microorganisms was HPC method. Membrane filter procedure would be recommended to monitor *E. coli* and total coliform in terms of being complied with regulatory purposed.

In Figure 4-3, the log value of the survival ratio of those two methods was almost overlapped at first phase. Since second phase, the Biomass Growth ATP Method was several orders of magnitude less sensitive than the HPC method. However, it still showed a similar decreasing trend with HPC method in a smaller scale. At third phase, there was no significant change of microbial survival ratio with even higher UV doses.

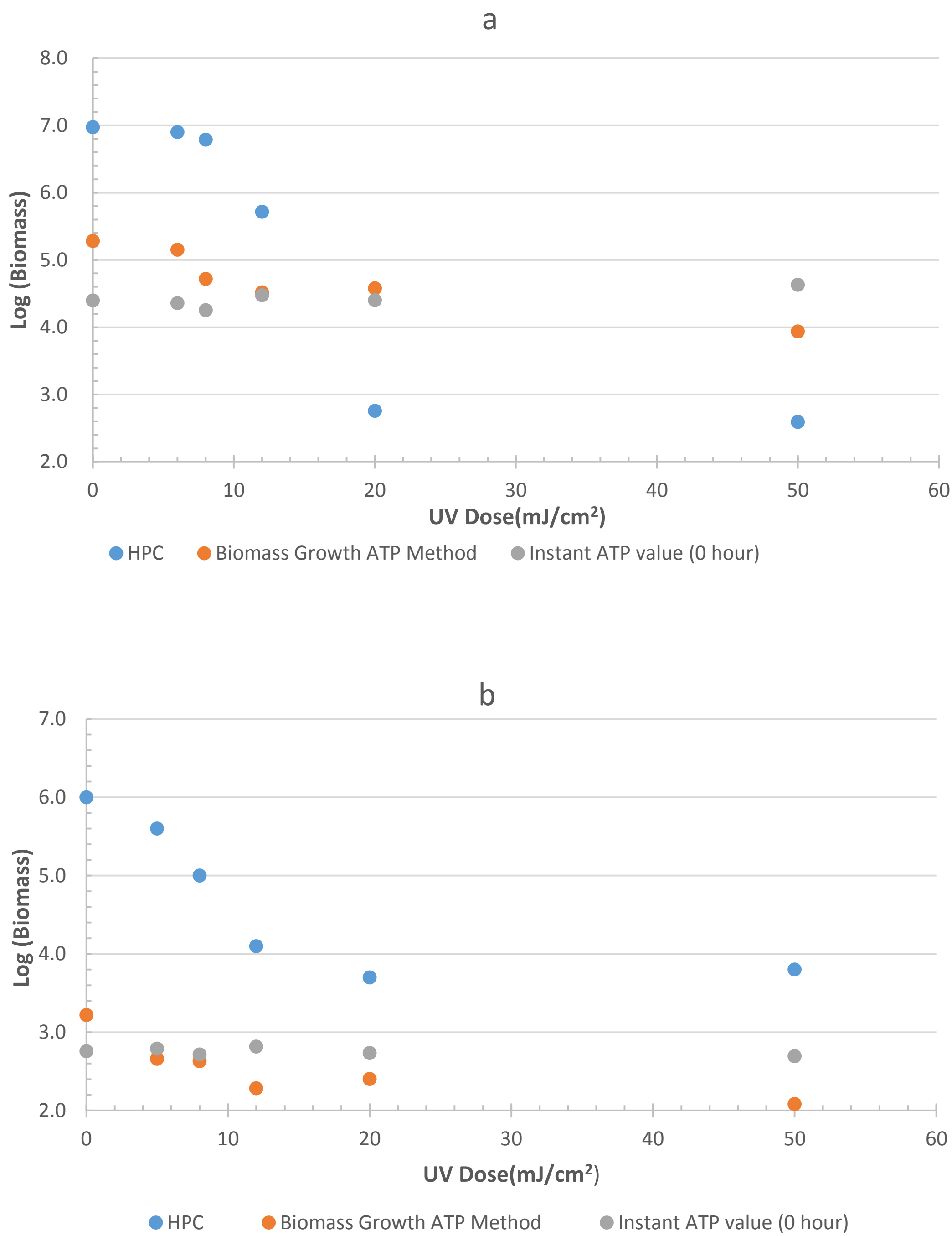


Figure 4-2 Comparison of the response of microorganisms to various UV doses using different monitoring methods (HPC method, Biomass Growth ATP Method and Instant ATP method): a) pure *E. coli* culture; b) secondary effluent. Plots display the biomass concentration in common logarithm value. Unit of HPC method: CFU/mL; unit of Biomass Growth ATP Method and Instant ATP test: pgATP/mL.

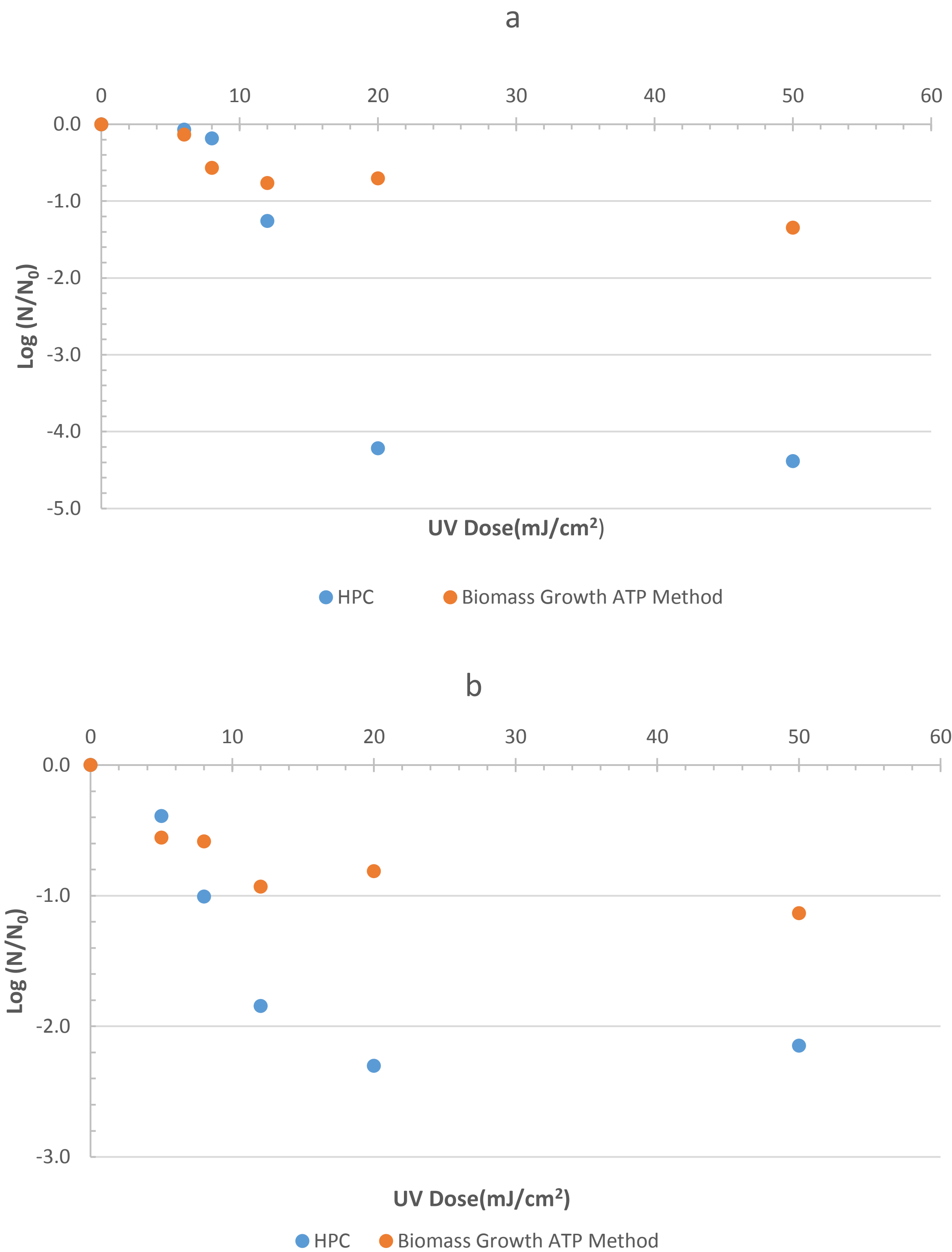


Figure 4-3 Comparison between HPC method and Biomass Growth ATP Method in common logarithm of survival ratio: a) pure *E. coli* culture; b) secondary effluent. Unit of HPC method: CFU/mL and unit of Biomass Growth ATP Method: pgATP/mL.

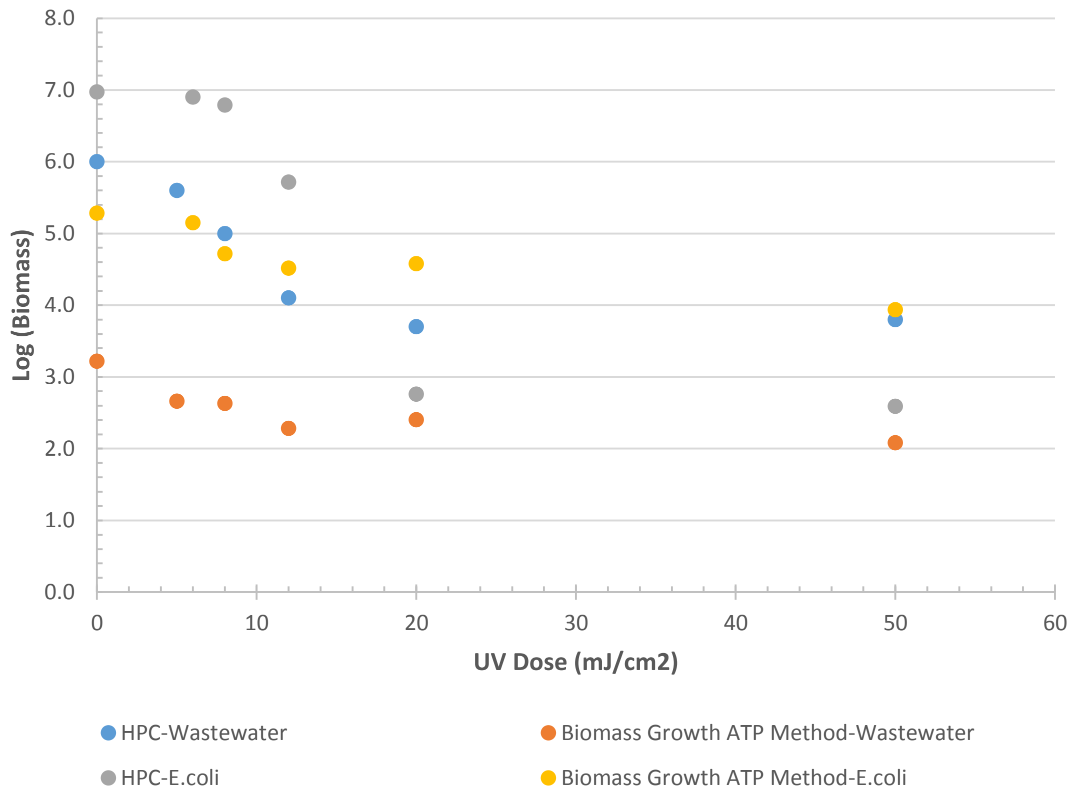


Figure 4-4 Comparison of microbial response to various UV doses between controlled samples and wastewater samples using either HPC method or Biomass Growth ATP Method. Plots display the biomass concentration in common logarithm value. Unit of HPC method: CFU/mL and unit of Biomass Growth ATP Method: pgATP/mL.

