Statistical Evaluation of Rapid Biochemical Oxygen Demand Test for Monitoring Municipal Wastewater Quality

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of

Master of Applied Science

in Environmental Systems Engineering

University of Regina

December 2013

Takaya Ono

© 2013: Takaya Ono

UNIVERSITY OF REGINA

FACULTY OF GRADUATE STUDIES AND RESEARCH

SUPERVISORY AND EXAMINING COMMITTEE

Takaya Ono, candidate for the degree of Master of Applied Science in Environmental Systems Engineering**,** has presented a thesis titled, ***Statistical Evaluation of Rapid Biochemical Oxygen Demand Test for Monitoring Municipal Wastewater Quality,*** in an oral examination held on December 10, 2013. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

External Examiner:	Dr. Andrei Volodin, Department of Mathematics & Statistics
Supervisor:	Dr. Dena McMartin, Environmental Systems Engineering
Co-Supervisor:	Dr. Roy Cullimore, Adjunct
Committee Member:	Dr. David deMontigny, Environmental Systems Engineering
Committee Member:	Dr. Stephanie Young, Environmental Systems Engineering
Chair of Defense:	Dr. Lisa Watson, Faculty of Business Administration

ABSTRACT

The conventional standard methods for measuring wastewater quality include the 5-day biochemical oxygen demand (BOD_5) and carbonaceous BOD ($CBOD_5$). This thesis presents an alternative wastewater quality test that requires much less time to achieve valid results. This research addresses the precision and statistical validity of an alternative methodology consisting of a two-phased testing approach: an enhanced total adenosine triphosphate (E-tATP) process and percent confirmatory bacterial reduction (%CBR) process that evaluates the aggressiveness of heterotrophic aerobic bacteria (HAB) communities as a means to determining the overall wastewater quality. Primary influent (PI) and final effluent (FE) samples from two municipal wastewater treatment plants (WWTPs) in Saskatchewan were collected between September and December of 2012 for analysis using the proposed E-tATP and %CBR techniques. Results indicated that the E-tATP and %CBR methods can be completed in between 0.25 to 28 hours, significantly less than the typical 5-day test.

The mean values of measured ATP for the E-tATP test ranged from approximately 24 000 to 79 000 pg/ml for PI, and 3 000pg/ml to 19 000 pg/ml for FE. The relative standard deviation (RSD) of the means ranged from 8.5 to 10.3% and 8.1 to 27.1%, for PI and FE, respectively. The analysis of the %CBR data also indicates that bacterial metabolic activity was directly impacted by incubation temperature (tested at 20, 22, and 28°C).

Both E-tATP and %CBR results were significantly different among the months of September, November, and December in 2012, thus indicating the test is sensitive to detect the effect from changes in climate during fall and early winter seasons on wastewater. Moreover, analysis of the E-tATP and %CBR methods demonstrated they were statistically repeatable and reliable across all 180 E-tATP and 576 %CBR tests that were performed. The relationship between average values from the 15-minute E-tATP test and average %CBR values resulted in an R^2 value of 0.898 based on exponential regression. These results indicate that a more practical approach to monitoring and managing wastewater discharge in a more timely fashion is available to wastewater regulators, managers and operators.

ACKNOWLEDGEMENTS

This research was funded and all technical assistance and materials were supplied by Droycon Bioconcepts Inc. and Luminultra Technologies Inc.

I would like to express my sincere gratitude and appreciation to Dr. Roy Cullimore, the president of Droycon Bioconcepts Inc. and adjunct Professor at the University of Regina for his guidance, profound knowledge in the field of microbiology and source of inspiration for innovation. I am profoundly grateful for Dr. Dena McMartin from the Faculty of Engineering the University of Regina for her patience and supervision on this research project. I am sincerely appreciative of Mr. Pat Whalen from Luminultra Technologies Inc. for supplying invaluable materials for the experiments for this research. This research would not have been possible without Mr. Derek Ross, Mr. Ahmed Sultan, Ms. Myrna Petri and other staff at Droycon Bioconcepts Inc. who collected samples from wastewater treatment plants and executed and maintained numerous replicates of tests on a daily basis. I also thank the staff at the City of Regina Wastewater Treatment Plant and the City of Moose Jaw Wastewater Treatment Plant for providing the samples for this experiment, without which none of this would have happened. Moreover, I sincerely thank the University of Regina for providing me with the opportunity to further pursue my academic endeavour.

