AN AUTOMATED AND HIGH-THROUGHPUT (HTP) METHOD FOR ADENOSINE TRIPHOSPHATE (ATP) QUANTIFICATION

by

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DEDICATION PAGE

I dedicate my dissertation work to my elder sister, Aji Kodou Secka, who not only financially supported my undergraduate degree in Canada but always motivated me from when we were very young. I have always strived to be as hardworking, as smart and dedicated as you have always been. I thank you from the depth of my heart and I will always appreciate all you have done.

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ABSTRACT

Exposure to microbial contamination through drinking water is a major global health concern, therefore, it requires rapid detection equipment to maintain microbial stability at the tap. Currently, microbial quality is monitored through time-consuming laboratory methods which causes delayed response. This study demonstrates the development of an automated and high-throughput (HTP) method for the measurement of microbiological activity in water, through the quantification of cellular adenosine triphosphate (ATP). The developed method was able to efficiently and accurately quantify cATP in raw water samples from treatment plants simultaneously. In addition, it proved to be 5x faster and as accurate ($p= 0.911$) as the Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water (ASTM D4012). This developed method has potential to represent a significant advancement for microbial monitoring and could benefit utilities interested in monitoring the biological activity of water, the health of biofilters, and the effectiveness of disinfection strategies.

LIST OF ABBREVIATIONS AND SYMBOLS USED

°C	Degree Celsius
µl	Micro liters
ATP	Adenosine Triphosphate
ALRA	as low as reasonably achievable
BSC	Biological safety cabinet
cATP	Intracellular ATP
CCL	Candidate Contaminant List
CFU	colony forming units
CV	Coefficient of variation
dATP	Extracellular ATP
E. coli	*Escherichia coli*
HPC	Heterotrophic plate count
HTS	High-throughput Screening
HTP	High-throughput method
MPR	Microplate reader
MDL	Minimum detection limit
MAC	Maximum acceptable concentration
PMT	Photomultipler tube
pg	Picograms per mL
PVC	polyvinyl chloride
QGA	Quench gone aqueous
R2A	Reasoner's 2A agar
RLU	Relative light units
TSB	Tryptic soy broth
WHO	World health organization
UN	United Nations

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CHAPTER 1. INTRODUCTION

1.1. Microbiological Contamination of Drinking Water

Waterborne pathogen contamination from human and animal wastes, sewers and septic systems are, globally, a major water quality concern (World Health Organization, 2017). From a drinking water quality context, microbiological water quality is commonly evaluated by monitoring the concentration of heterotrophic bacteria and indicator organisms (such as fecal coliforms and *Escherichia coli* (*E. coli*)) in finished drinking water before reaching consumers' taps (Rauch, Mackie, Middleton, Xie, & Gagnon, 2018).

The United States Centre for Disease Control and Prevention has reported a total of 42 drinking water related outbreaks which resulted in at least 1000 cases of illness, 124 hospitalizations, and 13 deaths during its most recent surveillance in 2013-2014 (Waterborne Disease & Outbreak Surveillance Reporting, 2017) . The ability of water utilities to rapidly gather information on the total concentration of bacteria throughout its treatment plant is vital as plant operators can optimize treatment processes to ensure that the water being delivered meets the country's proposed water quality guidelines (National Research Council (US) Safe Drinking Water Committee, 1980).

1.2. Water Contamination and Human Health

Microbial contamination is the most critical risk factor in water contamination, and it is a major water quality concern around the world. Harmful waterborne pathogens from

human and animal wastes, sewers and septic systems can all pollute water bodies and spread diseases such as cholera and Hepatitis A (World Health Organization, 2017). Through pathogen intrusion, water can also be contaminated within drinking water distribution systems during main breaks/repairs and pressure losses (Ashbolt, 2015). The United States Environment Protection Agency (USEPA), through its 5 Candidate Contaminant List (CCL), has identified more than 500 waterborne pathogens of potential concern that can be found in drinking waters (US Environment Protection Agency (USEPA), 2016).

Inadequate access to safe drinking water is a major cause of death in many parts of the world, children being the most vulnerable (Levallois & Villanueva, 2019). The World Health Organization estimates that almost 10% of the population in the world do not have access to improved drinking water sources; at least 2 billion people use a contaminated drinking water source (World Health Organization, 2017). Further study shows that up to 80% of illnesses are as a result of inadequate water sanitation (World Health Organization, 2017), and the lack of adequate and efficient resources to detect microbial contamination.

1.3. Traditional Methodologies for Microbial Detection and Concerns

Traditional methods for the detection of microbial communities in water rely heavily on microscopy and/or culture-based methods. These methods make use of broth for enriching bacterial communities, selective media for the isolation of colonies,

biochemical identification and the pathogenicity (Gugliandolo, Lentini, Spanò, & Maugeri, 2010). The enumeration methods which are culture based determine bacteria growth after long incubation times, as such it requires 24 hrs at minimum to obtain results (Rauch et al., 2018). This delay increases the risk of public exposure to waterborne pathogens and will also require time dependent remedial actions including characterization for remediation, decontamination and clearance (United States Environmental Protection Agency, 2018). There is also the possible existence of unculturable organisms which will leave some bacteria undetected when using these conventional methods (Kaeberlein, Lewis, & Epstein, 2002).

The most widely used method for microbial enumeration is the heterotrophic plate count (HPC) method, which estimates the number of live and culturable heterotrophic organisms present in a water sample (Rice, Baird, Eaton, & Clesceri, 2012). The number of bacteria is determined by spreading a water sample on Rasoner's 2A (R2A) agar, which is then incubated at room temperature for seven days (Rauch et al., 2018) (Rice, Baird, Eaton, & Clesceri, 2012). This allows culturable bacteria to grow into quantifiable colony-forming units (CFU) (Rice, Baird, Eaton, & Clesceri, 2012).

Viable but non-culturable bacteria that are present do not grow into colonies and as a result the HPC test does not give a true measure of the overall microbiological content in a water sample (Xu, et al., 1982). The time it takes to complete an HPC test also delays an operator's response to a contamination event (Lee & Deininger, 2001).

1.4. Bioluminescence ATP assays for Microbial Detection and Concerns

Alternative real-time or near real-time methods for control of microbiological quality can improve monitoring of water resources and be important in the implementation of early corrective actions and water operator intervention. Adenosine triphosphate (ATP) analysis, a rapid method for the quantification of microorganisms, has become popular as a fast and early microbial detection technique within water utilities (Rauch et al., 2018; Delahaye et al., 2003). ATP is an energy carrying molecule that is present in all living organisms and is the primary energy source for metabolism (Fan & Wood, 2007). ATP monitoring has been used as a real-time monitoring system for the cleanliness of food contact surfaces in the food industry (Osimani, Garofalo, Clementi, Tavoletti, & Aquilanti, 2014), to evaluate the presence and growth of microorganisms in the oil and gas industry (Dodos & Zannikos, 2013) and to assess the cleanliness of surfaces within hospitals (Amodioa & Dino, 2014) (Nante, Ceriale, Messina, Lenzi, & Manzi, 2017). The ATP method is actively used by water utilities alongside of the HPC method (Tracey, 2017).

Developed ATP assays are used for assessing and characterizing the microbiological state of water and have since been adopted for efficiency and the time it takes for processing and analyzing results (5mins) (Rauch et al., 2018). The ATP assay is based on the firefly luciferase bioluminescence reaction, an enzyme-catalyzed reaction, which is a light emitting chemical process in which an enzyme (a mixture of luciferin (D-LH2) and luciferase) breaks down a substrate (ATP) in the presence of magnesium and oxygen (Equation 1) (Fan & Wood, 2007). The light emitted during the reaction, when

measured with a luminometer, is directly proportional to the amount of biological concentration present in the sample (Younès, Lukyanenko, Lyashkov, Lakatta, & Sollott, 2011).

$$ATP + O_2 + \text{luciferin} \xrightarrow[\text{luciferase}]{Mg++} AMP + PPi + \text{Oxyluciferin} + \text{light}$$

Equation 1: ATP bioluminescence reaction

As opposed to the culture based HPC method, the ATP test is able to quantify the overall viable biological activity which includes all intact and damaged cells (Cangelosi & Meschke, 2014) regardless of whether they can be cultured or not. ATP commercial test kits, that follow the ASTM D4012-15 (Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water), have been developed. Briefly, bacterial cells are filtered onto a membrane and a lysing reagent is added to lyse the cells and extract the intracellular ATP (cATP). Luciferase enzyme is then added to the extracted sample in a 1:1 ratio (v/v) and the light emitted is measured using a luminometer. The light output measured in relative light units (RLU) is converted to pg/mL of ATP, which is a quantitative measure of the biological activity in the cell. The ASTM D4012-15 is a single-sample ATP assay (SSAA) method that was developed to measure the ATP concentrations in one sample at a time.

ATP assays make it possible for water managers to quickly and easily assess biological activity as they do with other water quality parameters including temperature, chlorine residual and pH. It reduces the response time of water operators to contamination events as it is a quick way to monitor the bacterial concentration. An ATP assay can, however,

only be used to analyze one sample at a time. As a per-sample based method, it ends up being time consuming and labour intensive depending on the total number of samples being processed.

1.5. High-throughput ATP bioluminescence assay for Microbial Detection

High-throughput screening (HTS), a scientific method that allows large number of compounds, genes or antibodies to be tested in an automated manner, has significantly contributed towards the pharmaceutical industry and is used extensively in drug discovery (Mayr & Furest, 2008). The process involves the use of a control software, automated equipment to handle liquids, and detectors to rapidly quantify active compounds of interest (Michael, et al., 2008).

An ATP high-throughput (HTP) microbial monitoring system for drinking water is yet to be developed. If developed, it would allow water managers and operators to proactively detect and respond to contamination events within distribution systems in a shorter amount of time. It would also enable water utilities to sample at multiple locations within a plant on a regular basis. In addition, water utilities can regularly sample at multiple locations within a plant to facilitate early detection and rapid identification of contamination from different sources including aging drinking water infrastructure, biofilm growth and contaminant intrusion (Rasekh & Brumbelow, 2013) (Flemming, Percival, & Walker, 2002). Automated methods have been shown to reduce user error during sample processing and data recording (Barchard & Pace, 2011) and is expected to increase accuracy and precision as all samples would be subjected to the same

experimental conditions. Furthermore, data is recorded by a software which can be exported to a data management and visualization software (e.g., Microsoft Excel, R studio) for analysis, storage and distribution.

1.6. Research Objective

The overall objective of this research was to evaluate and develop an automated and high-throughput (HTP) method for ATP quantification, as a monitoring microbial tool, by adapting the standard single-sample ATP assay test method. In addition, the potential and applicability of the HTP method is also investigated. Precisely, the aim of this research was to;

1. Investigate the possibility of implementing the ASTM D4012-15 as a HTP method for continuous monitoring of microbial water quality by using a Microplate reader (MPR) and a commercially available ATP assay (QGA, LuminUltra).

2. Investigate the applicability of using the assay reagents in a HTP setting.

3. Asses the accuracy and reliability of the developed ATP method in quantifying the total microorganisms in different environmental water samples.

4. Evaluate the prospects of applying the HTP for further drinking water experiments.

A bench-scale experimental plan was used for the aforementioned aims. The ASTM D4012-15 was applied as a HTP method by replacing the ATP extraction equipment and the luminescence detection equipment used for single-sample ATP assay (SSAA) methods.

1.7. Thesis Organization

The organization of this thesis is as follows;

- Chapter 1- Gives an introduction to water contamination and the importance and concerns of microbial monitoring procedures. The objective of the research and the thesis organization are also provided.
- Chapter 2 – Presents background information on basic principles, history, need for microbial detection in water systems, and an overview of available and used detection methods. The purpose of this section is to put the presented work into context and provide the study rationale
- Chapter 3 - Provides an overview of the method development process, the materials and equipment used for this work, and the experiments performed.
- Chapter 4 – Explains the results of the performed experiments and an evaluation of the method using a selection of environmental samples.
- Chapter 5 – Presents the use of the developed method to perform a chlorination study. The purpose of this section was to demonstrate the applicability of the method that was developed.
- Chapter 6 – Provides a conclusion of the major findings from this research and Recommendations

CHAPTER 2. BACKGROUND

2.1. Drinking Water Sources

The state of a drinking water supply can be quantified by four important characteristics: quality, quantity, reliability, and cost (Sullivan, Agardy, & Clark, 2005). The World Health Organization (WHO) and various national agencies have drinking water quality standards that specify the acceptable microbial, chemical, and radiological characteristics of safe drinking water. Consequently, drinking water utilities need to constantly monitor water quality in order to determine the efficacy of their disinfection processes and to ensure that the water meets the country's proposed water quality guidelines (National Research Council (US) Safe Drinking Water Committee, 1980).

Microbial contamination being the most critical risk factor in drinking water heightens the importance of ensuring the availability of efficient detection and decontamination, as described herein.

2.2. Monitoring Microbial Water Quality through Indicator Organisms

Indicator organisms in water are used as an indication of the presence of pathogens in a water, which when consumed by a person can result in serious health effects (World Health Organization (WHO), 2000). The consensus is that, if indicator organisms are detected, then pathogens, including viruses, and other sources of infection could also be present and adequate mitigation measures are required. According to (Herwaldt et al., 1992), the number of illness from chemical contamination is generally negligible when

compared to the total number of people that get sick from microbial pathogens in drinking water. Virulence is said to be a genetic trait and can vary markedly from one strain of bacteria to another (Beceiro, Tomás, & Bou, 2013). The World Health Organization (WHO) came up with definitions for indicator and index micro-organisms that are of public health concern (Ashbolt, Grabow, & Snozzi, 2001)

Table 1: Definitions of organisms that are of public health concern

Group	Definition
Process indicator	A group of organisms that demonstrate the efficacy of a process, such as total heterotrophic bacteria or total coliforms for chlorine disinfection.
Fecal indicator	A group of organisms that indicate the presence of faecal contamination, such as the bacterial groups thermotolerant coliforms or *E. coli*. Hence, they only infer that pathogens may be present
Index and model organisms	A group/or species that are indicative of pathogen presence and behaviour respectively, such as *E. coli* as an index for Salmonella and F-RNA coliphages as models of human enteric viruses.