It can be observed from Figure 4-4 that the secondary wastewater effluent samples treated in the laboratory using the UV collimated beam unit showed consistent results of log difference between non-irradiated and UV irradiated samples with the pure *E. coli* cultures using Biomass Growth ATP Method. There was a better log reduction of pure *E. coli* cultures compared to wastewater samples with HPC method. There were several possible reasons for this. First, *E. coli* cultures were more sensitive to UV irradiation than the wastewater samples. Also, the microbial situation in municipal wastewater was more

complicated than the pure bacterial culture, which is a complex mixture of microorganisms. There were certain kinds of microorganism existing in the wastewater, which were more resistant to UV light. Additionally, microorganisms associated with particles might shield the UV light and dodge the irradiation.

Pearson Correlation Coefficient

The visual observation of the relationship between two methods was also proved statistically by calculating the Pearson correlation coefficient. This coefficient is a measure of the degree of linear relationship between two variables. The coefficient and the p-value displayed in Table 4-12 indicate a statistically significant direct correlation between HPC method and Biomass Growth ATP Method for both *E. coli* sample and wastewater sample.

Table 4-12 Pearson correlation between HPC method and Biomass Growth ATP Method.

Variables	Pearson Correlation Coefficient	P-value
HPC-*E. coli* and ATP-*E. coli*	0.919	0.001
HPC-wastewater and ATP-wastewater	0.966	0.002

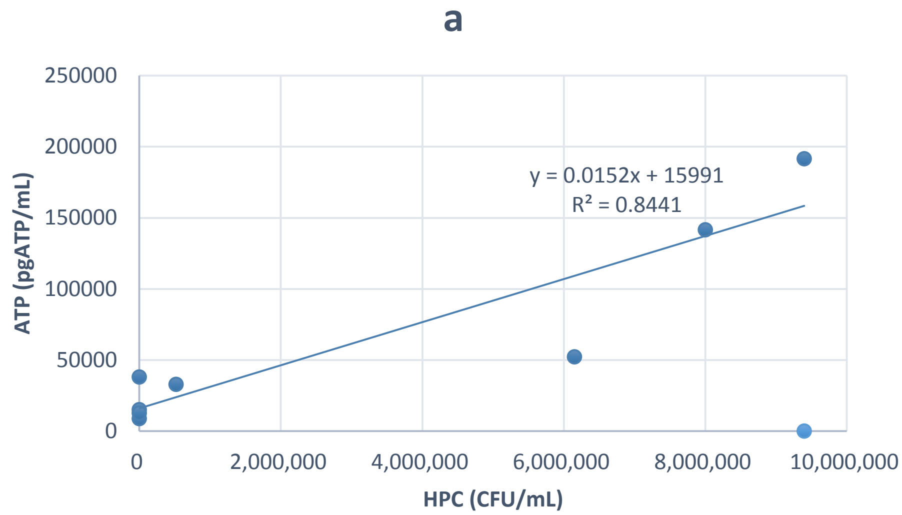
[1] p-value < 0.05, significant
[2] p-value <0.001, highly significant
[3] The Pearson Correlation Coefficient ranges from -1.0 to +1.0, where:
-1.0 is a strong inverse relationship;

0 indicates no relationship;
+1.0 is a strong direct relationship.

Regression Analysis

Regression analysis with a confidence interval set at 95% was performed using Minitab. In a qualitative sense, R^2 near 1 implies a good fit of the data to the regression line and R^2 near zero means a poor fit. A t-test was also performed to determine whether or not a linear correlation existed between HPC method and Biomass Growth ATP Method, which can be seen in Appendix A.

The result of Biomass Growth ATP Method versus HPC method tested on pure *E. coli* sample had a strong positive linear correlation which had a R^2 of 84.4 %, and a statistically significant slope of 0.0152 (T= 5.7, p=0.001). The regression result of wastewater sample was even better compared to the *E. coli* sample. It had a stronger linear relationship (R^2 = 93.3%). The slope 0.0015 was statistically significant which had a t-value of 7.47 and p-value of 0.002. Regression plots are displayed in Figure 4-5. The regression equation can be shifted as the size of data increases in the future and the testing procedure modifies.



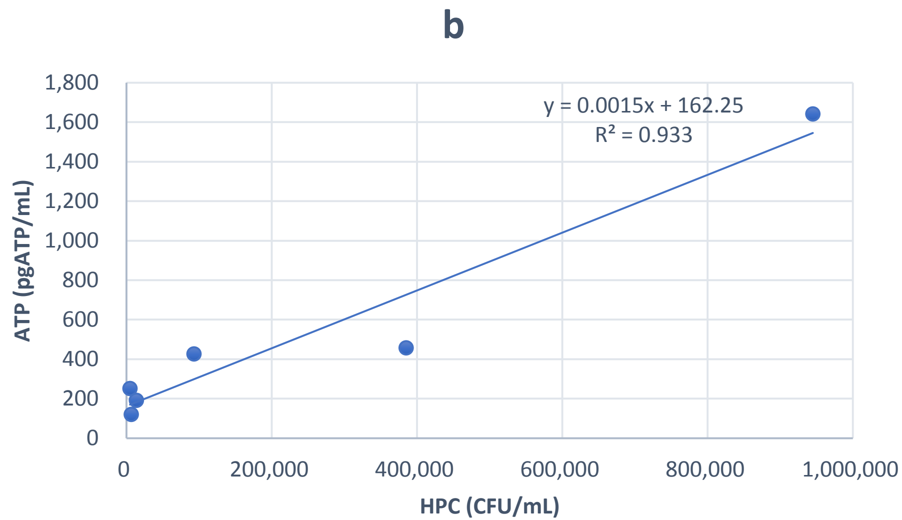


Figure 4-5 Regression plots of the results of Biomass Growth ATP Method versus the results from HPC method: a) pure *E. coli* culture; b) secondary effluent. Unit of HPC method: CFU/mL; unit of Biomass Growth ATP Method and Instant ATP test: pgATP/mL.

4.5 Field Scale Evaluation of Biomass Growth ATP Method

Wastewater Flow Characterization

Three municipal wastewater treatment plants were chosen according to the wastewater source in this research. All three wastewater facilities receive primarily residential waste flows. The flow characteristics were recorded at the plant during each sampling and presented in Table 4-13. In general, flow rates fluctuated as well as UVT%. Eastern Passage WWTF and Mill Cove WPCC were comparable in terms of the design daily treatment capacity, but Mill Cove had almost as twice as much of the influent of Eastern Passage. Fall River WPCP is a small-scale facility. Eastern Passage WWTF has a better UV light penetration of the secondary effluent than that of Mill Cove WPCC. Fall River WPCP isn't equipped to monitor UVT%. The real-time UV dose was monitored online at Eastern Passage and Mill Cove, and the average UV dose applied to the secondary influent annually were 34 mJ/cm^2 and 8-10 mJ/cm^2 respectively. Fall River was not equipped to monitor the UV dose.

Table 4-13 Flow rate and UVT% data from WWTP monitoring

Groups	Flow Rate (m^3/d)				UVT%			
Date	May 22nd	May 29th	June 6th	June 23rd	May 22nd	May 29th	June 6th	June 23rd
Eastern Passage	12,182	11,002	19,500	15,545	66	68	57	58
Mill Cove	18,720	21,120	38,448	27,480	43	33	42	62
Fall River	159	137	141	147	-	-	-	-

Influent and Effluent Quality Monitoring

In Figure 4-6 and 4-7, the microbial loads before-UV treatment and following UV treatment samples were measured by using HPC method and Biomass Growth ATP Method. These figures show the microbial quantities of influent entering into the UV facility and disinfection efficacy for the three WWTPs at each sampling date. In the graphs, each colour represents a WWTP. The columns in dark blue, dark red and dark green are the before UV treatment results and the light colours represent the post UV results.

In general, Eastern Passage had a superior UV treatment performance (an average microbial log reduction of 2.15), which can be observed from the microbial reduction measured by HPC method in Figure 4-6. Mill Cove WWTP has a higher microbial loading rate. Accordingly, the largest log difference in microbial removal between before

and after UV treatment was found for the Mill Cove wastewater samples in spite of lower UVT and UV doses. (Figure 4-7).

The Biomass Growth ATP Method is less sensitive compared to HPC method in quantifying total microorganisms in wastewater samples. It seems that the Biomass Growth ATP Method (Figure 4-7) is more effective to detect the microbial difference after UV treatment when the quantity of influent biomass is no less than 5 logs measured by HPC method (Figure 4-6). This suggests the potential applicability of Biomass Growth ATP Method in the WWTPs with large quantities of biomass entering the UV treatment facility.

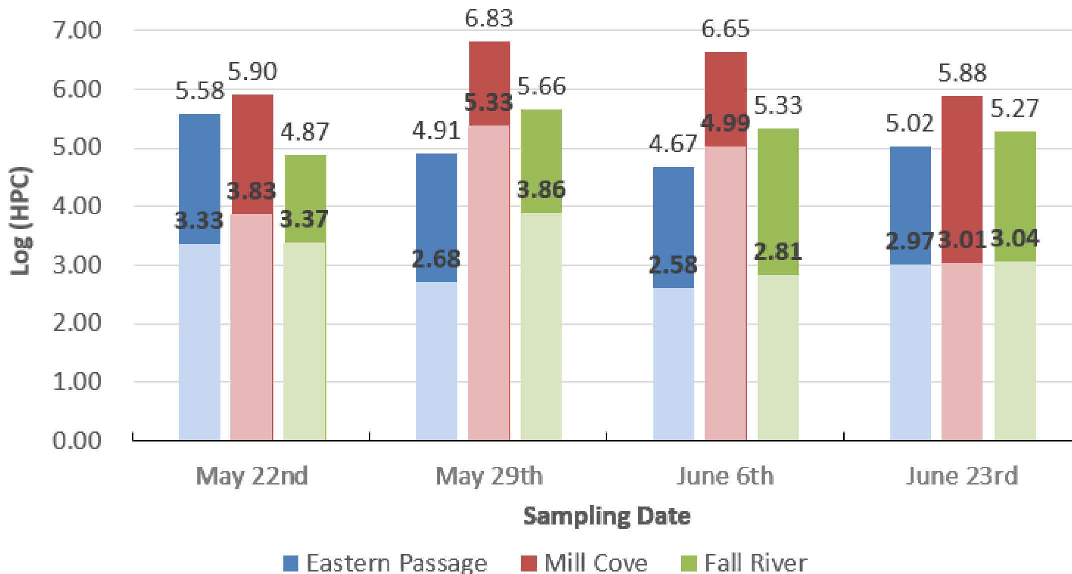


Figure 4-6 The difference between before and following UV treatment at the three WWTPs measured by HPC Method. Graph displays the biomass concentration in common logarithm value. Unit of HPC method: CFU/mL.

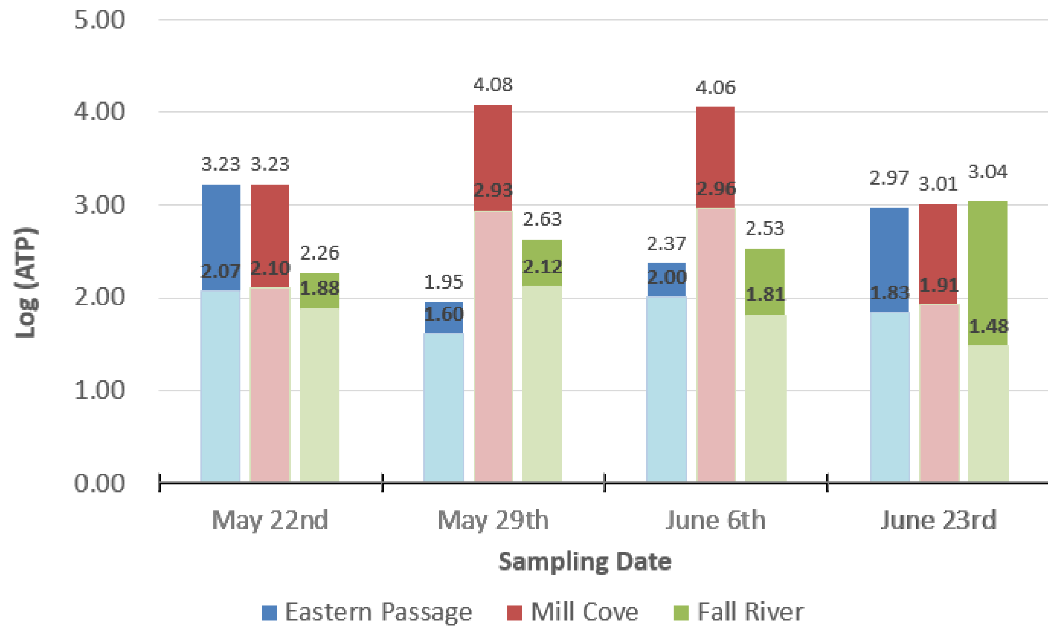


Figure 4-7 The difference between before and following UV treatment at the three WWTPs quantified by Biomass Growth ATP Method. Graph displays the biomass concentration in common logarithm value. Unit of Biomass Growth ATP Method: pgATP/mL.