DEDICATION

I dedicate this thesis to my wife, Kristina, and two sons, Joshua and Luke, for their inspiration, support, patience and understanding while I pursued this Masters. I am truly blessed to have them as my family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

NOMENCLATURE

ATP	adenosine triphosphate
BART	biological activity reaction test
BOD	biochemical oxygen demand
CBOD	carbonaceous biochemical oxygen demand
CCME	Canadian Council of Ministers of the Environment
DO	dissolved oxygen
ENH- BART™	enhanced BART
E-tATP	enhanced total adenosine triphosphate
HAB	heterotrophic aerobic bacteria
%CBR	percent confirmatory bacterial reduction
PI	primary influent
RBOD	rapid BOD
RLU	relative light unit
RSD	relative standard deviation
TE	tertiary, or final, effluent
U.S. EPA	United States Environmental Protection Agency
WWTP	wastewater treatment plant
WHO	World Health Organization

1.0 INTRODUCTION

National and provincial regulatory bodies mandate the management of wastewater effluent quality as a means for protecting public health and as well as the surrounding environment from deleterious substances that may be contained in raw wastewaters (Holeton et al., 2011). In Canada, wastewater is managed through the collaboration of federal, provincial and municipal governments. Federally, the Wastewater Systems Effluent Regulations (SOR/2012-139) under the Fisheries Act is most relevant (Environment Canada, 2012). Moreover, the Canada-Wide Strategy for the Management of Municipal Wastewater Effluent was established by the Canadian Council of Ministers of the Environment (CCME) in 2006 to further strengthen management of municipal wastewater discharge. At the provincial and municipal level, Saskatchewan's Ministry of Environment requires the discharge into Wascana Creek to contain less than 25 mg/L of the monthly average $CBOD_5$ and total suspended solids (TSS), less than 100 to 200 (averaged) per 100mL of *E. coli*, less than 1 mg/L of total phosphorus, and pH that is between 6.0 and 9.0. Moreover, the Ministry of Environment requires the Regina WWTP operators to conduct $CBOD_5$ test for five days per week to assess and monitor organic loading in the effluent (City of Regina, 2013).

Raw sewage is typically comprised of a complex and inconsistent matrix. As such, a variety of unit processes within the WWTP for treatment are required, along with a variety of water quality monitoring requirements to achieve high quality effluent for discharge (Tetreault et al., 2012). The quality of such wastewater effluents can vary among facilities and is influenced by many parameters, including choice of unit processes, seasonal variability, climate change effects, spring run-off, mechanical or treatment failure, stormwater drainage patterns, changing

population, and inclusion of industrial waste streams. Financial constraints as well as conducting regular monitoring and maintenance activities to meet both regulatory and environmental quality requirements for discharge add to the challenges of producing high quality effluent. The performance of WWTPs is assessed by a variety of water quality parameters including pH, turbidity, total suspended solids (TSS), oxygen demanding substances (measured as BOD and CBOD), and bacterial load. The BOD and CBOD methods are currently required by regulation and are considered as standard methods for the examination of wastewater (CCME, 2006; Environment Canada 2012). Because of the 5-day duration for BOD and CBOD testing, there is a significant delay in receipt of data and discharge of wastewater effluent to receiving waters, which may allow for inadequately treated wastewater to enter aqueous environments for several days prior to data confirmation. As a result of the potential for environmental contamination to occur when relying on BOD and CBOD analyses, several alternatives have been investigated. They focus on dissolved oxygen (DO) concentration, organic matter content, bacterial populations, and relationships with turbidity or total dissolved solids (TDS).

In the research presented herein, an alternative test method focuses on two aspects of bacterial community behaviour, specifically heterotrophic aerobic bacterial (HAB) associable with BOD and the level of high energy metabolic storage as adenosine triphosphate (ATP). Since HAB are essentially responsible for the degradation of organic matter, this parameter can be considered representative of the overall water quality of a water sample as it relates to organic material decomposition (Razban et al., 2012). Measurements of ATP provide indicators of the presence and activity level of microorganisms. According to Deininger et al. (2001), a correlation among ATP to bacterial cell numbers exists.