Note: Information on organisms of public health concern present in the environment. Reproduced from WHO (Ashbolt N. J., 2015), retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25821716

As shown in Table 1, *E. coli* and coliform bacteria are often used as indicator organisms for fecal pollution in water. As stated by the US Environmental Protection Agency (EPA) 2006, an indicator organism must fulfil the following criteria for it to be considered an ideal indicator of fecal contamination:

1. The indicator organism should be found in the feces of both humans and warm-blooded animals

2. The indicator organism should be more abundant than pathogenic organisms and should be present in high numbers in contaminated waters
3. The presence and persistence of the indicator organism in water, along with water removal by treatment, should be similar to those of the actual pathogens.
4. The indicator organism should be isolated easily, identified and enumerated

2.2.1. Coliform bacteria

Coliform bacteria are present in the environment and feces of all warm-blooded animals and humans and are part of the Enterobacterceae family. They are defined as aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas formation within forty hours at 37°C (Smith, 2001).

Total Coliform bacteria – Total coliforms are bacteria that are present in the soil, in water influenced by surface water, and in human or animal waste (Cabral, 2010). Thus, the presence of total coliforms may or may not indicate faecal contamination.

Fecal coliforms – Fecal coliforms are a group of total coliforms that are specifically found in the gut and feces of warm-blooded animals but are also found in the environment (Rice, Allen, & Eugene, 1999; Dufour, 1977). The presence of fecal coliform is an indication of fecal contamination, but it is mostly

used as an indication of treatment efficiency since they are also found in the environment (Smith, 2001).

E. coli - The sub-group *E. coli,* which has the ability to grow at elevated temperatures (49°C), is the most common member of fecal coliforms (Ontiveros, 2019) (Verhille, 2001). The Government of Canada (2019) mandates a water quality guideline of non detectable *E. coli* per 100 ml of water sample. The absence of fecal contamination in water distribution systems can also be a method of prevention of illnesses that are caused by viruses and parasites (such as Giardia and Cryptosporidium) in water (Smith, 2001).

Absence of both *E. coli* and coliforms does not, however, necessarily indicate the absence of other pathogenic organisms such as Enteroviruses, dormant Cryptosporidium or Giardia, which are more resistant to disinfection (Smith, 2001). As a result, using only specific indicator detection methods will not be a true measurement of the microbial quality within a drinking water distribution system.

2.3. Microbial Detection in Drinking Water

As previously stated, drinking water utilities with well-operated drinking water treatment systems need to constantly monitor water quality in order to determine the efficacy of their disinfection processes. This is done to ensure that the water is microbiologically safe and meets the country's proposed water quality guidelines (National Research Council (US) Safe Drinking Water Committee, 1980).

2.3.1. Heterotrophic Plate Counts (HPC)

The HPC test is a culture-based test which is intended to recover a wide range of microorganisms in water. Universally, heterotrophs are referred to as groups of microorganisms, such as bacteria, moulds and yeast, that use organic carbon sources to grow, and can be found in all types of water (Bartam, Cotruvo, Exner, Fricker, & Glasmacher, 2002). The HPC method only estimates live and culturable organisms that are present in water, however, it does not specify the type of bacteria that is present (Bartam, Cotruvo, Exner, Fricker, & Glasmacher, 2002). Bacteria cells are quantified as colony forming units (CFU) that appear in different shapes and sizes (single cells, clusters, chains etc.) (Ontiveros, 2019). According to (Hancock, 2016), the CFUs represent single, live bacteria that were able to rapidly multiply to be observed on the agar plate.

Depending on the study being conducted, the HPC method involves the use of different test conditions such as; temperature ranges from 20°C to 40°C, incubation time of a few hours to seven days, and the use of different nutrient conditions (Hancock, 2016). In general, the HPC test involves putting a known volume of water sample on a plate that contains nutrient that will stimulate bacteria to grow, the nutrient media that is more commonly used is Reasoner's 2A agar (R2A agar). Typically, the plate is incubated at room temperature for 5-7 days after which the number of colony cells that grow on the plate are counted and quantified (Rice, Baird, Eaton, & Clesceri, 2012). Standardized methods recommend only taking into account plates with colonies between 30 and 300

CFU (Standard Methods of Examination of Water and Wastewater, 2011). The CFU/mL is quantified using Equation 2;

$$CFU/mL = \frac{Number\ of\ colonies\ x\ dilution\ factor}{Volume\ of\ sample\ plated}$$

Equation 2: Formula to calculate the colony forming unit per millilitre of a sample (CFU/mL). (Rice, Baird, Eaton, & Clesceri, 2012)

The HPC method cannot be used as a concluding factor on the specificity of the type of bacteria present in a water system but it does help to determine filtration and disinfection efficiency and as an alarm for potential breakthrough of microbial contaminants (Hancock, 2016).

The disadvantages of the HPC method are; it is time consuming and that only 0.01% to 1% of bacteria can grow in a laboratory setting (Watkins & Jian, 1995) (Ontiveros, 2019).

2.3.2. Adenosine Triphosphate

2.3.2.1. ATP as a Nucleotide

ATP is an energy carrying molecule that is used as an intracellular energy source by all living organisms (Ashbolt N. J., 2015). It is generated through respiration and it fuels cellular functions that are necessary for survival, growth and replication (Ashbolt N. J., 2015). ATP is a nucleotide with three main structures: adenine (nitrogenous base), ribose (sugar) and three phosphate groups (Figure 1). The bonds between two of the

phosphate groups are high energy phosphoanhydride bonds. When one of these bonds are broken through the process of hydrolysis, more than 30KJ of energy is released (Vang, 2013).

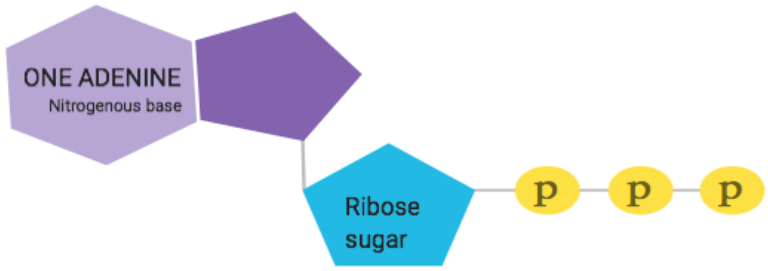

Figure 1: Chemical structure of ATP. Figure shows the components that make up the chemical compound

2.3.2.2. *ATP levels in drinking water distribution networks*

In drinking water distribution systems, the concentration of ATP is generally low when compared to other water matrices such as wastewater (Hammes, Goldschmidt, Vital, Wang, & Egli, 2010; El-Chakhtoura, et al., 2015; Vang, Corfitzen, Smith, & Albrechtsen, 2013) . The range of cATP in waterworks, distribution network and tap water (stagnant) is in the range of <1 to 27 pg/mL (Vang, 2013). The differences in concentration observed amoung drinking water distribution systems is as a result of distribution systems receiving water from different sources and also subjected to different treatment processes. The quality of drinking water also changes with season, providing an ideal environment for bacteria growth (Vang, 2013).

2.3.3. ATP Bioluminescence Assay

Bioluminescence is the emission of energy from a cell in the form of visible light as a result of chemiluminescence within the cell. The ATP bioluminescence assay is used for assessing and characterizing the microbiological state of drinking water and has

since been adopted for its efficiency and the time it takes for processing and analyzing results (5 mins) (Rauch et al., 2018).

The contents of a typical water sample include non-biological solids, healthy biomass and unhealthy biomass. The ATP contents associated with the microbial contents are divided into two;

1. Intracellular ATP (cATP) – ATP that are found contained within healthy and living biological cells.
2. Extracellular ATP (dATP) – These are dissolved ATP molecules that were released from dead and/or stressed organisms and are found outside the walls of biological cells.

In principle, the ATP assay is based on the firefly luciferase bioluminescence assay, an enzyme-catalyzed reaction, which is a chemical process (chemiluminescence) in which an enzyme (a mixture of luciferin (D-LH2) and luciferase) breaks down a substrate (cATP) in the presence of magnesium and oxygen (Fan & Wood, 2007), Equation 5. This reaction produces light which when measured using a luminometer is directly proportional to the amount of biological energy present in the sample (Fan & Wood, 2007).

There are two reactions involved in the assay. The first reaction involves the hydrolysis of ATP to AMP (adenosine monophosphate), were the enzyme-bound intermediate D-luciferyl-adenylate ($Luc._D$-LH_2-AMP) and inorganic pyrophosphate (PP_i, i.e. $P_2O_7^{4-}$) bound to Mg^{2+} are generated, Equation 3 (Fraga, 2008).

$$Luc + {}_{D}\text{-}LH_2. + ATP\text{-}Mg^{2+} \leftrightarrow Luc \cdot {}_{D}\text{-}LH_2\text{-}AMP + PP_i\text{-}Mg^{2+}$$

Equation 3: The hydrolysis of ATP to AMP to generate the enzyme-bound intermediate D-luciferyl-adenylate (${}_{D}LH_2$-AMP) and inorganic pyrophosphate (PP_i, i.e. $P_2O_7^{4-}$)

The second reaction , Equation 4, is the oxidation and decarboxylation of the intermediate ${}_{D}\text{-}LH_2$-AMP which produces AMP, CO_2 and oxyluciferin $OxyLH_2$ and the light emitter (hv) with photons of yellow-green light (550-570 nm) (Vang, 2013)

$$Luc \cdot {}_{D}\text{-}LH_2\text{-}AMP + O_2 \longrightarrow Luc + AMP + CO_2 + OxyLH_2 + hv$$

Equation 4: The second step of the assay which produces hv and the light emiiter

Research has shown that one photon of light is produced for one molecule of ATP consumed (Lasko & Wang, 1996). The firefly luciferase reaction, Equation 4, reaches its maximum intensity within half a second (Corey, 2009). The relative stability of the assay is highly dependent on the conditions of the assay (Vang, 2013); high luciferase concentrations will have a rapid maximum rise in light intensity in the first few seconds and subsequently start to decay. Low luciferase concentrations on the other hand gives a relatively stable light emission, where "decay is proportional to the ATP concentration at ATP <1 µmol/L ($ATP << K_m$)" (Vang, 2013) (Guardigli, Lundin, & Roda, 2011). The luciferase bioluminescence assay is assumed to follow the Michaelis-Menten kinetics, where the reaction rate (v) or rate of ATP degradation is expressed as;

$$\frac{v}{V_{max}} = \frac{S}{(S + K_m)}$$

v: reaction rate

S: substrate concentration (ATP)

V_{max}: maximum rate and maximum substrate concentration

K_m: Michaelis-Menten constant

2.3.3.1. *Factors that affect ATP assays*

A reduction in concentration of ATP in a microbial cell can occur as a result of physiological stress and/or the nutritional status of the environment (Yau & Potenza, 2014). A study conducted by (Kurath & Morita, 1983) showed the bacteria *Pseudomonas sp.* adapt and regenerate ATP to its initial level in a low nutrient environment (Vang, 2013). This observation showed that cells can regenerate ATP, after adaptation, to the initial level when starved (Vang, 2013). Jones & Simon, 1977 further showed that centrifugation and filtration had the potential to decrease the ATP content of cells (Kurath & Morita, 1983) and vacuum filtration was especially shown to decrease total ATP levels which increased with an increase in the total volume filtered (Vang, 2013).

Although luciferase, luciferin, oxygen and ATP-Mg^{2+} are required for the bioluminescence reaction to occur, pH and temperature are the two factors that have the most effect on the reaction (Fraga, 2008; Vang, 2013). Luciferase acts as a catalyst which affects the rate of a reaction by interacting with the light producing substrate, luciferin. The temperature at which the reaction occurs affects the rate of reaction, subsequently affecting the rate of light emission. Studies have shown that the optimum temperature for bioluminescence reactions is at 25°C and at a temperature range of 18-23°C for commercial assays (Vang, 2013). The optimum pH for bioluminescence reaction is 7.8 (Guardigli, Lundin, & Roda, 2011)

2.3.4. Mechanism of bioluminescense detection

Bioluminescence is the emission of energy from a cell in the form of visible light as a result of chemiluminescence within the cell. Through the measurement of light from chemiluminescense, the concentration of an unknown substance can be deduced from the rate and the intensity at which the light is emitted (Vang, 2013). The rate of light output is directly proportional to the concentration of the luminescent material present. As a result, light measurement is a relative indicator of the total amount of luminescent material present in the sample of interest (Vang, 2013). The light is measured using a lumimometer (Vang, 2013)

A luminometer is a sensitive instrument that is used for the detection of light. It is made up of the components below;

1. A sample chamber which holds the sample test tube – The bioluminescense reaction occurs in a light tight cuvette chamber Figure 2. The sample is prepared through filtration, which traps bacterial cells with trapped ATP molecules. To release the trapped ATP, a lysing reagent is used to release the ATP molecules, now contained in the cuvette. The cuvette is placed in the chamber, at which point luciferase is added and light is subsequently emitted.
2. A photomultipler tube (PMT) for light detection - The PMT detects and displays light photons by counting the individual excited photons or by registering the electric current. Light emitted during the reaction is

amplified by the PMT. The PMT also ensures uniformity of the light that is collected regardless of the size of the sample

3. Signal processing and a signal output display – The luminometer has a set integration when the signal at its highest and most stable peak is measured. The value is is reported in relative light units (RLU).