The logarithm of the number of surviving biomass after UV treatment (y-axis), measured in pictogram ATP per millilitre, is plotted against the logarithm of the biomass loading rate (x-axis), measured in pictogram ATP per millilitre to show the relationship between influent and effluent biomass. The green bar represents the current ATP measurement technique whereby the biomass concentration entering the plant equals the biomass concentration following UV disinfection. In contrast, the black line represents a regressed line for the three plants studied. Figure 4-8 demonstrates that there is a potential relationship between influent and effluent with the Biomass Growth ATP Method that could be expanded to other wastewater facilities. Although more data is required to

populate and confirm this regression trend, what it is beginning to indicate is that at higher microbial loading rates, the Biomass Growth ATP Method can detect a decrease of 1 to 2 orders of magnitude of ATP. This would be important because the WWTPs with high microbial loading rates on UV would be the ones that may need this new method to help improve compliance monitoring.

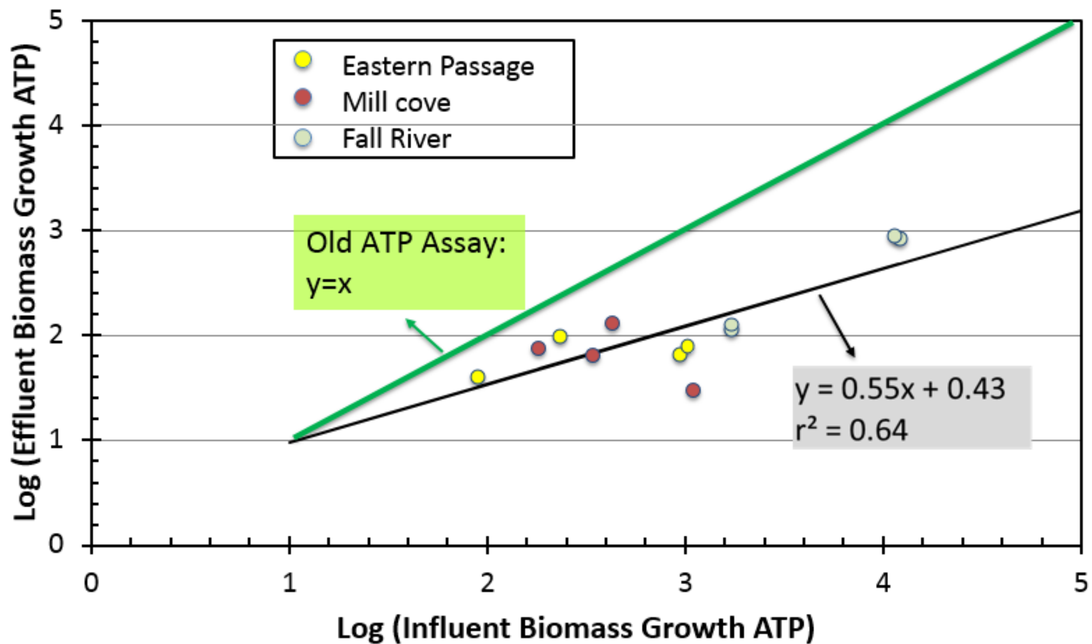


Figure 4-8 Comparison of detection ability of the microorganisms in wastewater samples between current ATP technique without pre-treatment and Biomass Growth ATP Method. Plot displays the biomass concentration in common logarithm value. Unit of Biomass Growth ATP Method: pgATP/mL.

Pearson Correlation Coefficient Analysis

Two-way ANOVA analysis was applied to the raw data to examine the influence of two

different categorical independent variables: the plant and sampling date on one dependent variable: microbial quantification. The main effect of contributions of each independent variable was determined. The details of Two-way ANOVA analysis and hypothesis testing are described in Appendix B. The analysis showed that the effect of sampling dates is indistinguishable on the composition and quality of the wastewater samples when the total microorganisms in the samples were examined by both the HPC and Biomass Growth ATP methods. Whereas the factor of plant has an effect merely on the microbial quantification using HPC method with before UV treatment sample. The treatment plant has no effect on the total microorganisms presented in the wastewater samples which were quantified by the Biomass Growth ATP Method or in the after UV treated wastewater samples examined by HPC method, as is showed in Table 4-14. Therefore, the "HPC method - Before UV" data of different WWTPs cannot be regarded as equivalent data to be merged as one group by ignoring the potential effect of plant purposely. Thus the relationship analysis between HPC method and Biomass Growth ATP Method could be applied to the data of after UV treated wastewater samples ("HPC method - After UV" data and "Biomass Growth ATP Method - After UV" data).

The analysis results are showed in Table 4-15. The post UV treatment data showed very promising results. The strong direct correlation between HPC method and Biomass Growth ATP Method was statistically significant ($p<0.05$).

Table 4-14 Two-way ANOVA analysis results of plant and date

Groups	Factor of Plant	Factor of Date
HPC method - Before UV	√ [a]	×
HPC method - After UV	×	×
Biomass Growth ATP Method - Before UV	×	×
Biomass Growth ATP Method - After UV	×	×

a – Significant effect of wastewater treatment plant indicating differences in the influent microbial loading rates.

Table 4-15 Pearson correlation of different sampling dates between HPC method and Biomass Growth ATP Method

Variables (HPC &ATP)	Pearson Correlation Coefficient	P-value
After UV	0.903	0.000

[1] p-value < 0.05, significant
[2] p-value <0.001, highly significant
[3] The Pearson Correlation Coefficient ranges from -1.0 to +1.0, where:
-1.0 is a strong inverse relationship;
0 indicates no relationship;
+1.0 is a strong direct relationship.

CHAPTER 5 DISCUSSION

The pre-treatment procedure or Biomass Growth ATP Method has been developed and used in combination with ATP assay to address the issue that there is no detectable difference in cellular ATP between pre and post UV treated municipal wastewater. The concentration of microorganisms was magnified by culturing the samples in the optimum chemical reagent at the optimum temperature for 4 hours, after which a 1-2 logs of inactivation was achieved with ATP test. The Biomass Growth ATP Method which functioned well in the controlled laboratory environment was also proved to be robust when tested with field samples, despite the slight deficiency in detection sensitivity of inactivation.

5.1 Applications of ATP assay for Evaluating UV Disinfection Performance

The research conducted by Villaverde et al. (1986) examined the effect of UV irradiation on ATP content. Several mutants of *E. coli* K12 were treated by UV doses in the range of 0-80 mJ/cm^2 with increments of 10 mJ/cm^2 and then incubated for 20 minutes in AB minimal medium with glucose and casa amino acid at 37℃. Results from Villaverde et al. (1986) showed that the increased UV irradiation produced a continuous increase of the

ATP production and the maximal ATP level achieved was varied according to the different types of the strain. This increase of ATP content following UV treatment could lead to an overestimation in the ATP-based quantification of the total microorganisms presented in the sample.

In another research, the ATP assay was employed to determine the UV disinfection efficacy in ballast water (First & Drake 2013). Both ambient microorganisms from an oligotrophic marine environment and laboratory cultures of marine algae were tested subsequent to different UV exposures: 0, 50, 100 and 200 mJ/cm^2. ATP assay was performed directly to the post-UV water samples without being processed by any forms of pre-treatment. The response of microorganisms to UV irradiation didn't vary significantly with any of the doses applied which corroborates with the results of instant ATP assay tested on wastewater samples in Chapter 4.1 of this thesis that there was no detectable difference in ATP concentration between pre and post UV treated samples.

As well, Linklater and Ormeci (2014) investigated the ATP bioluminescence assay to monitor UV disinfection efficacy at laboratory using pure *E. coli* culture (Strain ATCC 23631). Pure *E. coli* samples were irradiated with UV dose ranged from 0 to 100 mJ/cm^2 and increased in increments of 20 mJ/cm^2. ATP assay was performed immediately to the post-UV pure *E. coli* sample without any pre-treatment. The results as well as the

statistical analysis (ANOVA and post-ANOVA Tukey test) found that the UV dose did not cause a consistent increase or decrease in the ATP content and no clear trend was observed, whereas over 4-log reduction in membrane filtration method was achieved . The fluctuation of the *E. coli* quantities measured by ATP assay in ATP Technology section of Chapter 4.1 supported the conclusion of this study.

The outcome from the assessment of current ATP technology in Chapter 4 agrees with the conclusions of the aforementioned two studies, which suggested that the lack of sensitivity to UV-treated cells inhibited the ATP assay from being utilized as a reliable and rapid monitoring tool to detect the survival organisms after UV disinfection.

To overcome this limit, Cho and Yoon (2007) tried to amplify the detection sensitivity of luminescence assay by examining three potential methods: 1) amplification by enzymatic method, 2) concentration of cells by membrane filtration and 3) amplification of cell concentration by culturing. Among the three methods by Cho and Yoon (2007), the third culturing technique in which the low *E. coli* concentration sample was incubated in nutrient broth for 4 hours was proved to be the most efficient. The culturing method was able to improve the detection limit of *E. coli* measurement by 200 folds and the results were not affected by the state of *E. coli* growth. Then Cho and Yoon's (2007) culturing method was applied to UV disinfection to complete the integrity analysis of luminescence

assay. It was found that the inactivation span of *E. coli* was enlarged to 3-log reduction with the UV dose around 33 mJ/cm^2. The findings in this thesis supported the outcome of Cho and Yoon's work that amplification of *E. coli* following UV disinfection can reach a detectable extent. However, the results obtained from Cho and Yoon (2007) displayed a superior microbial difference (3-log) compared to that of the ATP assay with Biomass Growth ATP Method (about 1 log) at a similar UV dose and thus it is plausible that further method development could be considered..

This could probably be due to the difference in handling the incubation process, initial concentration of *E. coli* sample and the sample volume tested. In the culturing procedures developed by Cho and Yoon (2007), the cultivation took place in no more than 0.6 mL nutrient broth with 1 mL of *E. coli* suspension (3.0×10^0 - 3.0×10^4 CFU/mL) and then incubated in an shaking incubator at 37℃. Correspondingly, in ATP assay with Biomass Growth ATP Method 5 mL *E. coli* solution with an initial concentration of 10^7 CFU/mL was added into 45 mL of the optimum chemical reagent and then incubated without any agitation at the optimum temperature. In addition, the bioluminescence assay also followed different protocols in the two studies. Cho and Yoon (2007) mixed 0.1 mL *E. coli* with 0.1 mL detergent lysing agent in a 1.6 mL tube. After being vortexed for 10 minutes, 0.1 mL of the content was transferred to 96-well luminometer cuvettes containing 1/10 ATP assay mix diluted with dilution buffer. The reagents were provided

by Sigma Co., USA, and the luminometer was Lumin Ascent from Thermo Electron Co., USA. Whereas, the ATP assay in this thesis used the QGA test kit provided by LuminUltra Technologies Ltd., and the detailed protocol was depicted in Chapter 3. All those factors could contribute to the difference between Cho and Yoon's (2007) method and the Biomass Growth ATP Method.