1.1 Purpose of Research

The purpose of this research is to assess the precision and effectiveness of the proposed alternative test method for wastewater quality in a rapid and precise manner, and with more ease and flexibility of use for WWTP operators.

1.2 Scope of Research

The proposed alternative test method for wastewater quality, or Rapid BOD (RBOD) method, developed by Droycon Bioconcepts Inc. and Luminultra Technologies Inc., is an integrated analytical protocol for rapidly measuring BOD surrogates. In short, the RBOD method consists of an E-tATP process which measures adenosine triphosphate (ATP) levels produced by bacteria in a novel accelerated application and, if required based on exceeding the threshold value obtained in the E-tATP process, a percent confirmatory bacterial reduction (%CBR) process is performed that measures HAB respiration and oxygen consumption in terms of a time lapse reaction .

E-tATP levels are measured using a proprietary analytical protocol developed by Droycon Bioconcepts Inc. The E-tATP protocol was designed to determine ATP concentration using relative light units (RLU) in a 15-minute test. The E-tATP converts RLU values for calculation of E-tATP levels in picograms per ml (pg/ml). In the event that the E-tATP value is greater than 20 000 pg/ml, the RBOD analytical protocol specifies that the %CBR process be required as a complementary confirmatory test.

The %CBR protocol generates time lapse (TL) data in 15 minute increments over a 28-hour period. The biological activity reaction testers (BOD-testers) used for %CBR analyses contain proprietary media for assessing bacteriological activity using colourimetric output. For this research, BOD-testers were used to assess HAB activity in primary influent (PI) wastewater

samples, that is received as new sewage by the WWTP, and final effluent (FE) wastewater samples, that is discharged to receiving waters. The testers were monitored via CBR software that provides automated data collection of reactions within the 28-hour period. The tests were performed at three different temperatures in order to assess the impact of varying incubation temperatures had on the results.

Previous research demonstrated the feasibility and accuracy of using BOD-testers to analyze the bacteriological content of wastewater effluent (Razban et al., 2012). The BOD-testers have been in use for a number of years in both laboratory and field scenarios illustrating the ability to detect different types of communities of bacteria in drinking water, wastewater, and groundwater (Cullimore, 2013). Because the RBOD testing method is intended for application in a variety of settings and water sources, the scope of this research includes analysis of BOD-tester performance under varying temperature, season, wastewater stream (PI and FE), and WWTP source.

1.3 Research Objectives

The objectives of this research are to assess and verify the performance of the RBOD integrated analytical protocol as an alternative means to determine wastewater quality using effluent discharged from two municipal WWTPs, in Moose Jaw and Regina, Saskatchewan. Specifically, the objectives include:

(1) to examine the relationship between behaviour of E-tATP and %CBR methods, specifically using E-tATP values and time lapse values from the %CBR reactions;

(2) to statistically determine the level of precision of the analytical protocol, RBOD, consisting of the E-tATP and %CBR methods;

(3) to examine the statistical significance of the data from the analytical protocol at three incubation temperatures namely 20, 22, and 28°C;

(4) to apply statistical methods to assess the rigour and reliability of the proposed analytical process for providing unique indicators of changes in wastewater effluent quality, including variables associated with climate effects in September, November, and December, 2012;

(5) to assess the statistical significance of results from two WWTPs to confirm the application of the proposed analytical protocol as providing unique indication of treatment performance; and

(6) to assess the reliability and repeatability of the complete RBOD analytical protocol as it is introduced into the commercial sector as a management tool for monitoring wastewater effluent quality. This involves a comprehensive statistical analysis of the data generated from the E-tATP and %CBR results.