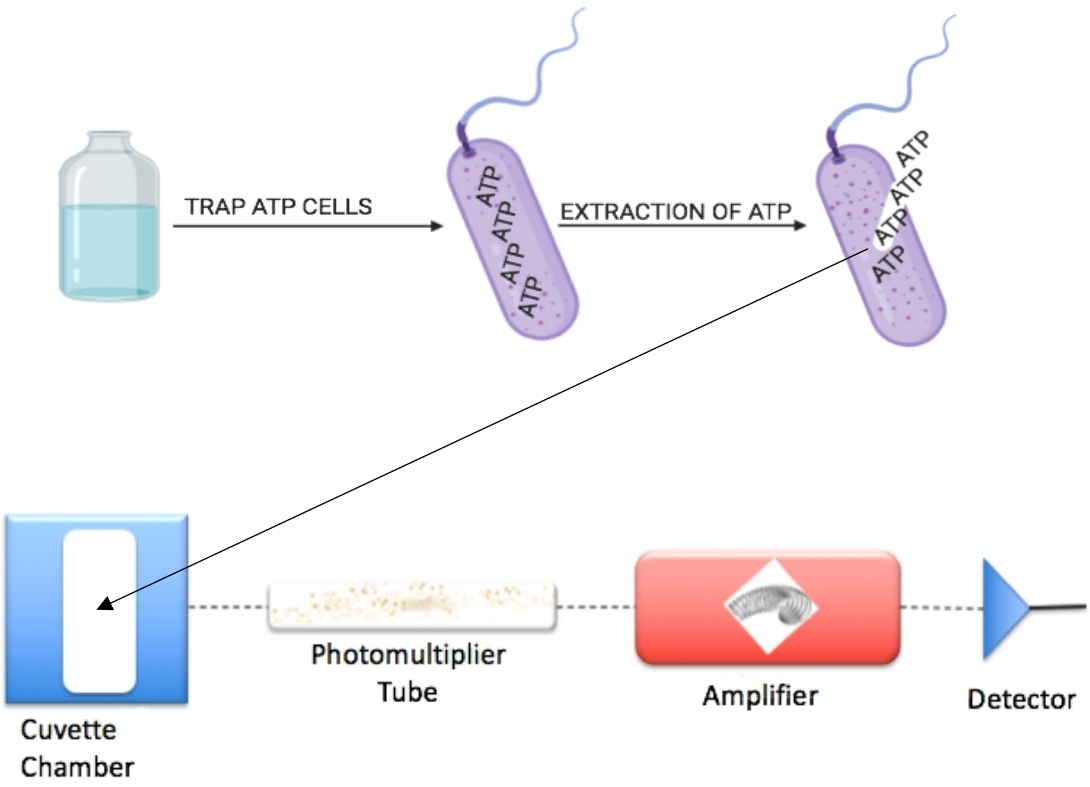

Figure 2: Bioluminescence ATP reaction. The figure shows the process of extracting ATP cells from bacteria in water samples and the production of light after the addition of luciferase in chamber

Factors that have an effect on sample light emission include, sample volume, reaction rates, concentration of reactants, reagent injection and mixing, cuvette material and size, and temperature (Vang, 2013).

2.3.5. Applications of the ATP bioluminescence assay

The ATP bioluminescence assay is considered to be an effective biosensor and has been employed for a range of applications including microbiological research, environmental monitoring and industrial applications (Vang, 2013).

The ATP assay is currently used to determine the concentration of microorganisms in marine and freshwater environments, after it was first used for the determination of ATP of microorganisms in marine water in 1966 (Vang, 2013). The ATP assay is used alongside the HPC test to determine and quantify the concentration of microorganisms in drinking water samples (Ferreira et. al, 2017; Deininger & Lee, 2001; Frundzhyan & Ugarova, 2007; Coallier, Prévost, Rompré, & Duchesne, 1994)). The assay can also be used for specific detection of bacteria or yeasts, which is done by separating the species through filtration (Stanley, 1989; (Vang, 2013).

The ATP bioluminescence assay has been used for hygiene monitoring in the food industry to asses and monitor the cleanliness of the surfaces in contact with food during production and also in the assessment of the cleanliness of surfaces in healthcare institutions (Syguła-Cholewińska, Lech, Szostak-Kot, Błyskal, & Sawoszczuk, 2014).

Contrary to conventional methods for microbiological detection, the ATP assay has been considered to be superior due to its ability to rapidly measure the amount of all viable microorganisms, both culturable and non-culturable cells, in a water sample (Shimomura, 2006) (Table 2). The disadvantage with the ATP assay is that it also does

not indicate or distinguish between or specify the type of microorganisms that are present in a water sample (Vang, 2013). Detection of specific bacteria and yeasts can however be achieved by separating organisms through filtration before measuring ATP (Stanley P. , 1989; Vang, 2013). Additionally, it is a SSAA method that can only be used to process one sample at a time which results in processing time that is directly proportional to the number of samples.

Table 2: Advantages and limitations of the ATP Bioluminescence Assay.

Advantages	**Disadvantages**
Rapid method- Results obtained within minutes (5 minutes) (Rauch et al., 2018)	Results cannot be converted to cell count (Vang, 2013)
Quantitative - One photon of light is produced for one molecule of ATP consumed (Lasko & Wang, 1996)	Non-Specific – Does not indicate the type of bacteria present (Vang, 2013)
All viable cells quantified (Rauch et al., 2018)	

2.4. High-throughput methods

High-throughput screening (HTS) is a scientific method that allows a large number of compounds, genes or antibodies to be tested in an automated manner. The use of HTS has significantly contributed towards the pharmaceutical industry and extensively in drug discovery (Mayr & Furest, 2008). Recently, HTS technology has expanded into new areas such as applied research in academia and hospitals. HTS was invented in the early and mid-1990s for works such as combinatorial and multi-parallel chemical

synthesis that have tremendously increased in the number of compounds that need to be tested (Mayr & Furest, 2008).

To account for reproducibility and screening, 96 well plates were invented (Mayr & Furest, 2008). The plates, Figure 3, allow for a number of experimental samples to undergo testing under the same given conditions simultaneously. Each well on the plate can hold a total volume of up to 300 µL and this makes it reagent efficient without compromising detection capability and accuracy.

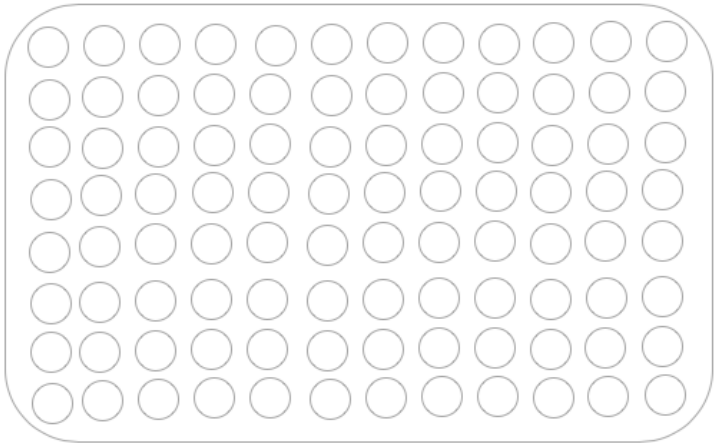

Figure 3: 96-well multi plate for highthroughput screening (HTS)

The development of any HTS experiment involves three basic steps; sample preparation, sample handling, and readouts and data acquisition (Springer instruments, 2015)

I. **Sample preparation-** Prior to analyzing any sample, the sample needs to be prepared in an arrayed format by filtering or pipetting a desired volume of sample into a multiwell plate.

II. **Sample handling** – Sample handling in HTS usually involves automatically dispensing known volumes of liquid reagents to the samples in the wells whilst the sample is being tested.

III. **Readouts & data acquisition**- Despite all the advancement in HTS, fluorescence spectroscopy has generally been the adopted methodology for sample readouts and data acquisition (Springer instruments, 2015). Fluorescence spectroscopy is a scientific method that is used to measure the concentration of different classes of compounds present in a sample (Diamandis, 1993). It works by passing a beam of light with a known wavelength through a sample which causes the sample to fluoresce through the emission of photons after absorbing the beam of light. The light that is emitted is directly proportional to the concentration of the target compound/analyte (Diamandis, 1993), although fluorescence is not always linear with conc.

The emission of photons can be achieved by both fluorescence and bioluminescence; in bioluminescence the energy that produces the emission of light results from exothermic chemical reactions (Fan & Wood, 2007), Equation 5. Bioluminescence is however yet to be used for HTS methods

A number of assays have been developed for HTS with potentially bioactive compounds. There is, however, no/limited evidence of an HTS ATP assay for drinking water sources.

2.5. Characteristics of an Efficient High-Throughput ATP assay

When developing an ATP method, there are a number of characteristics that the method should have for optimum ATP recovery. Below is a list of characteristics the method development was based on;

- Sensitive equipment for ATP measurement
- An efficient ATP extraction procedure
- Low interference ATP reagents
- A rapid, repeatable and accurate measurement

CHAPTER 3. MATERIALS AND METHODS

The method development was broken down into two steps; an equipment validation step (data readout and acquisition), where the MPR was validated and a measurement protocol developed, and an ATP extraction step (sample preparation and handling), which involved a method for filtering and extracting ATP from water samples.

3.1. Instrumentation

To account for reproducibility and screening for the developed HTP method, a configurable multifunctional microplate reader (BioTeK Synergy H1 Hybrid Multi-Mode, Winooski, Vermont, United States) with a monochromator-based module microplate reader was used to measure luminescence, Figure 7 (BioTek, n.d.). The microplate reader (MPR) was equipped with two reagent injection pumps used to dispense reagents into the wells of a plate.

A vacuum manifold (Anodized aluminum multi-well vacuum manifold with accessories, Pall Corporation, New York, United States), and 96-well multiwell filtration plates (AcroPrep™ Advance 96-Well Filter Plates for Aqueous Filtration – 2 mL, 1 μm, Pall Corporation, New York, United States) were used for sample preparation and handling.

3.2. Chemical reagents

A commercial SSAA test kit with associated reagents (Quench Gone Aqueous [QGA], LuminUltra Technologies, Fredricton, Canada) for low-solid water-based samples was used for developing and validating the HTP method. The test kit adhered to ASTM Standard Method D4012 which outlines the steps to measure intracellular ATP from

living organisms (cellular ATP (cATP)) in a fluid suspension, and can detect ATP concentrations as low as 0.1 pg/mL (ASTM D4012-15 Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water, 2015).

As per the manufacturer's instructions, a known volume of sample was filtered using a syringe filter system, trapping bacteria cells on a filter media bed (Figure 4, Step 1). The cells were lysed with 1 mL of lysing reagent (UltraLyse7, Luminultra Technologies) and released into 9-mL of buffer to dilute the filtrate (Figure 4 Step 2). Luciferase enzyme (Luminase, Luminultra Technologies, Fredericton Canada) was then added to the extracted sample in a 1:1 ratio (v/v) and the light emitted was measured using a stand-alone luminometer (PM) (Figure 4, Step 3&4). The PM used is a USB-operated luminometer that is coupled with a Bluetooth module which gives it real-time capabilities (PhotonMaster, LuminUltra Technologies, Fredericton Canada). The assay is rapidly introduced into the PM (Figure 4, Step 5). The light produced was reported in relative light units (RLU). A 1 ng/mL calibrant (UltraCheck™ 1, LuminUltra Technologies, Fredericton, Canada) was used to confirm the accuracy of the equipment in measuring within the confidence limit of the test. The cATP concentration of the analyzed sample was converted from RLU to pg cATP/mL as follows:

$$cATP\left(\frac{pg}{mL}\right) = \frac{RLU_{cATP}}{RLU_{ATP1}} X \frac{10{,}000\,(pg\ ATP)}{V_{sample}(mL)}$$

Equation 5: RLU conversion equation to ATP concentrations in pg/mL

Where:

RLU_{ATP1} = ATP of Standard Calibration (ATP1);

RLU_{cATP} = ATP of Sample

V_{sample} = Volume of sample that was filtered

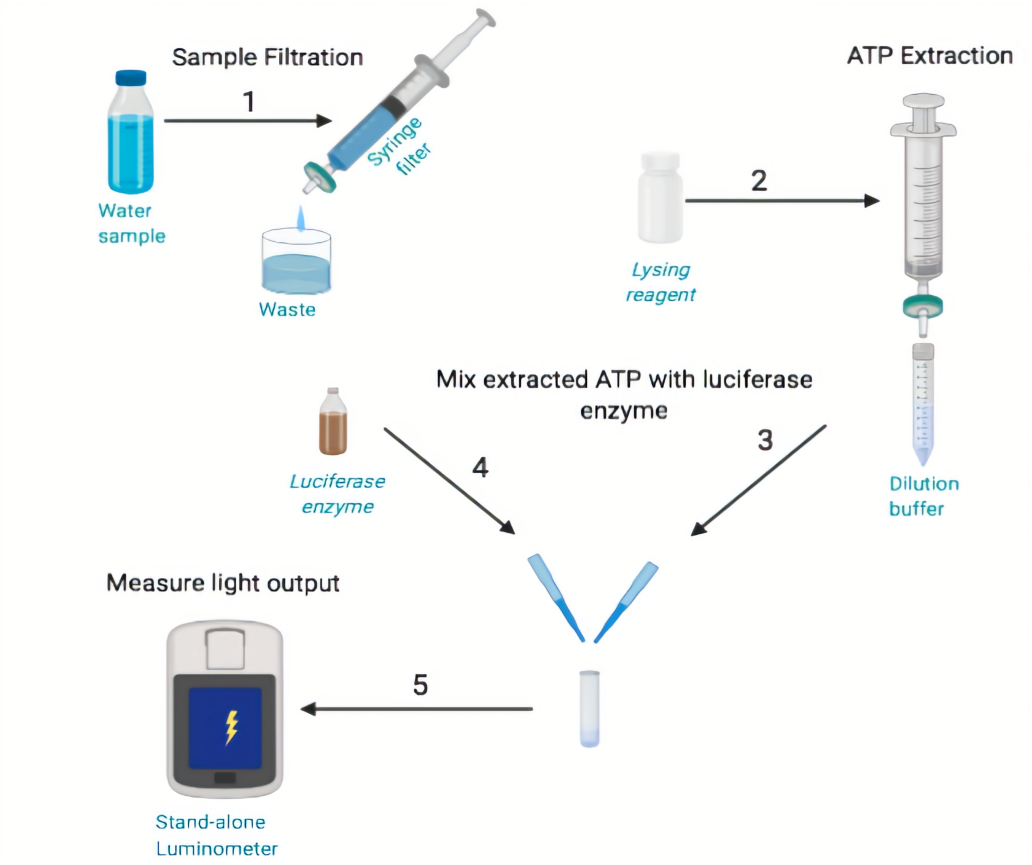

Figure 4: A schematic representation of the method for ATP analysis showing how cATP is extracted and measured using a syringe filter

3.3. Preparation of Standard Solutions

All ATP standards were prepared by serial dilution of a 100 ng/mL ATP stock solution (UltraCheck™ 100, LuminUltra Technologies, Fredericton, Canada) in 10 mL sterile tubes containing 9 mL of ATP stabilizing agent (Lumisolve, LuminUltra Technologies, Fredericton, Canada). All ATP standards were used for a maximum of 7 days as the concentration of the standards was found to remain stable for this duration (Table 3).