Table 5-1 Comparison between Cho & Yoon's (2007) culturing method and Biomass Growth ATP Method

	Parameter	**Cho & Yoon (2007)**	**Biomass Growth ATP Method**
Incubation	Incubator	Shaking	No shaking
	Temperature	37℃	the optimum temperature
	Medium	nutrient broth	the optimum chemical reagent
	Volume	1 mL *E. coli* with ≤ 0.6 mL NB	5 mL *E. coli* + 45 mL the optimum chemical reagent
	Initial *E. coli* concentration	3×10^0 - 3×10^4 CFU/mL	10^7 CFU/mL
ATP assay	Protocol	1. Mix 0.1 mL *E. coli* with 0.1 mL detergent lysing agent in a 1.6 mL tube. 2. Vortext for 10 minutes. 3. 0.1 mL of the content was transferred to 96-well luminometer cuvettes containing 1/10 ATP assay mix and diluted with dilution buffer.	10 mL of the *E. coli* and chemical reagent mixture were tested using 2nd generation QGA test kit and following the protocol instructed by LuminUltra Technologies Ltd..
	Materials	Reagents: Sigma Co., USA; Luminometer: Lumin Ascent from Thermo Electron Co., USA	LuminUltra Technologies Ltd., Canada

5.2 Detection Sensitivity of Biomass Growth ATP Method Combined with ATP Assay

The concept of viable but nonculturable (VBNC) state was originally defined and introduced by Colwell and co-worker's (Xu et al. 1982; Oliver 2005). Bacteria in the VBNC state fail to grow on the routinely employed bacteriological media on which they would normally undergo cell division and develop into colonies, but are alive and capable of renewed metabolic activity (Colwell 2000; Oliver 2000b; Oliver 2005).

The disinfection processes which are normally assumed to be bactericidal may instead result in cells remaining in the VBNC state (Oliver 2005). Oliver et al. (2005) found that a small portion (< 0.4 %) of the *Escherichia coli* and *Salmonella Typhimurium* populations in wastewater survived in the VBNC state after the chlorine disinfection.

In addition, the inactivation mechanism of UV irradiation would likely induce the metabolically active but nonculturable bacteria as described in Chapter 2 and proved in subtask 1 of this thesis. This was supported by the study that made the observation of VBNC bacteria following UV treatment for wastewater disinfection (Lazarova et al. 1998). Besides, several studies reported that although UV-treated *E. coli* lost culturability on nutrient media using culturing technique, they retained the metabolic capacity and

intact cellular integrity when the viability of the UV-treated *E. coli* was assessed by other methods involved with microscopic observation like direct viable count (DVC), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining technique and so on (Villarino 2000; Villarino 2003; Wilber & Oliver 2000).

Therefore, the conventional culturing technique like HPC method which relies on the yielding of visible cell replication on nutrient media to detect the presence of bacteria could lead to an underestimation of the total viable bacteria quantities. In subtask-5 of this thesis, when the Biomass Growth ATP Method was verified by being applied to three wastewater treatment plants, the microbial inactivation of HPC method appeared to be orders-of-magnitude superior than the ATP results of the Biomass Growth ATP Method. When there was a more than 100-fold drop in CFU/mL of the microbial densities in UV-treated secondary effluent, the ATP content contained in the UV-treated wastewater decreased by 10 folds. However, HPC method seemed to be a promising enumeration technique but in fact it biased the results by selectively giving a partial representation of the wastewater effluent microbial quantity without counting in the VBNC state bacteria. Whereas the seemingly lack of sensitivity of the Biomass Growth ATP Method combined with ATP assay can actually be explained by its ability to non-exclusively detect the ATP content from all the viable microorganisms, including the VBNC state bacteria. Thus compared to HPC method, the responsiveness of Biomass Growth ATP Method combined

with ATP assay would possibly give an illusion of being less sensitive since the detection scope of ATP assay encompasses that of HPC method.

5.3 Specificity Enhancement with Current ATP Assay

In water and wastewater treatment, *E. coli* is an indicator of fecal coliform contamination acted as a regulatory tool to ensure the effectiveness of inactivating pathogenic microorganisms achieved in disinfection process. Future studies could include the monitoring of *E. coli* and total coliform using the standard membrane filter procedure suggested by the American Public Health Association (2005). This culture-based counting technique could be performed as parallel tests to Biomass Growth ATP Method to establish a benchmark of comparison.

However, ATP technology is a non-specific measurement which can quantify the overall amount of viable microorganisms present in the water sample (Shimomura 2006). It cannot distinguish or enumerate any particular microbial strain. Several studies have been conducted to isolate the target microorganism prior the ATP assay to address this issue.

Squirrell et al. (2002) mentioned the examination of using antibodies in immunomagnetic separation and the use of bacteriophage to lyse targeted cells. The research found that the

two methods working in combination maximized the benefits in improving specificity and sensitive identification of living cells in a mixed sample when using luminescence assay.

Lee and Deininger (2004) investigated the immunomagnetic separation (IMS) for selective capture of target bacteria combined with ATP assay to quantify the bacterial population. Uniform superparamagnetic polystyrene beads (diameter of 0.6 μ m) coated with anti-bodies used in this research are capable of binding to the desired bacteria to form a bead–bacteria complex that is easily separated from the sample solution by exposure to a magnetic field. The concentration of trapped bacteria were then measured by ATP assay. The IMS-ATP procedure can be finished within 1 hour and showed a strong direct correlation with membrane plate count method.

Another study following a similar research idea conducted by Cheng et al. (2009) also showed promising results, which utilized 20 nm-sized biofunctional magnetic nanoparticles (BMNPs) in combination with ATP bioluminescence for the assay of *E. coli*. The BMNPs formed by covering the magnetic nanoparticles with a specific anti-*E. coli* antibody were able to attach to *E. coli* in high efficiency. Thus, the detection of *E. coli* in UV disinfection would be plausible using ATP assay when it is combined with potential methodologies to separate *E. coli* and the Biomass Growth ATP Method to pre-

treat the samples. This will allow the potentiality of applying the ATP assay to UV disinfection monitoring in terms of conforming to pertinent regulations.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In summary, a robust Biomass Growth ATP Method (optimum chemical reagent, optimum incubation temperature, 4 hours) was developed to provide an alternative rapid tool to monitor the UV disinfection efficacy in municipal wastewater treatment.

The response of the Biomass Growth ATP method was tested on pure *E. coli* culture and secondary wastewater effluent. Results showed a strong positive correlation with the degree of microbial inactivation observed in traditional heterotrophic plate count methods (i.e., nutrient agar, 35℃, 48 hours or R2A agar, 28℃, 7 days). The linearity analysis indicated a statistically strong direct correlation between HPC method and Biomass Growth ATP Method for both controlled pure *E. coli* sample (Pearson correlation coefficient = 0.919, p-value = 0.001) and secondary wastewater sample Pearson correlation coefficient = 0.966, p-value = 0.002). The outcome of laboratory experiments suggested that the Biomass Growth ATP Method is potentially applicable in field scale systems.

The Biomass Growth ATP Method was tested in field samples to validate practicality. The post UV treatment data obtained from three WWTPs showed statistically significant

direct correlation between HPC method (R2A agar, 28℃, 7 days) and Biomass Growth ATP Method (Pearson correlation coefficient = 0.903, p value = 0.000). The result that Biomass Growth ATP Method could detect a 1-2-order of magnitude of inactivation indicated a prospective adoption of this new method in WWTPs with high microbial loading rates to evaluate UV disinfection performance.

This pre-treatment method combined with ATP assay represents the first demonstration of a same-shift method to assess the germicidal effects of UV treatment. Besides, the application of ATP technology to wastewater treatment process quality control is an innovative research subject worth exploring. What's more important is this enlightening research project also serves as a pioneering endeavour to inspire more researchers to set foot in developing rapid monitoring methods for UV disinfection and other disinfection processes using ATP technology.

6.2 Recommendations

Due to the lack of pertinent studies in the same field and the limitations of time to repeat the experiments stated previously in the thesis, larger amount of data at laboratory scale is required in future research to have more statistically sound correlation between the measurement of ATP assay with Biomass Growth ATP Method and the conventional

culturing methods. Future work could include the application of the Biomass Growth ATP Method in more full-scale systems to demonstrate functionality and understand how these data could help wastewater operators in optimizing UV disinfection. During this, long-term and continuous monitoring of wastewater flow and exhaustive characterizations of wastewater inputs quality of all the sampling dates are required. The parameters regarding UV facility should be recorded in detail to build up a database for regular monitoring of UV treatment.

It is recommended to observe the behavior and status of viable but non-culturable bacteria during the incubation in the biomass growth reagents using microscopic methodologies. The effect of UV irradiation on ATP content should also be investigated. These fundamental studies would be very critical to better understand the mechanisms in a theoretical level which would help shed some light on further optimizing the Biomass Growth ATP Method.

In order to abide by the demands of pertinent regulations, *E. coli* and total coliform as pathogenic indicators are suggested to be measured by standard membrane filtration procedures as parallel monitoring method to ATP assay. *E. coli*-isolation techniques prior to the ATP assay are recommended to accomplish the specific detection of *E. coli* using ATP technology.

The Biomass Growth ATP Method could be potentially applied to detecting the microbial level in UV disinfection in ballast water treatment to prevent the marine bio-invasions. Other forms of disinfection processes could probably adopt this method with appropriate adjustments and optimizations to examine the disinfection performance.

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APPENDIX

APPENDIX A – Raw Data

Table A-1

Sampling date		RLU 1	RLU 2	RLU 3	AVG	%CV	cATP (pgATP/mL)	Log cATP
Nov. 2nd, 2012	Before UV	289,449	370,047	203,908	287,801	28.9	10,948	4.04
	After UV	333,828	449,225	328,216	370,423	18.4	14,091	4.15
Nov. 28th, 2012	Before UV	175,645	208,487	-	192,066	12.1	14,815	4.17
	After UV	119,152	153,320	-	136,236	17.7	10,509	4.02
Dec. 13th, 2012	Before UV	105,830	139,581	-	122,706	19.4	8,978	3.95
	After UV	103,503	120,756	-	112,130	10.9	8,204	3.91
Dec. 19th, 2012	Before UV	193,673	235,555	-	214,614	13.8	21,011	4.32
	After UV	239,066	136,558	-	187,812	38.6	18,387	4.26

Table A-2

Sampling date		CFU/mL	Log
Nov. 2nd, 2012	Before UV	1300000	6.11
	After UV	57500	4.76
Nov. 28th, 2012	Before UV	1380000	6.14
	After UV	29925	4.48
Dec. 13th, 2012	Before UV	540000	5.73
	After UV	10500	4.02
Dec. 19th, 2012	Before UV	1465000	6.17
	After UV	183000	5.26

Table A-3

	ATP assay			HPC method	
UV Dose(mJ/cm^2)	RLU1	cATP(pgATP/mL)	Log (cATP)	CFU/mL	Log
0	477,177	24,898	4.40	9,400,000	6.97
5	437,482	22,827	4.36	8,000,000	6.90
8	345,747	18,041	4.26	6,150,000	6.79
12	572,877	29,892	4.48	520,000	5.72
20	484,413	25,276	4.40	575	2.76
50	823,047	42,945	4.63	390	2.59
80	678,877	35,423	4.55	660	2.82
100	585,596	30,555	4.49	385	2.59

Table A-4

Reagent	RLU 1	RLU 2	AVG	%CV	cATP (pgATP/mL)	log cATP
Straight wastewater sample without reagent (0 hour)	54,004	45,082	49,543	12.7	2,564	3.41
A.0.0.2	29,278	33,444	31,361	9.4	1,623	3.21
A.0.2.2	22,174	29,816	25,995	20.8	1,345	3.13
A.1.0.2	61,814	35,136	48,475	38.9	2,509	3.40
A.1.1.2	68,540	51,460	60,000	20.1	3,106	3.49
B.0.0.2	76,303	65,702	71,003	10.6	2,911	3.46
B.0.2.2	69,126	69,425	69,276	0.3	2,840	3.45
B.1.0.2	88,650	96,378	92,514	5.9	3,793	3.58
B.1.1.2	324501*10	353190*10	338846*10	6.0	138,917	5.14

Table A-5

Before UV-Potential chemical reagent & potential temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[1]	-[1]	-[1]	-[1]	3,815[1]	3.58[1]
10	2	49,642	46,430	48,036	4.7	3,340	3.52
10	4	60,160	79,438	69,799	19.5	4,854	3.69
10	6	277,248	280,966	279,107	0.9	19,409	4.29

After UV-Potential chemical reagent & potential temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[1]	-[1]	-[1]	-[1]	3,109[1]	3.49[1]
10	2	41,951	37,356	39,654	8.19	2,156	3.33
10	4	26,880	29,997	28,439	7.75	1,546	3.19
10	6	30,689	21,746	26,218	24.12	1,426	3.15

[1] Results calculated from neat to correct for dilution in reagent.