2.0 LITERATURE REVIEW

No system or concept of wastewater management for urban areas existed until the latter part of the 19th century. Typically, all forms of waste were disposed directly onto public streets and pathways (Wiesmann, 2007). As such, numerous diseases and epidemics have been recorded throughout Europe prior to the 19th century (Aiello et al., 2008). Even today, there are significant public health problems in areas where there is no access to advanced industrial wastewater treatment management (Larsen, 2008). It is reasonable to state that the quality of human health may include dependencies that are affected by the condition of water resources that has depended on the management practices and procedures designed and developed by the interests of each era in history and geographic location. In a report published by the World Health Organization (WHO) in 2000, it is estimated that 2.2 million deaths mostly among children under 5 years of age and 4 billion cases of diarrhea annually attributes to poor or lack of proper wastewater management (World Health Organization [WHO], 2000). In Mexico, only 28.2% of total municipal wastewaters were treated in 2005 (de Anda, 2008).

Untreated wastewater contains both residential and industrial waste, and is a likely candidate for contaminating the environment through directly entering bodies of water or through penetrating the surface and entering ground water resources that may be subsequently used for human use. In this regard, the treatment of wastewater has become an essential part of municipal infrastructure in order to provide clean and disease-free water to the environment and subsequently for the consumption by the residents. As such, public wastewater works are required by government regulations to manage the final effluent quality of WWTPs for public health and environmental protection.

The risks and contaminants associated with untreated or under-treated wastewater are extensive and well documented. According to Metcalf & Eddy (2003), over 10,000 unrecognized organic compounds are found in various discharged wastewaters each year. Excess organic material causes bacterial overgrowth and a decrease in dissolved oxygen, required by other aquatic species to survive. Additionally, Ghafourian et al., 2004 reported that numerous pharmaceuticals and personal care products (PPCP), antibiotics and endocrine disrupting compounds (EDC) or substances (EDS) exist in the environment from wastewater effluent. For instance, an analysis of the effluent from the sewage treatment plant in Regina indicated that PPCPs are always present in the treated effluent in quantities that exceed Canadian and American water quality guidelines. Weiser et al. (2010) discovered that bacteria resistant to eryhthromycin, ampicillin, tetracycline, trimethoprim, and ciprofloxacins were found in the receiving waters of Wascana Creek even in areas that were exposed to low concentration of these antibiotics.

Excess or unwanted microorganisms caused the 2001 *Cryptosporidium* outbreak in the city of North Battleford, SK. One significant factor that contributed to the outbreak was that that the City's raw water intake for drinking water was located downstream of the wastewater discharge point. Thus, contaminants still present in the sewage outfall could still be present in the raw drinking water. Tests on human patients that fell ill with *Cryptosporidium* infection showed the source of the parasite was from human feces, and not cattle manure, thus indicating a bypass in raw sewage treatment. To prevent future outbreaks such as this, it is necessary to verify wastewater effluent quality in a timely manner. Therefore, a rapid and reliable water quality test for wastewater effluent is required for operators.

2.1 ATP

Adenosine triphosphate (ATP) is present in all forms of life, including microorganisms. Therefore, the presence of ATP can indicate the presence of microorganisms in sewage. ATP is detected when living organisms metabolize nutrients and produce energy. When a bacterial cell dies, the ATP level is quickly reduced as the level of energy metabolism subsides (Stoech et al., 2000). ATP has been a quantitative indicator of microbiological activity in biological and environmental systems as well as in food and hygienic industries (Lee et al., 2008; Luo et al., 2001). Furthermore, various rapid techniques for bacteria identification have been developed with a high level of accuracy and reproducibility using bioluminescent agents extracted from fireflies (Chapelle, 1968).

ATP tests have been developed for and used in monitoring potable water quality (Deininger et al., 2001; van der Wielen et al., 2010) and sludge produced from wastewater treatment plants (Brault et al., 2011; Dalzell et al., 2002). They operate on the same principle whereby the sample reacts with the luciferase, luciferin, magnesium and oxygen, and subsequently, light is emitted. The amount of light (i.e. the production of photons) can be correlated to the amount of ATP molecules present (Chapelle, 1968). However, a review of the literature demonstrates that there is no commonly used method involving ATP detection for monitoring wastewater quality.