Table 3: Degradation of ATP standards with time

Added Concentration (pg/mL)	Day	% Recovery	Precision (%CV)
10000	1	102	6
	3	101	5
	10	97	2
1000	1	99	2
	3	99	5
	10	85	2
100	1	107	1
	3	98	1
	10	89	5

A 1 ng/mL ATP stock solution (UltraCheck™ 1, LuminUltra Technologies) was used as a calibration solution for the MPR, and the calibration was performed to confirm the accuracy of the equipment in measuring within the confidence limit of the test.

3.4. Preparation of pure *E. coli* culture

Pure *E. coli* culture samples were prepared using non-pathogenic *E. coli* K12 (ATCC #47076 strain MG1655; Centre for Research in Environmental Microbiology, University of Ottawa) stock. For each experiment, a pure *E. coli* culture was prepared by inoculating laboratory prepared sterile tryptic soy broth (TSB) with frozen stocks of *E. coli*, then incubated overnight at 37°C with shaking at 250 rpm. A subculture was successively prepared the next day by incubating 0.1 mL of the overnight culture in 9.9 mL of TSB for 3-4 hours to ensure that the *E. coli* culture was always at a late exponential phase. The growth phase of the culture was tested by an *E. coli* growth curve (Figure 5)

An *E. coli* and OD600 growth curve versus time that was created using *E. coli* and TSB, Figure 6, was used to make an estimation of the concentration of *E. coli* in CFU/

mL at a given OD600 value. This allowed for an *E. coli* solution to be prepared at a desired and controlled concentration. A significant correlation with an $R^2 > 0.9$ was found between OD600 and the concentration of E. *coli* CFU/mL Figure 5.

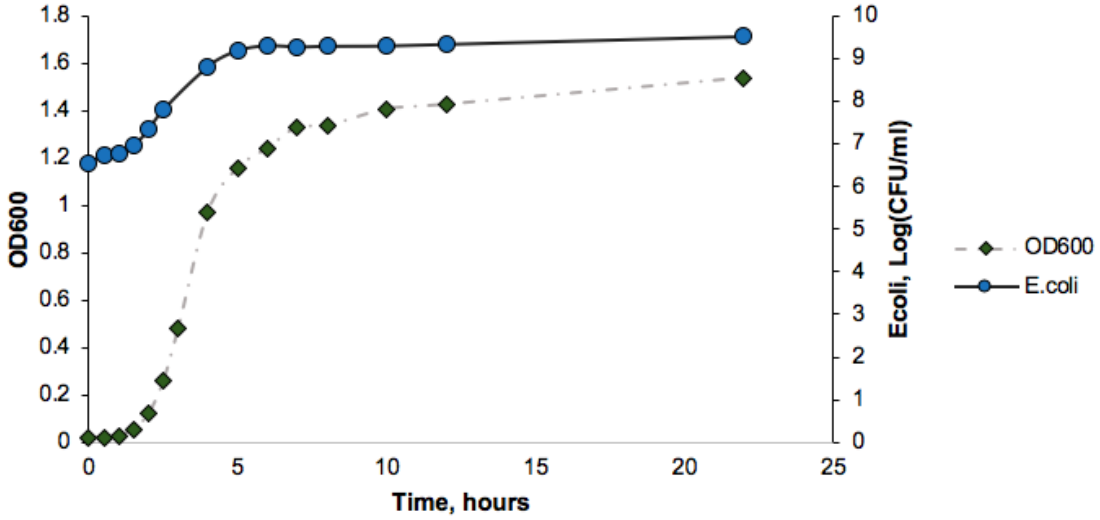

Figure 5: Relationship between *E. coli* (CFU/mL) and OD600

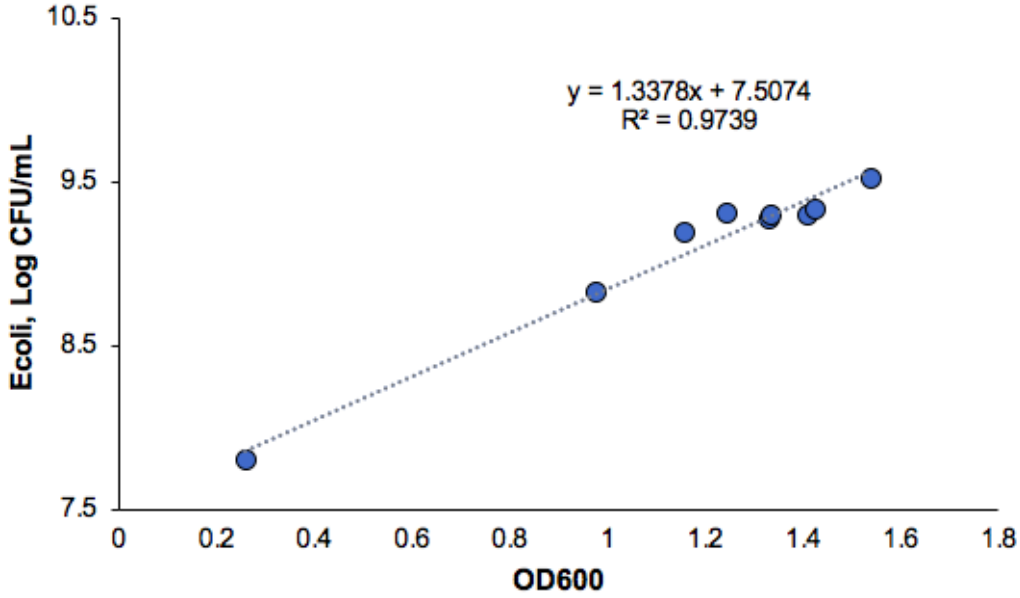

Figure 6:Linear relation between OD600 and *E. coli.* Figure shows a high correlation between the two parameters with an $R^2 > 0.9$ (Ontiveros, 2019)

To prepare samples at a desired concentration of *E. coli* cells, a known volume of laboratory prepared phosphate buffer solution (PBS) was spiked with a known volume

of the pure *E. coli* subculture. All solutions were prepared in sterile amber bottles and in a biological safety cabinet (BSC).

3.5. Preparation of Blank Solutions

Sterile deionized water (DI), used as a blank sample, was prepared by autoclaving DI water collected from a Milli-Q system (Reference A+, Millipore Corporation, MA, USA). Milli-Q water is ultrapure water purified through the process of reverse osmosis.

3.6. Water samples

All environmental samples used in the method development and validation were collected in accordance with the Standard Methods of Examination of Water and Wastewater. Water samples were collected and processed using glassware and pipette tips autoclaved at 121°C for at least 20 mins to guarantee sterility.

3.7. Method Validation

Laboratory tests were performed to validate and optimize ASTM D4012 for the MPR. Two rounds of laboratory tests were performed to verify that the MPR and the Gen 3.03 software program worked sufficiently in measuring luminescence. The MPR was equipped with two reagent injection pumps used to dispense reagents into the wells of a plate. In the first round, ATP standard samples at concentrations ranging from 0.5-10000 pg/mL were pipetted directly into a 96-well plate and each ATP concentration measured in triplicate, three different times. In addition, three ATP standard samples at three concentrations (10000, 1000, 10 pg/mL) were also measured to validate the MPR. The

validation parameters included: linearity, range, accuracy, precision, sensitivity (method detection limit (MDL)) and robustness.

A performance specification protocol for the MPR that efficiently performs luminescence measurements was used for the HTP method. All experiments for the MPR were performed at an internal temperature set-point of 25°C, a gain of 150 and an integration time of 10 s. A gain of 150 was used because a gain higher than 150 resulted in intense signals over the detector of the MPR which gave inappropriately high background readings. An integration time of 10 s was used because it achieved the maximum and most stable luminescence signal when measuring ATP. The MPR was programmed to dispense 100 μL of enzyme into a well, perform an orbital shake for 2 seconds and then read luminescence.

3.7.1. Method validation- Data Readout and Acquisition

The functionality of the MPR (Figure 6) was compared to that of the PM using environmental samples. Environmental water samples (wastewater, raw water, reservoir water and tap water) were filtered using only a syringe filter as per ASTM D4012-15. Luminescence of each filtered sample was measured using both the MPR and PM and then converted the RLU values to cATP concentrations in pg/mL. One of the reagent injection pumps was used to dispense luciferase enzyme (LuminUltra Technologies, Fredericton, Canada) into the wells of the plate. A linear regression curve was plotted to determine correlation (R^2) values between the PM and the MPR.

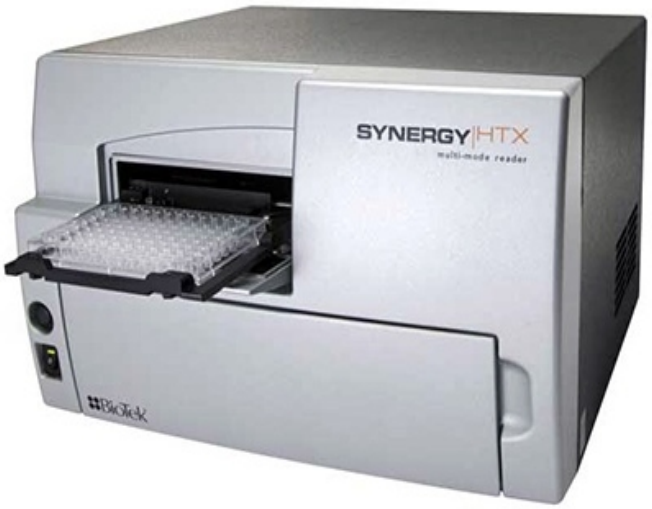

Figure 7: BioTeK Synergy H1 Hybrid Multi-Mode MPR that was used for data acquisition for the HTP method *(BioTek, n.d.)*

3.7.2. Method validation- Sample Preparation and Handling

In the second round, wastewater, raw water, reservoir water and tap water samples were syringe filtered as per the ASTM D4012 method and extracted into empty sterile assay tubes. The ATP concentration of each sample was measured with both the stand-alone PM and the MPR to compare the RLU outputs of the two instruments.

To validate the filtration step for the HTP method, the vacuum manifold was compared to the syringe filter using four environmental water samples (wastewater, raw water, reservoir water and tap water). A sample volume of 1.9 mL was filtered using each filter type. For ATP extraction, 170 µL of lysing reagent was used for the vacuum manifold and 1 mL for the syringe filter. The difference in lysing volume was accounted for when converting RLU to pg/mL Equation 6. Luminescence was measured using only the PM and then converted the RLU values to cATP concentrations in pg/mL.

$$\text{cATP}\left(\frac{\text{pg}}{\text{mL}}\right) = \frac{\text{RLU}_{\text{cATP}}}{\text{RLU}_{\text{ATP1}}} \text{ X } \frac{1000(\text{pg ATP})}{\text{V}_{\text{sample}}(\text{mL})} \text{ X V}_{\text{lysing reagent}}(\text{mL})$$

Equation 6: RLU conversion equation to ATP concentrations in pg/mL to account for difference in lysing reagent volume

3.8. Sample filtration method

A key aspect of the determination of cATP is the filtration step. To sufficiently automate the ASTM D4012 protocol for ATP determination, the filtration step must also be automated. The anodized aluminum multi-well vacuum manifold and accessories, and two multiwell filtration plates (0.45 and 1µm) were tested. Laboratory prepared *E. coli* pure culture and different environmental samples (wastewater, raw water, tap water and MilliQ water) were filtered using both the vacuum manifold and a syringe filter. The extracted ATP, filtered using both instruments, were then measured using the PM by mixing 100µL of sample with 100µL of enzyme

The multi-well vacuum manifold, attached to a pressure pump, and a 1-µm multiwell filtration plate were used to filter 1.9mL of each sample. Samples were mixed using 20-30 seconds of gentle agitation before using sterile pipette tips to transfer the samples into desired wells of the filtration plate; each well with a number ID. To maintain a constant pressure, **a sealant was used to seal the plate and a sterile knife** was used to poke holes only on the wells with samples; it was only possible to filter samples to waste with this step. The samples were filtered at a pressure of ~10 psi for 30 sec -1 min depending on the turbidity of the sample. The receiver plate of the vacuum manifold collected the filtrate to waste, (). To avoid cross contamination, a new pipette tip was used for each sample.