Table A-6

Before UV-Optimum reagent & potential temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[1]	-[1]	-[1]	-[1]	3,815[1]	3.58[1]
10	2	43,480	50,336	46,908	10.3	3,262	3.51
10	4	59,070	51,209	55,140	10.1	3,834	3.58
10	6	650,710	717,883	684,297	6.9	47,587	4.68

After UV- Optimum reagent & potential temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[1]	-[1]	-[1]	-[1]	3,109 [1]	3.49[1]
10	2	36,299	29,876	33,088	13.73	2,301	3.36
10	4	25,476	29,748	27,612	10.94	1,920	3.28
10	6	24,174	27,286	25,730	8.55	1,399	3.15

[1] Results calculated from neat to correct for dilution in reagent.

Table A-7

Volume（mL）	**Neat (straight water sample)**	**RLU 1**	**RLU 2**	**AVG**	**%CV**	**cATP(pgATP/mL)**
10	Before UV	600,832	496,437	548,635	13.5	38,153
10	After UV	426,080	468,093	447,087	6.6	31,091

Table A-8

Before UV-Potential chemical reagent & optimum temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[2]	-[2]	-[2]	-[2]	1,030[2]	3.01[2]
10	4	65,175	89,207	77,191	22.0	3,925	3.59

After UV - Potential chemical reagent & optimum temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[2]	-[2]	-[2]	-[2]	1,078[2]	3.03[2]
10	4	10,684	9,232	9,958	10.31	506	2.70

[2] Results calculated from neat to correct for dilution in reagent.

Table A-9

Before UV - Optimum reagent & optimum temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[2]	-[2]	-[2]	-[2]	1,030[2]	3.01[2]
10	4	159,395	277,826	218,611	38.3	11,116	4.05

After UV - Optimum reagent & optimum temperature							
Volume（mL）	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
	0	-[2]	-[2]	-[2]	-[2]	1,078[2]	3.03[2]
10	4	7,320	6,571	6,946	7.63	353	2.55

[2] Results calculated from neat to correct for dilution in reagent.

Table A-10

Volume(mL)	Neat (straight water sample)	RLU 1	RLU 2	RLU 3	AVG	%CV	cATP(pgATP/mL)
10	Before UV	187,615	283,417	136,783	202,605	33.4	10,303
10	After UV	236,130	188,001		212,066	16.0	10,784

Table A-11

Biomass Growth ATP Method-BeforeUV								
Volume(mL)		Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
10	Rf sample[3]	0	-[1]	-[1]	-[1]	-[1]	786[1]	2.90[1]
10	RT sample[4]	0	-[1]	-[1]	-[1]	-[1]	834[1]	2.92[1]
10	① Rf Sam[3]+RT Me[6]	2	34,644	32,800	33,722	3.9	1,428	3.15
10	② Rf Sam[3]+PW Me[5]	2	45,421	25,315	35,368	40.2	1,683	3.23
10	③ RT Sam[4]+RT Me[6]	2	46,172	25,480	35,826	40.8	1,705	3.23
10	④ RT Sam[4]+PW Me[5]	2	44,680	44,399	44,540	0.4	2,120	3.33
10	① Rf Sam[3]+RT Me [6]	4	121,270	143,291	132,281	11.8	6,295	3.80
10	② Rf Sam[3]+PW Me [5]	4	108,713	80,095	94,404	21.44	4,493	3.65
10	③ RT Sam[4]+RT Me[6]	4	147,048	207,022	177,035	23.95	8,425	3.93
10	④ RT Sam[4]+PW Me [5]	4	188,954	112,651	150,803	35.78	7,176	3.86
10	③ RT Sam[4]+RT Me[6]	6	-[2]	-[2]	-[2]	-[2]	70,851[2]	4.85[2]

[1] Results calculated from neat to correct for dilution in reagent.

[2] Results calculated from diluted RLU.

[3] Rf Sam = refrigerated sample

[4] RT Sam= room temperature sample

[5] PW Me = prewarmed medium

[6] RT Me = room temperature medium

Table A-12

Biomass Growth ATP Method-After UV								
Volume(mL)		Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)	log cATP
10	Rf sample[3]	0	-[1]	-[1]	-[1]	-[1]	476[1]	2.68[1]
10	RT sample[4]	0	-[1]	-[1]	-[1]	-[1]	549[1]	2.74[1]
10	⑤ Rf Sam[3]+RT Me[6]	2	17,313	12,957	15,135	20.4	641	2.81
10	⑥ Rf Sam[3]+PW Me[5]	2	10,766	9,365	10,066	9.8	479	2.68
10	⑦ RT Sam[4]+RT Me[6]	2	18,904	13,930	16,417	21.4	781	2.89
10	⑧ RT Sam[4]+PW Me[5]	2	8,391	12,326	10,359	26.9	493	2.69
10	⑤ Rf Sam[3]+RT Me [6]	4	11,906	9,735	10,821	14.2	515	2.71
10	⑥ Rf Sam[3]+PW Me [5]	4	10,912	8,016	9,464	21.64	450	2.65
10	⑦ RT Sam[4]+RT Me[6]	4	10,083	9,050	9,567	7.64	455	2.66
10	⑧ RT Sam[4]+PW Me [5]	4	8,169	9,299	8,734	9.15	416	2.62
10	③ RT Sam[4]+RT Me[6]	6	-[2]	-[2]	-[2]	-[2]	13,028[2]	4.11[2]

[1] Results calculated from neat to correct for dilution in reagent.

[2] Results calculated from diluted RLU.

[3] Rf Sam = refrigerated sample

[4] RT Sam= room temperature sample

[5] PW Me = prewarmed medium

[6] RT Me = room temperature medium

Table A-13

Volume(mL)	③RT Sam+RT Me	Time (hour)	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)
10	Before UV	6	142,656	155,111	148,884	5.92	7,085
10	After UV	6	33,194	21,558	27,376	30.06	1,303

[1] Original results were overscaled. 1mL extracted samples in Ultralute was diluted in 9mL Ultralute.

Table A-14

Volume (mL)	Neat-Before UV	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)
10	Refrigerated-sample	201,392	169,688	185,540	12.1	7,855
10	Room Temperature-sample	186,992	207,177	197,085	7.2	8,344

Volume (mL)	Neat-After UV	RLU 1	RLU 2	AVG	%CV	cATP(pgATP/mL)
10	Refrigerated-sample	104,670	120,063	112,367	9.7	4,757
10	RoomTemperature-sample	116,772	142,744	129,758	14.2	5,493

Table A-15

Before UV - Fresh Sample + RoomTemp Medium		
Time (hour)	cATP(pgATP/mL)	log cATP
0	1,030	3.01
4	11,116	4.05

After UV - Fresh Sample + RoomTemp Medium		
Time (hour)	cATP(pgATP/mL)	log cATP
0	1,078	3.03
4	353	2.55

Table A-16

Biomass Growth ATP Method-BeforeUV			
Time (hour)	0	2	4
Rf sample	2.90	-	-
RT sample	2.92	-	-
①Rf Sam+RT Me	-	3.15	3.80
②Rf Sam+PW Me	-	3.23	3.65
③RT Sam+RT Me	-	3.23	3.93
④RT Sam+PW Me	-	3.33	3.86

Biomass Growth ATP Method-After UV			
Time (hour)	0	2	4
Rf sample	2.68	-	-
RT sample	2.74	-	-
①Rf Sam+RT Me	-	2.81	2.71
②Rf Sam+PW Me	-	2.68	2.65
③RT Sam+RT Me	-	2.89	2.66
④RT Sam+PW Me	-	2.69	2.62

[1] Rf Sam = refrigerated sample

[2] RT Sam= room temperature sample

[3] PW Me = prewarmed medium

[4] RT Me = room temperature medium

Table A-17

E. coli solution: 15mL *E. coli* Tryptic Soy Broth solution +~500 mL phosphate buffer.

Incubation detail: Biomass Growth ATP Method.

UV Transmittance% = 73.5%

Before UV - 4h							
UV Dose	Volume(mL)	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
0	10	5,276,700	4,622,030	4,949,365	9.35	211,892	5.33

After UV - 4h							
UV Dose	Volume（mL）	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
10 mJ/cm^2	10	442,309	303,263	372,786	124.18	15,960	4.20
50 mJ/cm^2	10	239,290	147,290	193,290	33.66	8,275	3.92
100 mJ/cm^2	10	177,295	134,722	156,009	19.30	6,679	3.82
150 mJ/cm^2	10	194,894	146,720	170,807	19.94	7,313	3.86

Table A-18

E. coli solution: 12mL *E. coli* Tryptic Soy Broth solution +~330 mL phosphate buffer. UV Transmittance% = 62.8%

0h							
UV Dose	Volume（mL）	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
Before	10	476,628	477,725	477,177	0.16	24,898	4.40
5 mJ/cm^2	10	437,482	-	437,482	-	22,827	4.36
8 mJ/cm^2	10	345,747	-	345,747	-	18,041	4.26
12 mJ/cm^2	10	572,877	-	572,877	-	29,892	4.48
20 mJ/cm^2	10	484,413	-	484,413	-	25,276	4.40
50 mJ/cm^2	10	823,047	-	823,047	-	42,945	4.63
80 mJ/cm^2	10	678,877	-	678,877	-	35,423	4.55
100 mJ/cm^2	10	585,596	-	585,596	-	30,555	4.49

4h							
UV Dose	Volume（mL）	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
Before	10	3,917,940[1]	3,427,470[1]	3,672,705[1]	9.44[1]	191,636[1]	5.28[1]
5 mJ/cm^2	10	2,142,360[1]	3,287,460[1]	2,714,910[1]	29.82[1]	141,660[1]	5.15[1]
8 mJ/cm^2	10	814,680[1]	1,184,220[1]	999,450[1]	26.14[1]	52,150[1]	4.72[1]
12 mJ/cm^2	10	721,064	542,017	631,541	20.05	32,953	4.52
20 mJ/cm^2	10	714,086	746,554	730,320	3.14	38,107	4.58
50 mJ/cm^2	10	155,013	177,472	166,243	9.55	8,674	3.94
80 mJ/cm^2	10	297,661	283,488	290,575	3.45	15,162	4.18
100 mJ/cm^2	10	235,835	245,641	240,738	2.88	12,561	4.10

[1] Original samples are scaleover, Extracted samples are diluted x10 then measured the cATP.

Table A-19

E. coli solution: 12mL *E. coli* Tryptic Soy Broth solution +~400 mL phosphate buffer.

UV Transmittance% = 68.6%

0h							
UV Dose	Volume（mL）	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
Before	10	228,305	182,175	205,240	15.89	13,408	4.13

4h							
UV Dose	Volume（mL）	RLU1	RLU2	AVG	%CV	cATP(pgATP/mL)	log cATP
Before	10	3,468,450[1]	2,216,400[1]	2,842,425[1]	31.15[1]	185,688[1]	5.27[1]
5 mJ/cm^2	10	3,446,550[1]	4,115,720[1]	3,781,135[1]	12.51[1]	247,012[1]	5.39[1]
8 mJ/cm^2	10	834,844[1]	917,826[1]	876,335[1]	6.70[1]	57,249[1]	4.76[1]
12 mJ/cm^2	10	313,913	163,713	238,813	44.47	15,601	4.19
20 mJ/cm^2	10	355,771	336,585	346,178	3.92	22,615	4.35

[1] cATP of original samples are scaleover. Extracted samples are diluted x10 then measured the cATP.