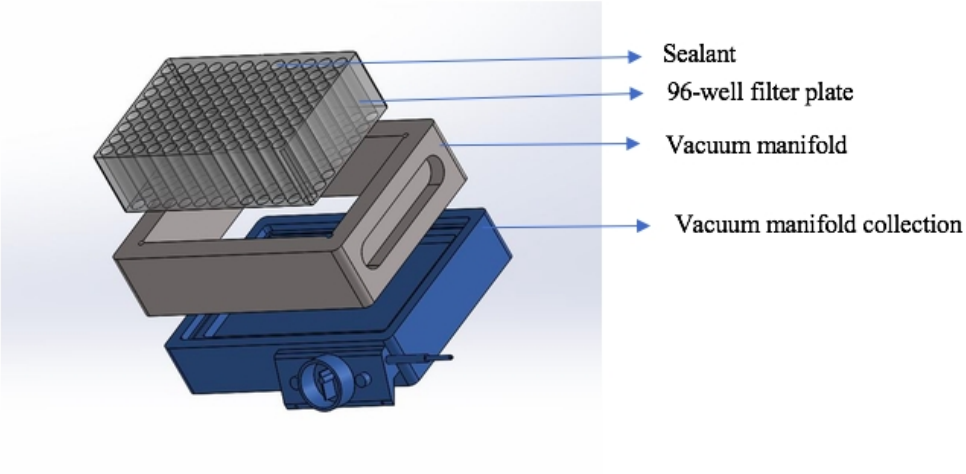

Figure 8: Equipment set-up of the vacuum manifold for sample filtration

3.9. ATP extraction method

A plate spacer block and a 96-well plate (to collect the extracted cATP) were placed into the receiver plate. The filtration plate was reattached and 120 µL of a lysing reagent was added to the wells used to filter the samples. cATP was subsequently released during lysis of the trapped microbial cells, (Figure 9).

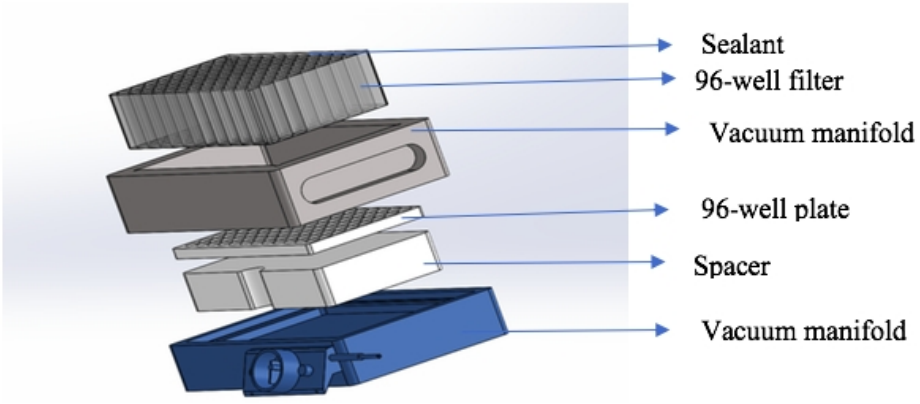

Figure 9: Equipment set-up for ATP extraction set by introducing a 96-well collection plate

The filtration plate was sealed with a new sealant and the pressure pump at ~10 psi was turned on for 40 seconds. A duration of 40 seconds was chosen to prevent foaming of the reagent, and subsequent overflow and cross contamination between wells. The extracted cATP was collected in the wells of the multiwell plate with a final volume of 100 µL; there was a holdup volume of approximately 20 µL per well. The plate was removed, and standards at concentrations of 10000, 1000, 100, and 10 pg/mL and/or calibrant (1000pg/ml) (RLU_{ATP1}) were then added to individual empty wells. The plate was incubated in the dark for 10 minutes whilst setting up the MPR for luminescence measurement (RLU_{cATP}). An incubation step was necessary because light exposure can cause delayed fluorescence which results in the emission of weak light by pre-illuminated molecules (Berden-Zrimec, Drinovec, & Zrimec, 2010).

A batch of 48 samples could be filtered at a time because the waste collection plate could only hold 100 mL of waste and most importantly, processing more than 48 samples resulted in foaming of the lysing reagent and cross contamination between wells.

3.10. Linearity

To evaluate the linearity and range of the method, ATP standard (std) curves were created using standard concentrations ranging from 0.5-10000 pg/mL. Each standard was manually pipetted directly into a 96-well plate in triplicate and the ATP concentrations were measured with the MPR by automatically injecting luciferase enzyme into each well. Each concentration was analysed under the same conditions in triplicate. Linear

regression analysis was used to evaluate the linearity of the calibration curve by using the least square linear regression method.

3.11. Minimum Detection Limit (MDL)

The MDL was determined at a target cATP standard concentration of 0.9 pg/L. Each sample was measured in triplicate and a blank sample of sterile Milli Q water was measured in triplicate after each set of three samples. The MDL was calculated with a total of 7 sets of replicates using the formula below.

$$MDL_s = t_{(n-1,\ 1-\alpha=0.99)} S_s$$

Equation 7: Equation used to compute method detection limit
Note: Retrieved from (United States Environment Protection Agency, 2016)

Where;

- MDLs = method detection limit based on spiked samples
- $t_{(n-1,\ 1-\alpha=0.99)}$ = *t*-value appropriate for a single-tailed 99th percentile *t* statistic and a standard deviation estimate with n-1 degrees of freedom.
    - For 7 replicates, $t_{(n-1,\ 1-\alpha=0.99)}$ = 2.821
- Ss= sample standard deviation of the replicate spiked sample analyses.

3.12. Accuracy and Precision

The accuracy and precision of the method were determined through a recovery studies using three different standard concentrations (10, 100 and 1000 pg/mL), analysed under the same conditions. The percent recovery (accuracy) and coefficient of variation (% CV) (precision) of each ATP standard were calculated for each of the replicate samples.

3.13. Robustness

A performance specification protocol for the microplate reader (MPR) was formulated for efficient luminescence quantification by evaluating the influence of different system parameters.

Gain. The term "gain" refers to the control of voltage across the detector of the MPR which adjusts the sensitivity of the MPR to the signal coming from the measured well (BioTek, 2015). A gain value was chosen by measuring the RLU of individual reagents, the plate only, the luciferin-luciferase reagent, and the blank (sterile ultrapure water, [Milli-Q]) at a gain of 255 (maximum gain setting for the MPR) and 150. The optimum gain for this protocol will need to be determined independently for other MPR instruments. All experiments for the microplate reader were performed at an internal temperature set-point of 25°C and a gain of 150.

Integration time. The MPR was programmed to dispense enzyme into wells, perform an orbital shake for 2 seconds and then read luminescence with an integration time of 10 seconds. Integration time was the time during which the voltage signal was held as it maximized and stabilized so it could be measured. To achieve the maximum and most stable luminescence signal, two integration times (5 and 10 seconds) were tested at four different ATP concentrations (1000, 526, 250 and 20 pg/mL). An integration time of 10 seconds was used because it achieved the most accurate and precise luminescence signal when measuring ATP. The PM also operates at an integration time of 10 seconds.

Enzyme volume. The luciferase-luciferin enzyme was needed for bioluminescence to be produced and was the costliest reagent in the ATP assay kit. To investigate if enzyme volume has an effect on ATP recovery, 100µL of enzyme was added to 100 µL of ATP standard. The experiment was repeated at a 1:2 ratio, were 50 µL of enzyme was added to 100 µL of ATP standard.

3.14. Selection of filter plate

The effect of filter plate type on ATP extraction was tested using two filter plates: a 0.45 µm GHP (universal hydrophilic polypropylene) membrane plate and a 1.0 µm GF (glass fiber) polypropylene membrane plate (AcroPrep™ 1 mL 96-Well Filter Plates, Pall Laboratory) using laboratory prepared *E. Coli* culture at a concentration of ~200 pg/mL. The *E. Coli* culture was filtered for 1 min at ~10 psi using the vacuum manifold and the cells were extracted using a 75 µl (trial 1) and 300 µl (trial 2) extraction reagent.

3.15. Method Efficiency

A time study was conducted to determine the efficiency of the HTP method by computing the time it took to prepare and process samples by using a clock timer. An environmental sample was processed with the HTP method by filtering and measuring the cATP concentration of 24 samples (trial 1) and 51 samples (trial 2), including 3 wells with standards, using the developed HTP method. Establishing the sampling time of the developed method was used to validate the high-throughput capacity of the method.

3.16. Statistical Analysis

Statistical analysis for this study was conducted using Microsoft Excel to determine correlation (R^2) values between mean readings. Log transformation plots for RLU and ATP values were used for some analysis because of highly skewed distributions with a long tail towards the higher values.

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CHAPTER 4. RESULTS AND DISCUSSION

4.1. Linearity and Range

The linearity of an analytical method is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range. The linearity study (Figure 10) showed that the relationship between ATP concentrations and RLU was linear in the specified range with $R^2 > 0.99$ for all standard curves. The results confirmed the method's sensitivity for cATP analysis within the chosen range of cATP concentrations. The range of cATP in waterworks, distribution network and tap water (stagnant) is in the range of <1 to 27 pg/mL (Vang, 2013) and the method can accurately measure cATP concentrations within 0-10000 pg/mL.

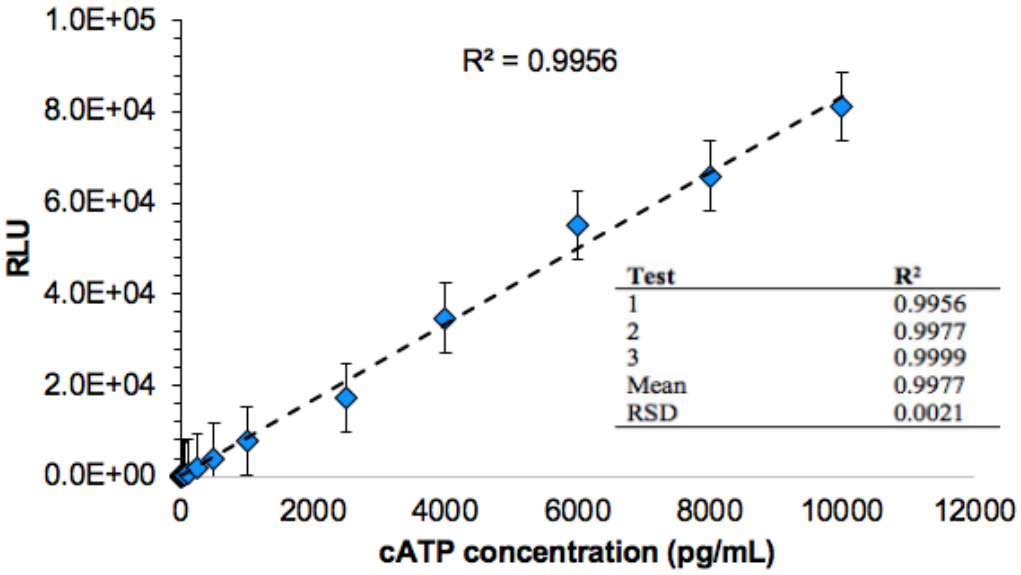

Figure 10: Linearity curve for ATP of a representative linearity check

4.2. Minimum Detection Limit (MDL)

The minimum detection limit (MDL) is the minimum concentration of a substance (greater than zero), that can be measured and reported with 99% confidence. The results showed that the method had an MDL of 1.00 pg ATP/mL for bioluminescence measurements (Table 4). This indicated that it can reliably measure samples with cATP concentrations ≥ 1 pg/mL but below this, the method will provide an unreliable estimate that could represents background noise.

Table 4: MDL Determination

Mean cATP Concentration
0.9 pg/mL
1.135
1.323
1.603
1.798
1.917
1.273
1.135
MDL = 1.00 pg/mL

4.3. Accuracy and precision

The accuracy and precision of the MPR was evaluated by measuring the pg/mL value obtained from three ATP standards, 10000, 1000 and 10 pg/mL, in triplicate. The percent recoveries were in a range of 98-102% proving that the MPR could accurately measure cATP concentrations in samples (Table 5). The %CV for all measurements was ≤ 5%, which indicated a low degree of variability between each measurement and that the MPR was precise in measuring ATP (Table 5).

Table 5: Accuracy and precision of the MPR in measuring ATP from three different standard solutions.

Added Concentration (pg/mL)	Day	Mean ATP Recovered (pg/mL)	% Recovery	Precision (%CV)
	1	10239	102	6
10000	3	10103	101	5
	1	992.5	99	2
1000	3	989.8	99	5
	1	107.3	107	1
100	3	98.1	98	1

4.4. Robustness

The robustness of the analytical method was established by analyzing the effect of different instrument conditions.

4.4.1. Effect of gain

A gain of 150 gave an acceptable background RLU range of 0-4.3, the normal range also observed with the PM. At 255, high background RLU values in the range 160-1816 were observed (Figure 11). The gain controlled the sensitivity of the MPR to the light that was emitted from the reaction between cATP and the luciferase enzyme; a high background, therefore, would subsequently increase the probability of crosstalk (cross contamination of light) between the wells and also increase the minimum detection limit of the equipment by raising the RLU values of standards. Crosstalk occurs when light signal from an already measured well contributes to the light output from an adjacent well (Wieczorek, Hooper, & Bjerke, 2019). A gain of 150 was set for all further luminescence measurements, and to further limit the occurrence of crosstalk, samples with low ATP concentrations (blanks, tap water) were not placed next to samples with high ATP concentrations (standards, wastewater).

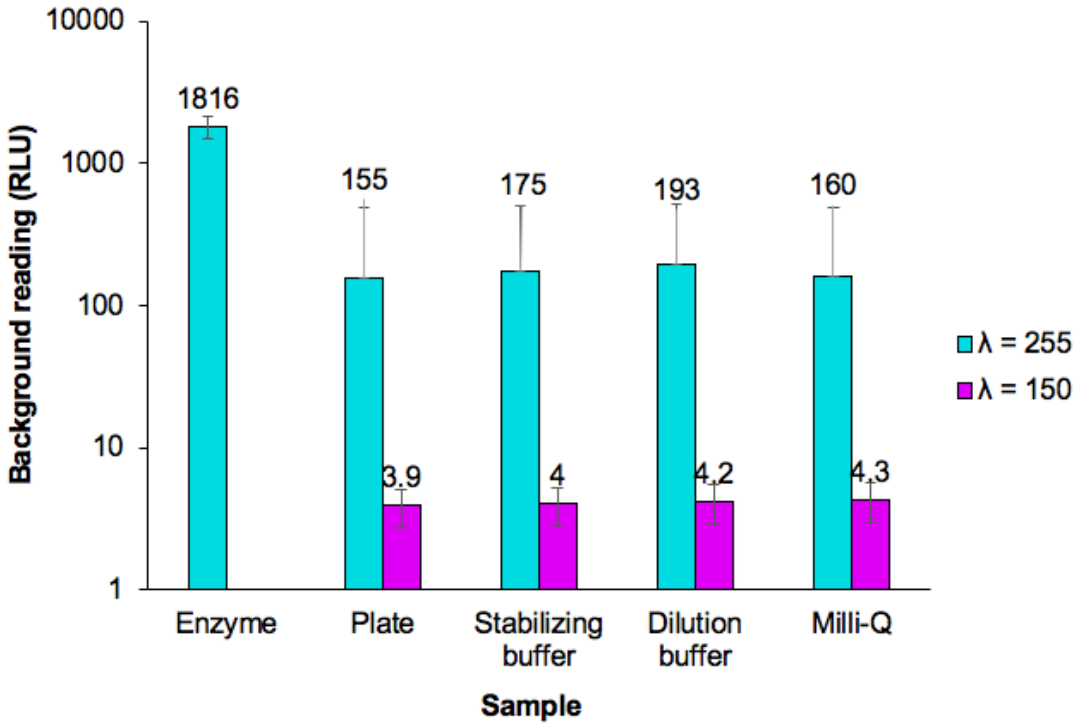

Figure 11: Selection of set gain for the microplate reader using individual reagents (luciferin-luciferase [enzyme], stabilizing buffer [LumiSolve] and extraction dilution buffer solution [UltraLute]) the plate (Plate) and a blank (Milli-Q).

4.4.2. Effect of integration time

An integration time of 10 seconds yielded ATP concentrations that were ≥ 94% of the actual concentration values (Figure 12), and a tighter coefficient of variation (%CV) (4-6%) (Table 6). A 5 seconds integration time yielded ATP recoveries 16-31% less than the actual ATP concentrations and %CVs at a range of 5-12%. The results show that luminescence signal was more accurate and more precise at an integration time of 10 seconds. The ASTM D4012-15 also stipulated an integration time setting of 10 seconds. As a result, all further experiments were conducted at an integration time of 10 seconds.

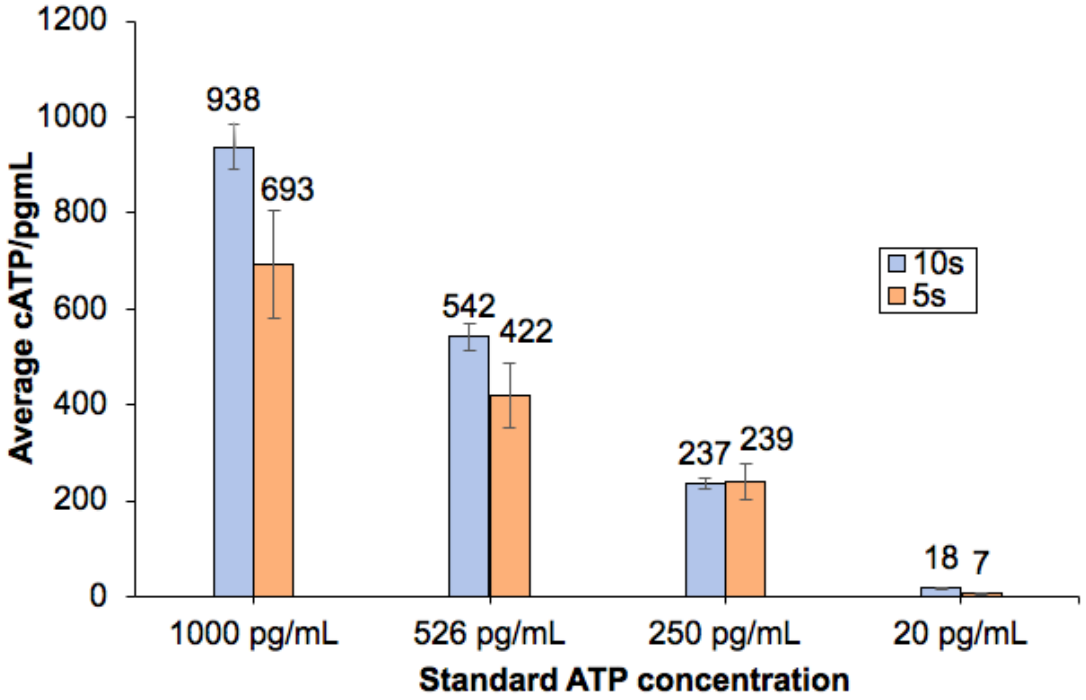

Figure 12: Effect of integration time on ATP recovery using four different standard ATP concentrations. Figure shows that light output stabilizes and reaches its maximum value at 10seconds

Table 6: Coefficient of variation of the measured ATP concentrations (triplicate measurements for each concentration) at two integration times (5 and 10 seconds)

ATP Concentration (pg/mL)	%CV at 10s	%CV at 5s
1000	4	5
526	4	12
250	6	5
20	4	11

4.4.3. Effect of enzyme volume

The rate of a chemical reaction is affected by both enzyme concentration and substrate concentration. A paired t-test resulted in a high p value of 0.99 ($p>0.05$), indicating that there was no difference between the ATP concentrations measured with 100 or 50 µL of enzyme Figure 13. The efficient use of reagents would increase

productivity and reduce costs, stretching budget further and improving efficiency simultaneously. The advantage of being able to control the gain of the MPR improves its sensitivity. It is assumed that this functionality enables the MPR to measure cATP concentrations with half the volume of enzyme.

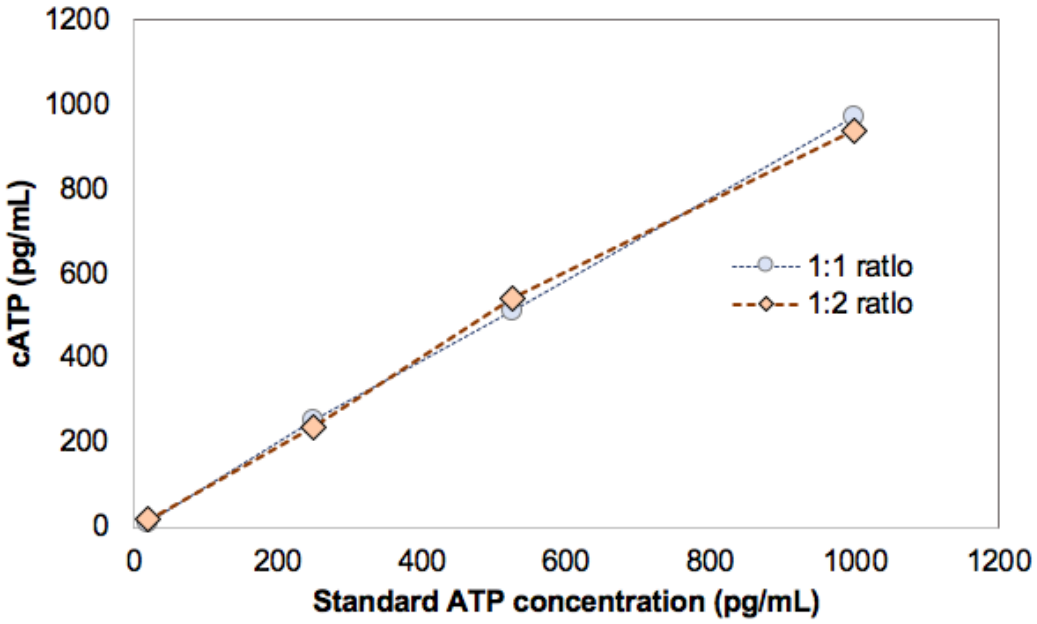

Figure 13: The concentration of ATP standard solutions incubated with Luminase enzyme in a 1:1 (100µL) or 1:2 (50 µL) ratio.

4.4.4. Selection of filter plate

Under the set experimental conditions, the 1.0 µm GF polypropylene resulted in a higher cATP recovery (Figure 14). Although the ASTM D4012 for ATP determination in all types of water samples includes a filtration process that uses a 0.7 µm pore size, the 0.45 µm GHP polypropylene 96-well plate achieved a lower recovery. It was expected that the 0.45 µm plate would have a higher recovery since *E. coli* is 1–2 µm in length and about 0.25 µm in diameter and therefore less likely to pass through the smaller pores of the 0.45 µm GF polypropylene filter. However, the 1.0 µm GF polypropylene filter resulted in a

higher cATP recovery when compared to the 0.45 µm GHP polypropylene filter (Figure 14). The increased recovery may be due to a greater binding capacity of bacterial cells to the GF polypropylene filter than the GHP polypropylene filter. Glass fiber plates are reported to show high binding capacity and are primarily intended for receptor binding assays (Millpore SiGMa, n.d.).

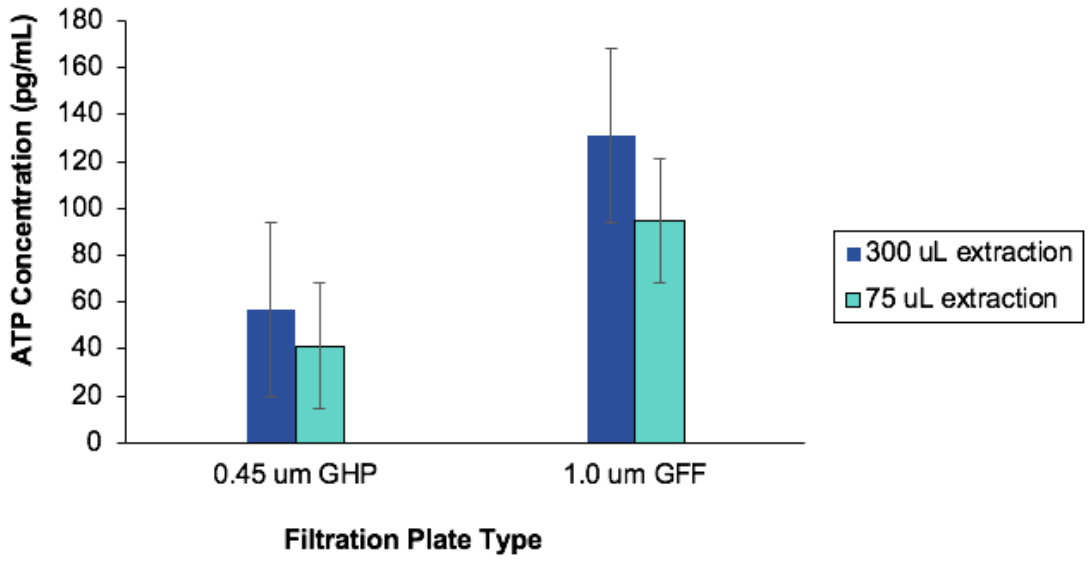

Figure 14: Recovery of ATP from a pure *E. coli* sample using a 0.45µm GHP filter and a 1µm GF filter

4.5. Method Validation: Data readout and acquisition

The results obtained from comparing the MPR to the PM show that the MPR detected a slightly higher intensity of light output (RLU) (Figure 15). It was assumed that this is due to the MPR having a greater sensitivity to light because of the set gain since the gain of the used PM is unknown. Also, the MPR was designed with broad brand filters to minimize interference (Simeonov & Davis., 2015) by using diffracting prisms to separate

light into variable wavelengths. This could be another reason for the higher ATP concentrations as opposed to the concentrations obtained with the PM.

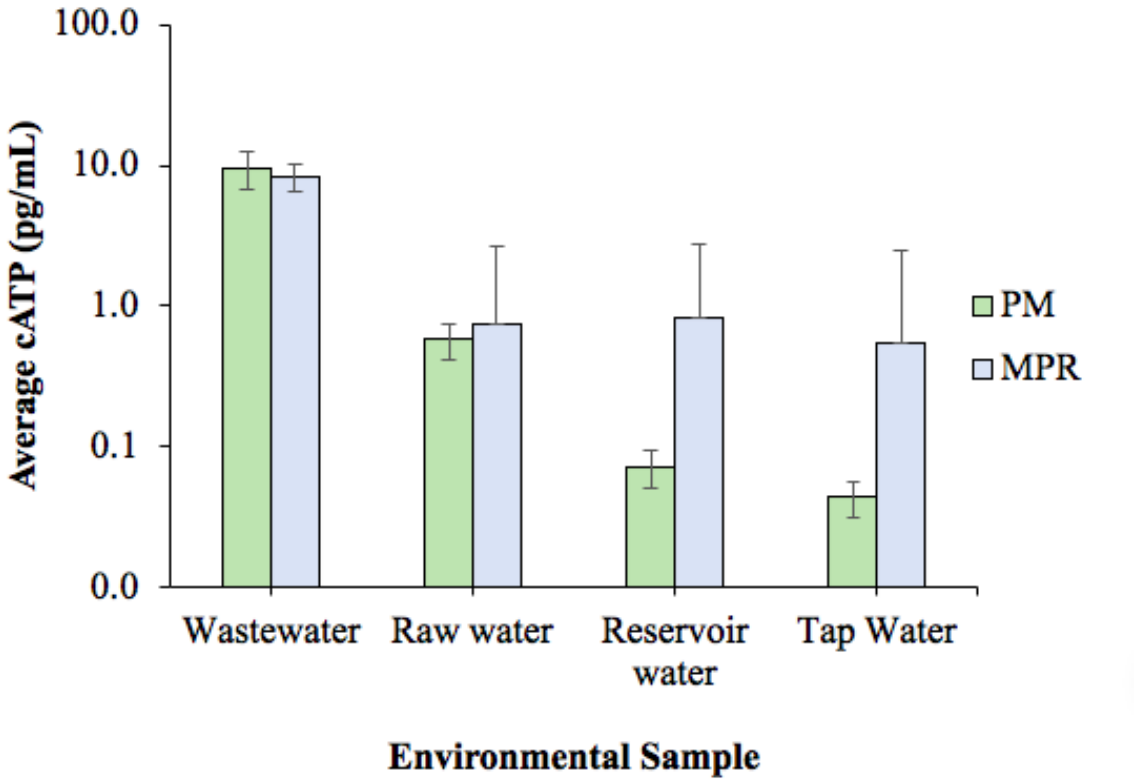

Figure 15: Comparing the microplate reader (MPR) to the PhotonMaster (PM) by measuring the cATP from different environmental samples. The samples were prepared with the syringe filter.