Table A-20

E. coli									
	Instant ATP assay			Biomass Growth ATP Method			HPC		
UV Dose	pgATP/mL	Log	Log (N/N_0)	pgATP/mL	Log	Log (N/N_0)	CFU/mL	Log	Log (N/N_0)
0 mJ/cm^2	24,898	4.40	0.00	191,636	5.28	0.00	9,400,000	6.97	0.00
5 mJ/cm^2	22,827	4.36	-0.04	141,660	5.15	-0.13	8,000,000	6.90	-0.07
8 mJ/cm^2	18,041	4.26	-0.14	52,150	4.72	-0.57	6,150,000	6.79	-0.18
12 mJ/cm^2	29,892	4.48	0.08	32,953	4.52	-0.76	520,000	5.72	-1.26
20 mJ/cm^2	25,276	4.40	0.01	38,107	4.58	-0.70	575	2.76	-4.21
50 mJ/cm^2	42,945	4.63	0.24	8,674	3.94	-1.34	390	2.59	-4.38
80 mJ/cm^2	35,423	4.55	0.15	15,162	4.18	-1.10	660	2.82	-4.15
100 mJ/cm^2	30,555	4.49	0.09	12,561	4.10	-1.18	385	2.59	-4.39

Table A-21

Wastewater									
	Instant ATP assay			Biomass Growth ATP Method			HPC		
UV Dose	pgATP/mL	Log	Log (N/N_0)	pgATP/mL	Log	Log (N/N_0)	CFU/mL	Log	Log (N/N_0)
0 mJ/cm^2	572	2.76	0.00	1,642	3.22	0.00	945,000	5.98	0.00
5 mJ/cm^2	618	2.79	0.03	458	2.66	-0.55	385,000	5.59	-0.39
8 mJ/cm^2	518	2.71	-0.04	428	2.63	-0.58	93,000	4.97	-1.01
12 mJ/cm^2	657	2.82	0.06	192	2.28	-0.93	13,500	4.13	-1.85
20 mJ/cm^2	544	2.74	-0.02	253	2.40	-0.81	4,700	3.67	-2.30
50 mJ/cm^2	494	2.69	-0.06	121	2.08	-1.13	6,700	3.83	-2.15

Table A-22

Locations		May 22nd				May 29th			
		HPC		Biomass Growth ATP Method		HPC		Biomass Growth ATP Method	
		CFU/mL	Log (HPC)	pgATP/mL	Log (ATP)	CFU/mL	Log (HPC)	pgATP/mL	Log (ATP)
Easter Passage	Before	381,250	5.58	1,692	3.23	81,000	4.91	90	1.95
	After	2,160	3.33	117	2.07	480	2.68	40	1.60
Mill Cove	Before	800,000	5.90	1,679	3.23	6,752,500	6.83	11,888	4.08
	After	6,800	3.83	127	2.10	215,000	5.33	858	2.93
Fall River	Before	74,000	4.87	183	2.26	457,500	5.66	427	2.63
	After	2,320	3.37	75	1.88	7,200	3.86	132	2.12

Locations		June 6th				June 23rd			
		HPC		Biomass Growth ATP Method		HPC		Biomass Growth ATP Method	
		CFU/mL	Log (HPC)	pgATP/mL	Log (ATP)	CFU/mL	Log (HPC)	pgATP/mL	Log (ATP)
Easter Passage	Before	46,500	4.67	236	2.37	104,000	5.02	943	2.97
	After	380	2.58	101	2.00	943	2.97	68	1.83
Mill Cove	Before	4,435,000	6.65	11,423	4.06	760,000	5.88	1,013	3.01
	After	97,000	4.99	916	2.96	1,013	3.01	82	1.91
Fall River	Before	216,000	5.33	339	2.53	187,750	5.27	1,098	3.04
	After	640	2.81	65	1.81	1,098	3.04	30	1.48

APPENDIX B – Statistical Analysis

Subtask-1 ATP test with before and after UV wastewater samples

- One-way ANOVA, Tukey test and Dunnett's test

Table B-1 Raw ATP results tested on before and after UV wastewater secondary effluent samples

Before UV (pgATP/mL)	After UV (pgATP/mL)
12543.4	14893.5
14815.3	10508.8
8978.2	8204.4
21010.7	18386.8

One-way ANOVA: Before UV-AVG, After U-AVG

Method

Null hypothesis All means are equal

Alternative hypothesis At least one mean is different

Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	2	Before UV-AVG, After U-AVG

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	3583530	3583530	0.16	0.707
Error	6	138507332	23084555		
Total	7	142090862			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
4804.64	2.52%	0.00%	0.00%

Means

Factor	N	Mean	StDev	95% CI
Before UV-AVG	4	14337	5056	(8459, 20215)
After U-AVG	4	12998	4539	(7120, 18877)

Pooled StDev = 4804.64

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
Before UV-AVG	4	14337	A
After U-AVG	4	12998	A

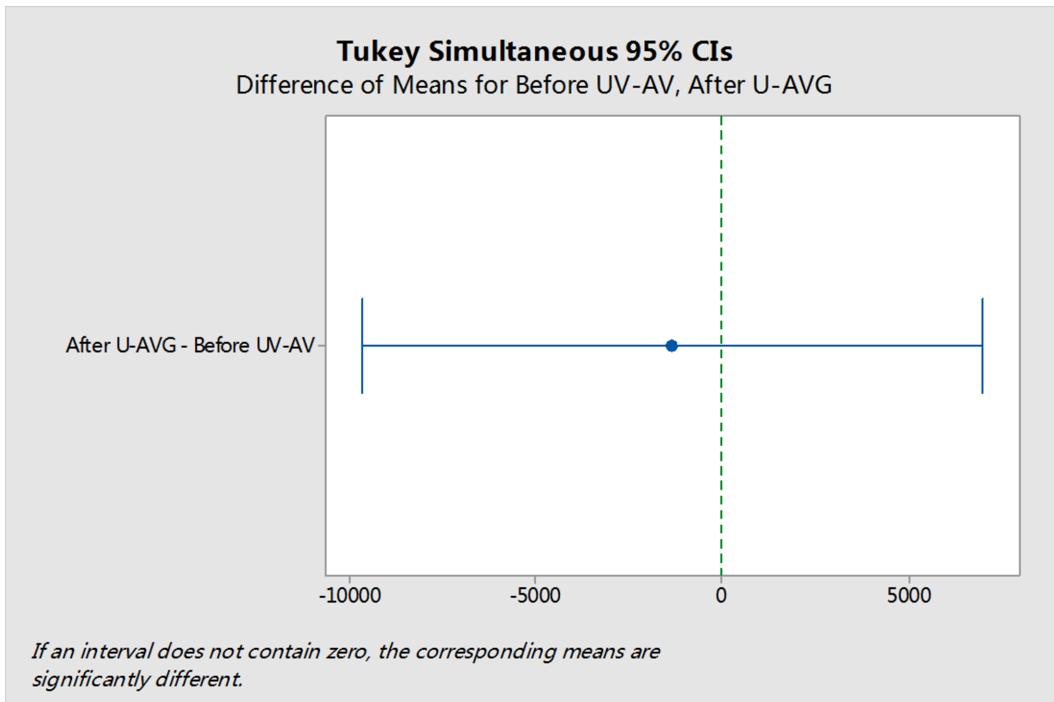
Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
After U-AVG - Before UV-AV	-1339	3397	(-9652, 6975)	-0.39	0.707

Individual confidence level = 95.00%

Tukey Simultaneous 95% CIs



Dunnett Multiple Comparisons with a Control

Grouping Information Using the Dunnett Method and 95% Confidence

Factor	N	Mean	Grouping
Before UV-AVG (control)	4	14337	A
After U-AVG	4	12998	A

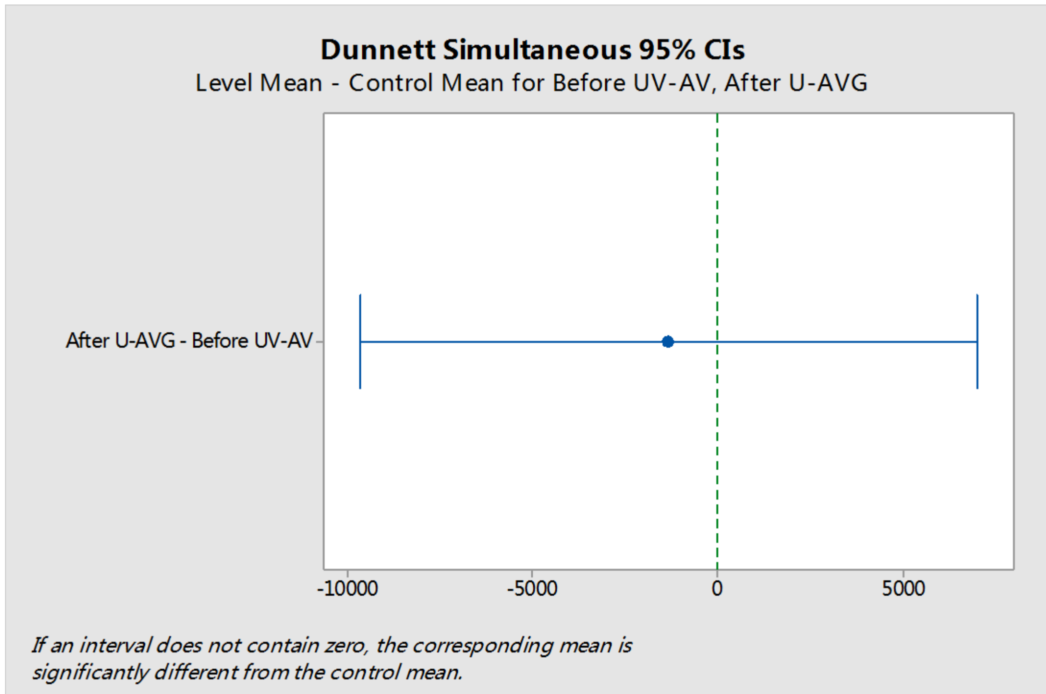
Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

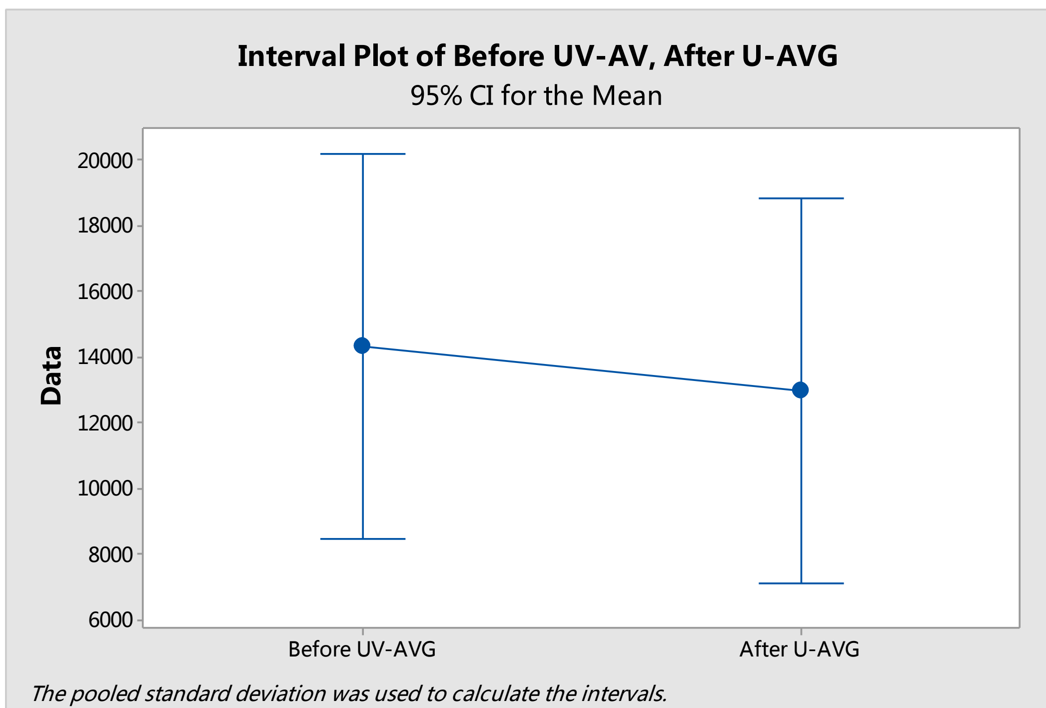
Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
After U-AVG - Before UV-AV	-1339	3397	(-9652, 6975)	-0.39	0.707