It is important to recognize that the cATP extracted from the samples was not diluted in buffer solution, (Figure 4, Step 2), which potentially concentrated the existing interference that resulted in artifactual activity. Omitting the buffer solution resulted in ATP values that were significantly low (Figure 15).

Artifactual activity arises when there is interference with the assay signal. The interference attenuated the excited light which reduced the RLU value reported, resulting in significantly lower cATP concentrations with both instruments. Research has shown

that approximately 5% of compounds among a 72377-member library showed luciferase interference through the inhibition of the luciferase enzyme (compounds such as indoprofen, pifithrin-R, flavonoid) or by competing with the luciferin substrate used in ATP bioluminescence assays (benzthiazole and quinoline compounds) (Auld & Inglese, 2016).

The experiment was repeated using new reagents formulated by LuminUltra Technologies to eliminate the observed interference (Figure 16). The results show expected cATP concentrations for the environmental water samples, indicating that the reagents and the omission of the buffer solution did concentrate interference.

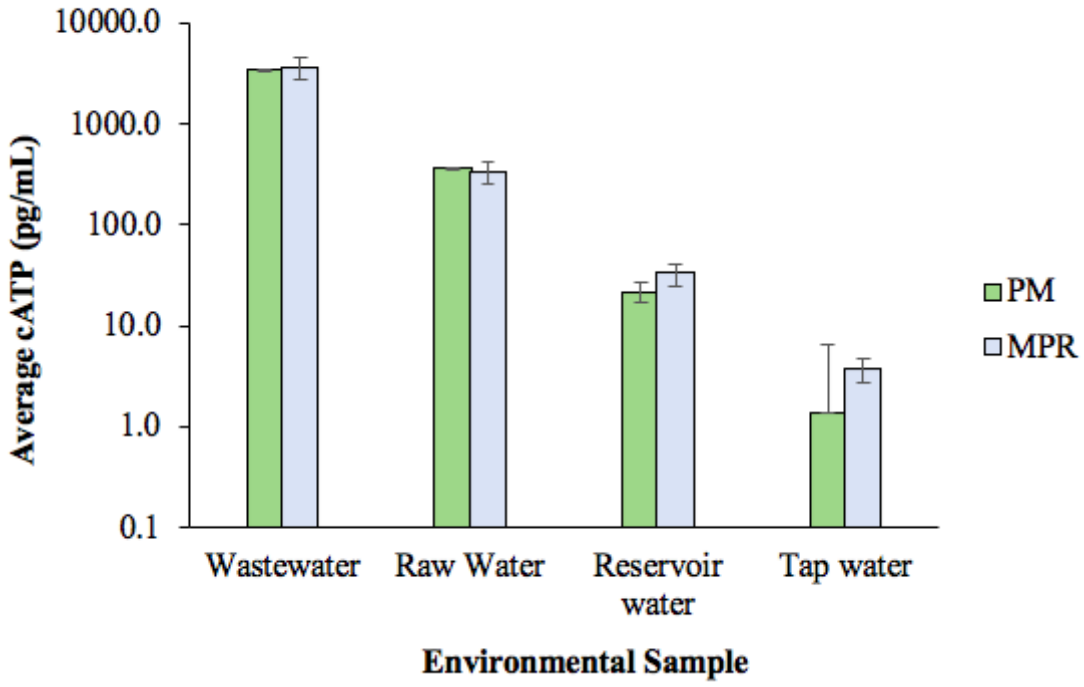

Figure 16: Comparing the microplate reader (MPR) to the stand-alone luminometer (PM) by measuring the cATP from different environmental samples prepared using the syringe filter and new reagents

A linear regression plotted for all samples that were measured using both the PM and the MPR, Figure 17, showed a strong correlation between the MPR and PM with an R^2 of 0.979. A paired t-test resulted in a high p value of 0.90 ($p>0.05$) and showed that there was no difference between the measurements of the two instruments.

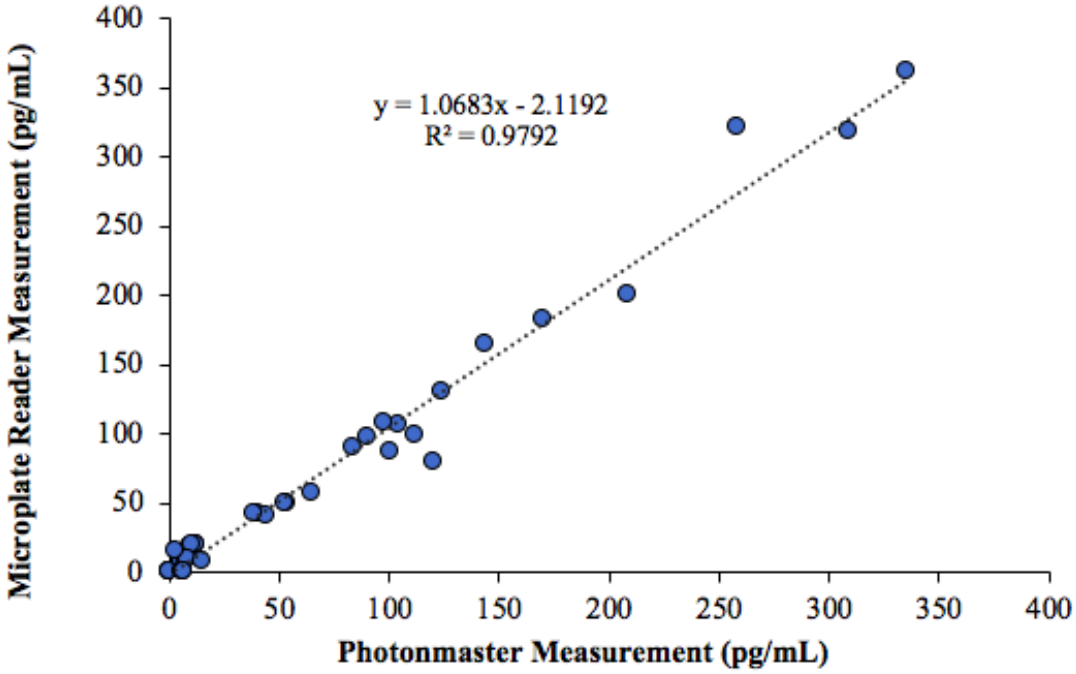

Figure 17: Linear Regression Relation Between Microplate Reader and Photonmaster. Figure shows cATP concentrations for samples using both measuring equipments (N=30).

4.6. Method Validation: Sample preparation and handling

The vacuum manifold showed the highest cATP recovery compared to the syringe filter for all water samples with an 8% difference (Figure 18). A paired t-test, p=0.54, showed that there was no significant difference between the two filtration methods. The results proved that the vacuum manifold could efficiently recover cATP in water samples.

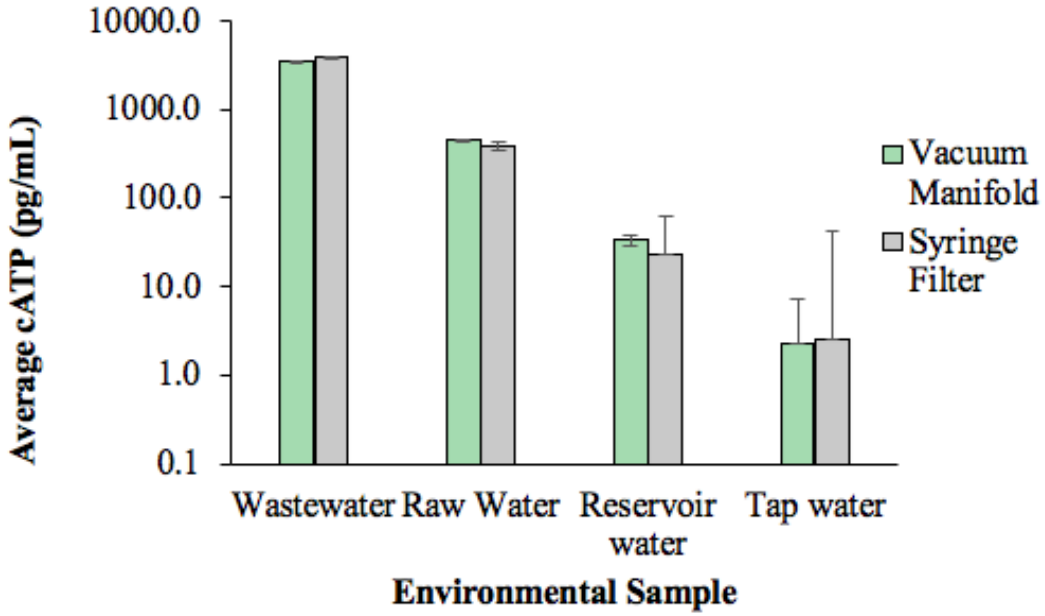

Figure 18: Comparing the vacuum manifold to the QGA syringe filter to assess the capability of the filter plate in extracting cATP. Figure shows that the vacuum manifold setup achieved a greater recovery of cATP compared to the syringe

4.7. Efficiency of HTP Method

A method is high throughput if it enables the evaluation of a high number of samples in a short amount of time. The time study, Table 7, highlighted that the HTP method would allow researchers to process multiple samples with minimal hands-on time, only during sample preparation and handling (approximately 30 seconds per sample). The second stage of the method, data readout and acquisition, was handled by the MPR and as such an operator/lab technician could focus on other work whilst the sample was being processed, saving operator time. In comparison, all the steps involved in the ASTM D4012-15 method are hands on. The results show that the HTP method could process a sample in approximately 1 min, while the ASTM method could process a sample in 5 mins.

Table 7: Efficiency of the HTP method; Evaluated by processing a sample in multiple wells on two consecutive days and also compared it to the ASTM D4012

	Trial 1	Trial 2
Number of Samples	24	51
Sample preparation and handling (minutes)	12	25
Data readout and acquisition (minutes)	8	23
Total Time (minutes)	**20**	**48**

4.8. Validity of the HTP method

The HTP method was compared to the ASTM D4012-15 that was used to develop the method. The cATP concentration of an effluent wastewater sample and a raw water sample was measured using the SSAA method (Figure 4) and also measured using the developed HTP method. Figure 19 shows that both methods were able to quantify the cATP concentrations of the samples. A paired t-test resulted in a significantly high p value of 0.91 ($p>0.05$) indicating that there was no significant difference between the two ATP test methods.

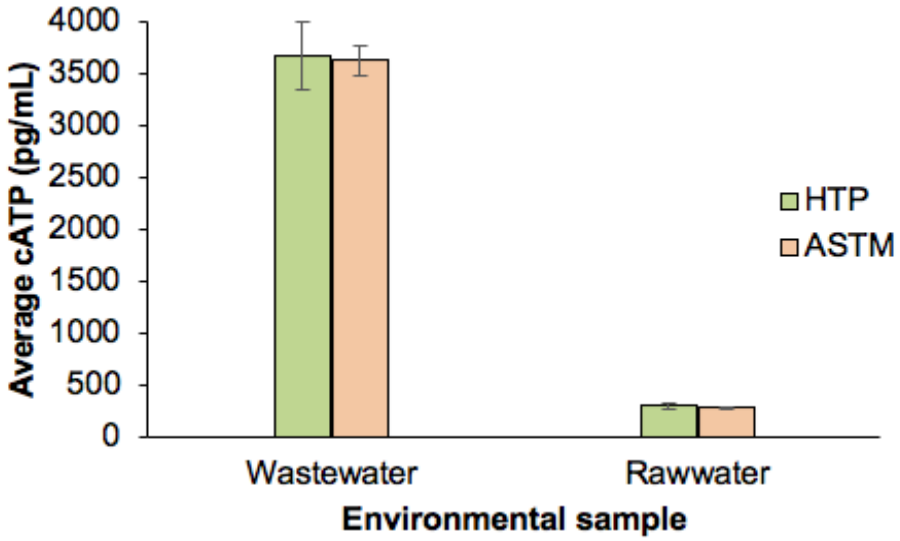

Figure 19: Accuracy of the HTP in measuring cATP concentration of a wastewater and raw water sample compared to the QGA method (N=5)

4.9. Advantages and Limitations of the HTP Method

4.9.1. Advantages

1. The HTP method is a fast method. It is possible to process a sample in approximately 1 min were only half of the processing time requires hands-on involvement and human presence (sample preparation and handling)

2. Highly accurate method that significantly reduces human error as it involves minimal manual pipetting. Results are automatically produced and can be exported to a data processing software

3. The method is reagent efficient – Samples are processed using 1/8 of the lysing reagent and by using ½ the volume of enzyme

4. Highly sensitive method with a low minimum detection limit and can measure low ATP concentrations.

4.9.2. Limitations

1. The omission of the buffer solution introduced interference. New reagents formulated to adapt to the HTP method should be used to eliminate interference.

2. Currently can only process batches of 48 samples at a time. When more than 48 samples are processed, the wells bubble and overflow which causes cross contamination among wells. New reagents formulated to reduce this effect could improve this in the future.