Individual confidence level = 95.00%

Dunnett Simultaneous 95% CIs



Interval Plot of Before UV-AV, After U-AVG



Subtask-1 ETS assay with pure *E. coli* culture

- One-way ANOVA, Tukey test and Dunnett's test

Table B-2 ETS results with pure *E. coli* culture irradiated by different UV doses

Absorbance - 0 mJ/cm^2	Absorbance - 10 mJ/cm^2	Absorbance - 50 mJ/cm^2
0.097	0.091	0.089
0.096	0.092	0.089

One-way ANOVA: ETS-1, ETS-2, ETS-3

Method

Null hypothesis All means are equal

Alternative hypothesis At least one mean is different

Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	ETS-1, ETS-2, ETS-3

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	0.000058	0.000029	87.50	0.002
Error	3	0.000001	0.000000		
Total	5	0.000059			

Model Summary

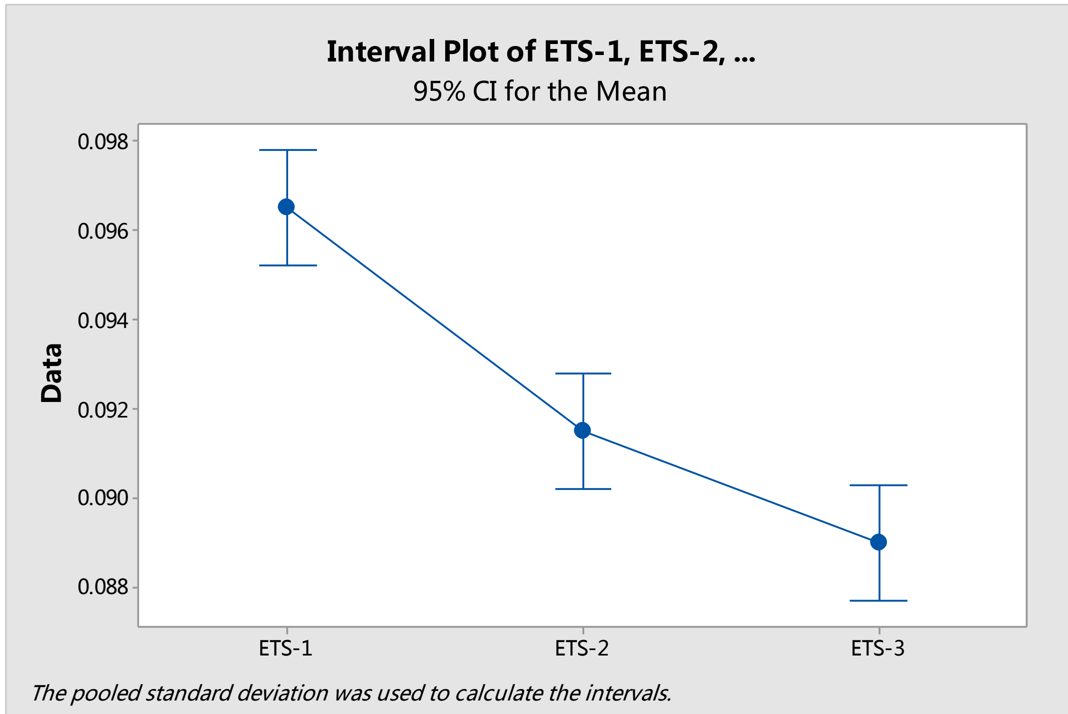
S	R-sq	R-sq(adj)	R-sq(pred)
0.0005774	98.31%	97.19%	93.26%

Means

Factor	N	Mean	StDev	95% CI
ETS-1	2	0.096500	0.000707	(0.095201, 0.097799)
ETS-2	2	0.091500	0.000707	(0.090201, 0.092799)
ETS-3	2	0.08900	0.00000	(0.08770, 0.09030)

Pooled StDev = 0.000577350

Interval Plot of ETS-1, ETS-2, ...



Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
ETS-1	2	0.096500	A
ETS-2	2	0.091500	B
ETS-3	2	0.08900	C

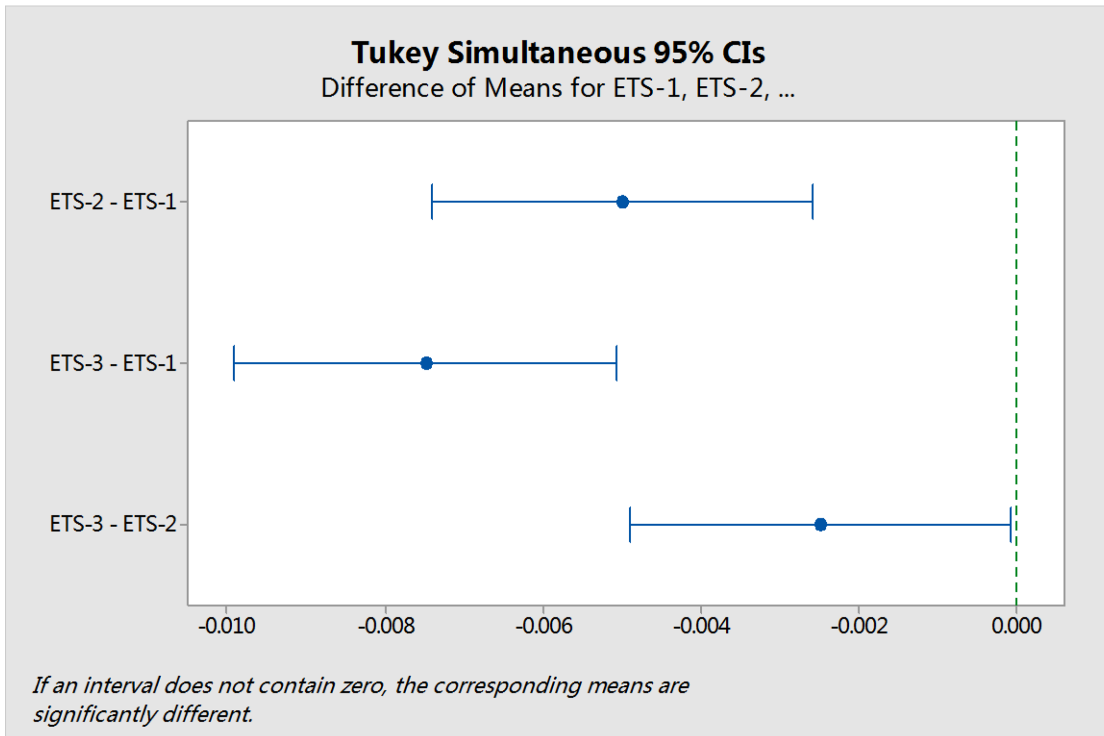
Means that do not share a letter are significantly different.

Tukey Simultaneous Tests for Differences of Means

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
ETS-2 - ETS-1	-0.005000	0.000577	(-0.007413, -0.002587)	-8.66	0.007
ETS-3 - ETS-1	-0.007500	0.000577	(-0.009913, -0.005087)	-12.99	0.002
ETS-3 - ETS-2	-0.002500	0.000577	(-0.004913, -0.000087)	-4.33	0.046

Individual confidence level = 97.50%

Tukey Simultaneous 95% CIs



Dunnett Multiple Comparisons with a Control

Grouping Information Using the Dunnett Method and 95% Confidence

Factor	N	Mean	Grouping
ETS-1 (control)	2	0.096500	A
ETS-2	2	0.091500	
ETS-3	2	0.08900	

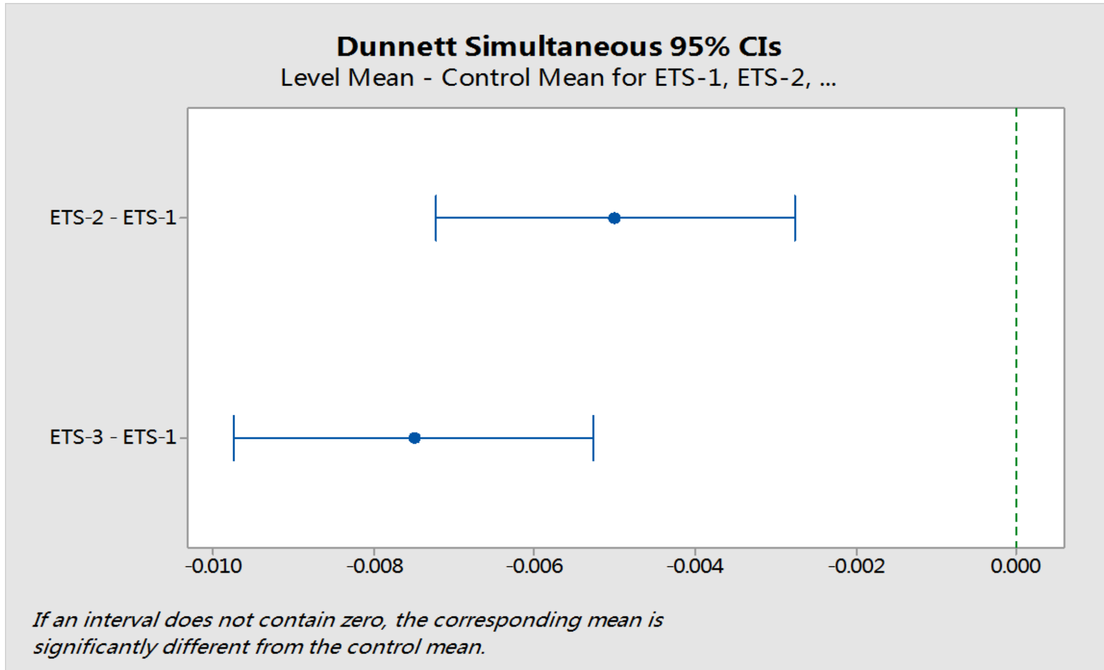
Means not labeled with the letter A are significantly different from the control level mean.