CHAPTER 5. AN INVESTIGATION ON THE EFFECT OF CHLORINATION ON cATP USING THE HTP METHOD

5.1. Introduction

Water from all sources are exposed to and at risk of contamination from disease-causing pathogens, which pose severe health risks. According to the World Health Organisation, all drinking water should be free from all strains of pathogenic organisms (World Health Organization (WHO), 2000). To make water safe for human consumption, the microbial level of the water, before it leaves the treatment plant, must be within limits set by health and water authorities (World Health Organization (WHO), 2006). Drinking water suppliers, therefore, disinfect water using chlorine, before reaching consumers' taps. However, as water travels through a distribution system, it runs the risk of declining in microbial water quality. As a result, a low-level amount of disinfectant, after a certain contact period after its initial application, is left remaining in the water (Environment Protection Agency , 2011). The residual disinfectant protects water as it travels through a distribution system against the risk of subsequent microbial contamination after treatment (Environment Protection Agency , 2011).

The most widely used method for disinfecting water supplies is chlorination and the chlorine dose that is added is determined by the raw water quality (concentration of pathogens in the water and turbidity), and disinfectant demand (amount needed for oxidation of reducing agents) (J.Brandt, K.Johnson, Elphinston, & Ratnayaka, 2017). Disinfection procedures are implemented to decrease the levels of pathogenic

microorganisms in the water to levels designated safe by public health standards which prevents the transmission of diseases.

5.2. Factors influencing disinfection

Disinfection efficiency is greatly affected by factors such as disinfectant concentration, contact time, temperature and pH. The two most important factors that highly influence disinfection kinetics and "the practical application of the CT concept (CT being the disinfectant concentration multiplied by the contact time)" are disinfectant concentration and contact time (World Health Organization, 2004).

5.3. CT concept and disinfection

The CT concept has been used to model the disinfection efficiency of many treatment systems (World Health Organization, 2004). CT represents the product of the concentration of a chemical disinfectant and the time at which the water is exposed to the disinfectant (West, Teska, Lineback, & Oliver, 2018). The product Ct has been normalized to microbial inactivation and widely used in regulation (Nova Scotia Environment, 2012).

In order to control microbial pathogens in water systems, the required disinfectant concentrations and exposure times to kill the pathogens have to be determined. Bench scale studies, using laboratory prepared *E. coli* samples and/or collecting wastewater samples from treatment plants are conducted before being implemented in a pilot scale

setting. The reason is, if water tests positive for e. coli bacteria, it is highly possible that it will test positive for viruses as well

5.4. Purpose of investigation

The purpose of this investigation was to establish that the developed HTP method can be used for different experiments such as, to investigate the concentration of chlorine and the required time for the disinfection of bacteria, specifically non-pathogenic *E. coli* K12 (ATCC #47076 strain MG1655; Centre for Research in Environmental Microbiology, University of Ottawa) stock.

5.5. Materials and Methods

The MPR, with two separate injection pumps, rendered it possible to use both injection pumps to inject two different reagents/liquids into the same well for the same experimental cycle. To demonstrate this functionality, a chlorination study was completed using the HTP method. Injection pump 1 was used to dispense a chlorine solution and injection pump 2 was used to inject luciferase enzyme.

A laboratory prepared E. *coli* sample, prepared as described on section 3.4, was filtered and its ATP extracted using the HTP method. The MPR was set-up to dispense 20µL of chlorine (0.4 mg/L free chlorine) into the wells that have the sample, have contact times that range from 0 -10minutes. After the set contact time, the second pump dispenses 100 µL of enzyme and then measure the light emitted in RLU. Three control wells with only

chlorine was used, and no chlorine was added to the wells at 0 seconds. The only varied condition in the first experiment was contact time.

The same experiment was repeated by increasing both the concentration of chlorine (1.7 mg/mL free chlorine) and the volume of chlorine injected, 0-50 μL. The contact time was constant at 2 seconds and only changed to 5 minutes on the last set of wells.

5.6. Results and Discussion

At a free chlorine concentration of 0.4 mg/L and contact times of 0-10 minutes, a steady increase in cATP concentration was observed. A steady increase in cATP from 2 seconds to 5 minutes was observed, after which it steeply increased (Figure 20). Normally, *E. coli* is killed when it is in contact with chlorine (Health Canada, 2019) (Zhao, Doyle, Zhao, Blake, & Wu, 2001), as a result, the concentration was expected to decrease with time. However, since extracted cATP molecules are being quantified and not the *E. coli* cells, studies have found ATP to be stable in sterile deionized water and also in chlorinated water until higher chlorine concentrations are used (Nescerecka, Juhna, & Hammes, 2011). In addition, the CT depends on the strength of the oxidation properties of the disinfectant; the stronger the oxidation properties, the less contact time required (Environmental Protection Agency, 2011).

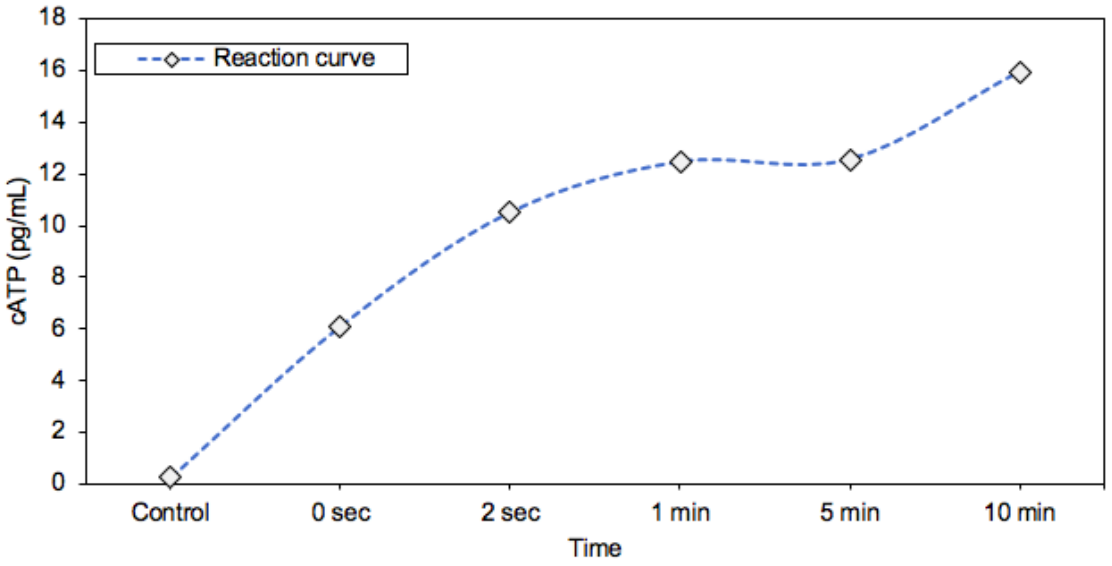

Figure 20: Effect of chlorine on ATP concentration at different contact times. The figure shows the concentration of ATP increasing with time.

A chlorine disinfection bench scale studies using *E. coli* and chlorine concentrations of 1.1 mg/L free chlorine and an exposure time of 1 min showed significant removal of pathogenic and wild strains *E. coli* 0157:H7 (Rice, Clark, & Johnson, Chlorine Inactivation of Escherichia coli O157:H7, 1999) (World Health Organization, 2004). Therefore, the experiment was repeated and a steep decrease was observed at a contact time of 5 minutes, volume of 50 µL and a free chlorine concentration of 1.7 mg/L Figure 21.

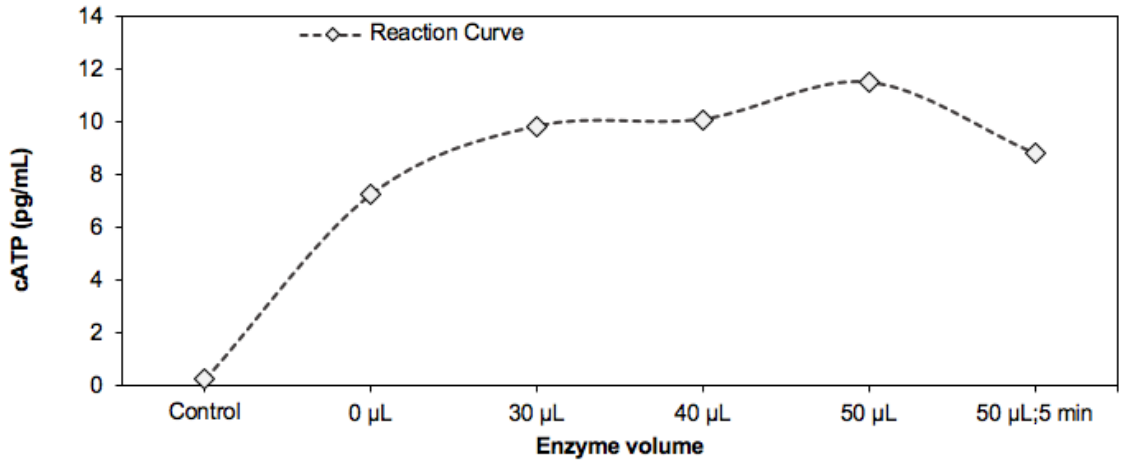

Figure 21: Effect of chlorine on ATP concentration at different chlorine volumes. The figure shows a decrease in cATP concentration at a chlorine volume of 50 µL and a contact time of 5 mins.

In both experiments (Figure 21 & 22), it is imperative to recognize that the prepared *E. coli* sample was filtered, and the trapped cells were lysed to release cATP molecules; chlorine was therefore not added to samples with bacteria cells, rather, it was added to samples with cATP molecules. An investigation that involved the addition of ATP standards to nanopure water before chlorination showed that ATP molecules were stable unless high concentrations of chlorine (> 11.2 mg-Cl^2 L^{-1}) was added (Hammes, Nescerecka, & Juhna, 2016). The results obtained in this study, Figure 21 & 22, differs from that observation; ATP molecules were not stable but increased with time.

There is evidence that damaged bactria cells can release ATP molecules with chlorine addition as a stress response (Hammes, Nescerecka, & Juhna, 2016). In this study, *E. coli* cells were separated through centrifugation and the cells were first diluted in filtered bottled water for a concentrated suspension (Hammes, Nescerecka, & Juhna, 2016). The suspension was then added to river water for a final concentration of approximately 3 x 10^6 cells mL^{-1}. Chlorine was subsequently added to the river water sample and then

incubated for 5 minutes (Hammes, Nescerecka, & Juhna, 2016). Flow cytometric total cell count and intracellular ATP measurements showed that intracellular ATP decreased considrably with chlorine dose (Hammes, Nescerecka, & Juhna, 2016). Contrarily, a dramatic increase in extracellular ATP was reported at a chlorine dose of 0.35 mg-Cl^2 L^{-1} (Hammes, Nescerecka, & Juhna, 2016), indicating that extracellular ATP was released by damaged *E. coli* cells as a stress response. The researchers observed that a five minute exposure to high concentrations of chlorine (> 0.35 mg-Cl_2 L^{-1454}) resulted in a considerable release of ATP from bacteria (Hammes, Nescerecka, & Juhna, 2016). Lower concentrations resulted only in a decrease of intracellular ATP without release of ATP (Hammes, Nescerecka, & Juhna, 2016).

While the study conducted by Hammes, Nescerecka, & Juhna, 2016 shows a link between chlorination and ATP release by *E. coli* cells (extracellular ATP), it does not explain why cATP (intracellular) molecules increased with chlorine exposure. An assumption is that, chlorine may have reacted with the lysing reagent and/or enzyme to produce a by-product that resulted in an immediate emmission of light. This increased the intensity of light (RLU) which contributed to the final cATP concentrations, Figure 21 & 22. Further studies should threfore be conducted to investigate why cATP concentration increased with chlorine addition.

The overall results of this study, however, underline the potential and success of using the developed HTP method to conduct additional experiments such as a chlorination study using different environmental samples.

CHAPTER 6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The standard test method for ATP (ASTM D4012), employed in a high-throughput setting, was successful in determining the total cATP concentration in multiple water samples simultaneously. While several conventional cATP-based methods have been developed for the quantification of cATP in water, they are all per-sample based, meaning only one sample can be measured at a time. When compared to traditional microbial methods that require a minimum of 24-hrs, the combination of a vacuum manifold multi-well plate set-up and a microplate reader enabled a rapid (1minute/sample) cATP quantification method. As an automated method, the HTP method is 5X faster than the ASTM for cATP quantification and significantly reduces human error in sample handling, data entry, and data analysis.

After evaluating the results obtained during both steps of the method development, artifactual activity was observed, which resulted in low cATP recoveries. However, after receiving modified reagents that were formulated to eliminate interference, the HTP method was found to corelate strongly to the ASTM method ($R^2 > 0.99$).

Principally, the HTP method provides superior accuracy with the ability to quantify the microbial concentration in water samples, by measuring cATP. All experiments for the MPR were therefore performed at an internal temperature set-point of 25°C, a gain of 150 and an integration time of 10 seconds. A gain of 150 was used because a gain higher than 150 resulted in intense signals over the detector of the MPR which gave extremely high

background readings. An integration time of 10s was used because it achieved the most accurate and precise luminescence signal when measuring cATP. The MPR was programmed to dispense 100 µL of enzyme into a well, perform an orbital shake for 2 seconds and then read luminescence. It was concluded that instrumentation has a large impact on the results obtained from the method. Therefore, those looking to replicate this method should determine specific settings that will achieve maximum and accurate results based on the type of equipment that is chosen.

6.2. Recommendations

This study presents the successful development of an HTP ATP method for drinking water evaluation. Despite the provision of all the conditions and steps for the experiment, it was concluded that instrumentation has a large impact on the results obtained from the method. Therefore, those looking to replicate this method should determine specific settings that will achieve maximum and accurate results based on the type of equipment that is chosen.

The work opens up opportunity for future researchers to investigate the applicability of the method for different water quality experiments that involve luminescence measurements. The method can be used for increased water quality monitoring to monitor contamination and to also determine the site of contamination.

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