Dunnett Simultaneous Tests for Level Mean - Control Mean

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
ETS-2 - ETS-1	-0.005000	0.000577	(-0.007232, -0.002768)	-8.66	0.005
ETS-3 - ETS-1	-0.007500	0.000577	(-0.009732, -0.005268)	-12.99	0.002

Individual confidence level = 96.94%

Dunnett Simultaneous 95% CIs



Subtask-4 Regression analysis of HPC method and Biomass Growth ATP Method tested on pure *E. coli* culture

Regression Analysis: ATP-*E. coli* versus HPC-*E. coli*

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	1	27130819143	27130819143	32.48	0.001
HPC-*E. coli*	1	27130819143	27130819143	32.48	0.001
Error	6	5012453916	835408986		
Total	7	32143273059			

Model Summary

S R-sq R-sq(adj) R-sq(pred)

28903.4	84.41%	81.81%	68.39%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	15991	12981	1.23	0.264	
HPC-*E. coli*	0.01516	0.00266	5.70	0.001	1.00

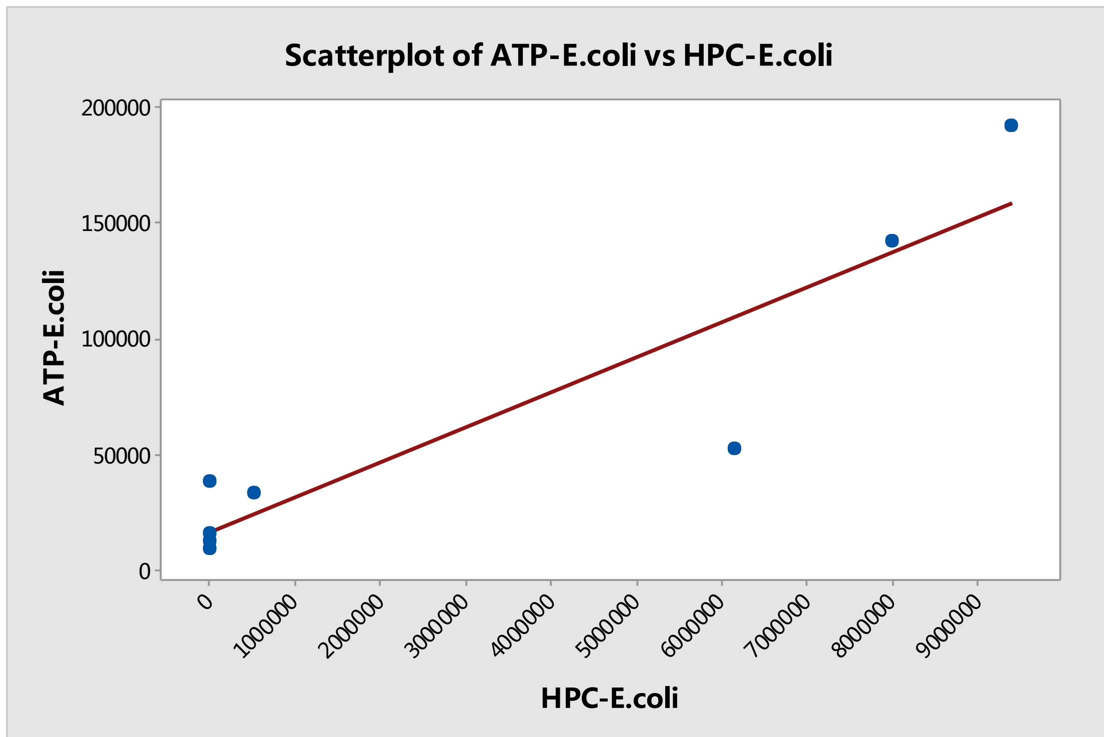
Regression Equation

ATP-*E. coli* = 15991 + 0.01516 HPC-*E. coli*

Fits and Diagnostics for Unusual Observations

Obs	ATP-*E. coli*	Fit	Resid	Std Resid	
3	52150	109236	-57087	-2.22	R

R Large residual



Subtask-4 Regression analysis of HPC method and Biomass Growth ATP Method tested on secondary wastewater effluent sample

Regression Analysis: ATP-WW versus HPC-WW

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	1	1501455	1501455	55.73	0.002
HPC-WW	1	1501455	1501455	55.73	0.002
Error	4	107771	26943		
Total	5	1609226			

Model Summary

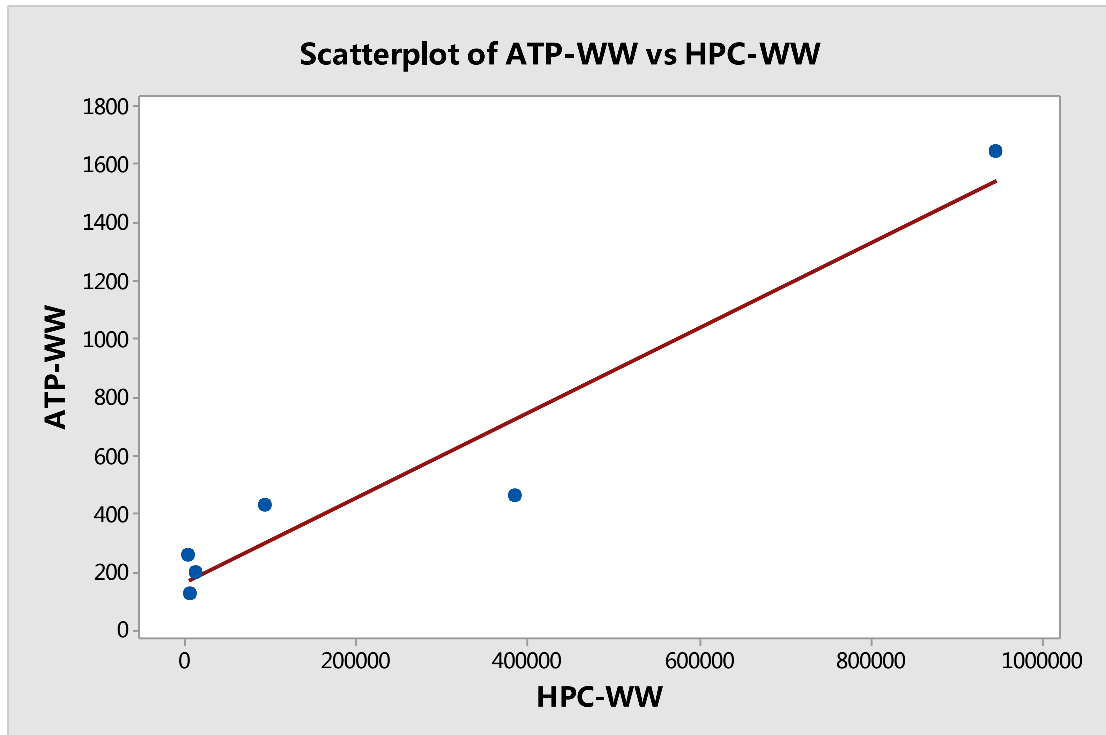
S	R-sq	R-sq(adj)	R-sq(pred)
164.143	93.30%	91.63%	54.46%

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	162.2	82.0	1.98	0.119	
HPC-WW	0.001464	0.000196	7.47	0.002	1.00

Regression Equation

ATP-WW = 162.2 + 0.001464 HPC-WW



Subtask-4: Performing a t-test on HPC method versus Biomass Growth ATP Method with pure *E. coli* cultures to determine the significance of simple regression

H0: There is no linear relationship between HPC method and Biomass Growth ATP

Method tested on pure *E. coli* cultures

H1: There is a linear relationship between HPC method and Biomass Growth ATP

Method tested on pure *E. coli* cultures

Test statistic: $|t|$ =5.70

T distribution with a degree of freedom of n-1=7

$T_{0.05/2} = 2.365$

Reject H_0 if $|t| > t_{\alpha/2}$

Conclusion: Reject H_0. There is a linear relationship between HPC method and Biomass

Growth ATP Method tested on pure *E. coli* cultures.

P-value is 0.001, which means the linear correlation is significant.

Subtask-4: Performing a t-test on HPC method versus Biomass Growth ATP Method with wastewater samples to determine the significance of simple regression

H0: There is no linear relationship between HPC method and Biomass Growth ATP

Method tested on wastewater samples.

H1: There is a linear relationship between HPC method and Biomass Growth ATP

Method tested on wastewater samples.

Test statistic: $|t| = 7.47$

T distribution with a degree of freedom of n-1=5

$T_{0.05/2} = 2.571$

Reject H_0 if $|t| > t_{\alpha/2}$

Conclusion: Reject H_0. There is a linear relationship between HPC method and Biomass

Growth ATP Method tested on pure *E. coli* cultures.

P-value is 0.002, which means the linear correlation is significant.

Subtask-5: Two-way ANOVA analysis on HPC-Before UV, HPC-After UV, ATP-Before UV and ATP-After UV versus Plant and Date, tested using wastewater secondary effluent samples

- **Two-way ANOVA: HPC-Before UV versus Plant, Date**

Table B-3 HPC method - Before UV raw data tested on secondary wastewater samples from 3 WWTPs

HPC-Before UV (CFU/mL)	Plant	Date
381250	EP	May 22nd
800000	MC	May 22nd
74000	FR	May 22nd
81000	EP	May 29th
6752500	MC	May 29th
457500	FR	May 29th
46500	EP	June 6th
4435000	MC	June 6th
216000	FR	June 6th
104000	EP	June 23rd
760000	MC	June 23rd
187750	FR	June 23rd

```
Analysis of Variance

Source   DF       Adj SS       Adj MS  F-Value  P-Value
  Plant   2  2.39071E+13  1.19536E+13     4.20    0.072
  Date    3  8.93890E+12  2.97963E+12     1.05    0.437
Error     6  1.70685E+13  2.84476E+12
Total    11  4.99146E+13


Model Summary

      S    R-sq  R-sq(adj)  R-sq(pred)
1686640  65.80%     37.31%       0.00%
```

Hypothesis testing:

- Plant:

F = 4.2

F critical: (α=0.05, v1=2, v2=11) = 3.98

F > F critical, the factor of plant has an effect on the wastewater composition and quality.

- Date:

F = 1.05

F critical: (α=0.05, v1=3, v2=11) = 3.59

F < F critical, the factor of date has no effect on the wastewater composition and quality.

- **Two-way ANOVA: HPC-After UV versus Plant, Date**

Table B-4 HPC method - After UV raw data tested on secondary wastewater samples from 3 WWTPs

HPC-After UV (CFU/mL)	Plant	Date
2160	EP	May 22nd
6800	MC	May 22nd
2320	FR	May 22nd
480	EP	May 29th
215000	MC	May 29th
7200	FR	May 29th
380	EP	June 6th
97000	MC	June 6th
640	FR	June 6th
943	EP	June 23rd
1013	MC	June 23rd
1098	FR	June 23rd

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Palnt1	2	16251718963	8125859481	2.47	0.165
Date1	3	10422974276	3474324759	1.06	0.434
Error	6	19717358421	3286226403		
Total	11	46392051659			

Model Summary

```
      S     R-sq  R-sq(adj)  R-sq(pred)
57325.6   57.50%     22.08%       0.00%
```

Hypothesis testing:

- Plant:

F = 2.47

F critical: (α=0.05, v1=2, v2=11) = 3.98

F < F critical, the factor of plant has no effect on the wastewater composition and quality.

- Date:

F = 1.06

F critical: (α=0.05, v1=3, v2=11) = 3.59

F < F critical, the factor of date has no effect on the wastewater composition and quality.

Two-way ANOVA: Biomass Growth ATP Method-Before UV versus Plant, Date

Table B-5 Biomass Growth ATP Method - Before UV raw data tested on secondary wastewater samples from 3 WWTPs

ATP-Before UV (pgATP/mL)	Plant	Date
1692	EP	May 22nd
1679	MC	May 22nd
183	FR	May 22nd
90	EP	May 29th
11888	MC	May 29th
427	FR	May 29th
236	EP	June 6th
11423	MC	June 6th
339	FR	June 6th
943	EP	June 23rd
1013	MC	June 23rd
1098	FR	June 23rd

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Plant2	2	92138258	46069129	3.36	0.105
Date2	3	26457777	8819259	0.64	0.615
Error	6	82270723	13711787		
Total	11	200866758			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3702.94	59.04%	24.91%	0.00%

Hypothesis testing:

- Plant:

F = 3.36

F critical: (α=0.05, v1=2, v2=11) = 3.98

F < F critical, the factor of plant has no effect on the wastewater composition and quality.

- Date:

F = 0.64

F critical: (α=0.05, v1=3, v2=11) = 3.59

F < F critical, the factor of date has no effect on the wastewater composition and quality.

Two-way ANOVA: Biomass Growth ATP Method-After UV versus Plant, Date

Table B-6 Biomass Growth ATP Method - After UV raw data tested on secondary wastewater samples from 3 WWTPs

ATP-After UV (pgATP/mL)	Plant	Date
117	EP	May 22nd
127	MC	May 22nd
75	FR	May 22nd
40	EP	May 29th
858	MC	May 29th
132	FR	May 29th
101	EP	June 6th
916	MC	June 6th
65	FR	June 6th
68	EP	June 23rd
82	MC	June 23rd
30	FR	June 23rd

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Plant3	2	464332	232166	3.45	0.100
Date3	3	220485	73495	1.09	0.421
Error	6	403434	67239		
Total	11	1088251			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
259.305	62.93%	32.04%	0.00%

Hypothesis testing:

- Plant:

F = 3.45

F critical: (α=0.05, v1=2, v2=11) = 3.98

F < F critical, the factor of plant has no effect on the wastewater composition and quality.

- Date:

F = 1.09
F critical: (α=0.05, v1=3, v2=11) = 3.59
F < F critical, the factor of date has no effect on the wastewater composition and quality.