2.2 Standard BOD_5 and $CBOD_5$ Tests

In 1898, the British government passed the Royal Commission of Sewage Disposal which eventually led to the selection of BOD_5 as the standard test for measuring pollution levels of organic materials in rivers (Kale and Mehrotra, 2009). The Biochemical Oxygen Demand (BOD) test measures the amount of oxygen demanded or consumed by microorganisms when they degrade and decompose the organic material present in the water as well as the oxygen

consumed through the chemical oxidation of inorganic matter. BOD_5 is the most commonly adopted technique for assessing the wastewater quality by treatment plants (Gutierrez et al., 2002). BOD_5 requires five days of incubation in the dark at 20°C as well as a significant amount of dilution, making the process more prone to operational errors and increased complexity (APHA, 1992). During the five days, the amount of oxygen demand is reduced to approximately 67%.

CBOD measures the amount of oxygen demanded for microorganisms to oxidize organic matter under aerobic conditions and is a widely used parameter to monitor the wastewater effluent for pollution or the potential for polluting the environment (Uludag-Deimirer, 2001). $CBOD_5$ tests are frequently used as substitutes for the BOD_5 in assessing wastewater effluent quality, especially when the likelihood of nitrification occurrence is high due to occasional suppression of nitrification during wastewater treatment process (Kobori et al., 2009).

The BOD_5 and $CBOD_5$ tests, despite being widely applied methodology for determining wastewater quality, still require a relatively long testing period of 5 days. During this period, poor quality effluent may be entering receiving water bodies and having adverse environmental and health effects. As such, alternative faster methodologies have been studied. However, according to a review of published literature, a limited numbers of available rapid techniques are available.

2.3 Alternatives to the BOD_5 Test

The primary alternatives to the BOD_5 test consider other aspects of microorganisms that can be measured. Respirometers measure bacterial consumption of oxygen but require significant lab space and is a lengthy technique to perform. Total organic carbon (TOC) and chemical oxygen

demand (COD) tests can determine the total amount of organic material but do not indicate the amount of biodegradable organic material present in the sample.

Chromatography and spectrophotometry are often used to determine the presence and levels of organic compounds but are not feasible for ongoing, real-time monitoring. As a result, the past two decades have seen an increase in research regarding more rapid and cost-effective techniques for assessing BOD levels and providing equivalent monitoring techniques.

Biosensors are one available technology that have the ability to detect specific substances in a sample. They are an integrated system comprised of biological components, a bioreceptor, and an electrical amplifier and transducer. Changes in the amount of proton concentrations, production and induction of gases, emissions of light, and electrical current differentials are used to determine BOD. However, a variety of microorganisms must be integrated and immobilized within the sensing media. Immobilized bacteria may react differently with various organic materials that could result in different levels of oxygen consumption. This may lead to large variability in final water quality results (Jia et al., 2003).

The use of an electron acceptor, such as ferricyanide, is another technique that was developed to produce BOD values in shorter time periods. It also measures electrical current to derive BOD values and is designed to generate BOD readings in 1 hour. However, a significantly large variability or inaccuracy could result depending on the substance that is used for incubation. (Kale et al., 2009).

Toshifumi used luminous bacterial cells on chips to develop onsite and rapid BOD monitoring system. It utilizes luminescence reduction or emission as a signal to analyze and derive BOD values. The sample must be diluted by 50 to 100 times in order for the test to function properly.

Moreover, the test process requires a high level of precision which would require skilled staff and more overhead for sufficiently performing this protocol (Sakaguchi et al, 2007).

One alternative method to determining wastewater quality effluent that is representative of BOD is measuring ATP levels of bacteria in combination with their ability to metabolize nutrients. Measuring ATP of wastewater samples to determine their current amount bacterial activity can be performed in a short time period and can accurately represent active bacterial populations. As well, BOD-testers (Droycon Bioconcepts Inc., Regina, SK) can be used to indicate the level of bacterial activity in terms of their metabolism of key nutrients. Previous research has demonstrated that there is a correlation between results from BOD-testers and BOD_5 values with R^2 value of 0.9154 at 28±1°C (Johnston, 1990; Kale and Mehrotra, 2009). Further research performed by Razban et al. (2012) indicated that BOD-testers could assess wastewater quality after different unit processes within a WWTP. The research described herein, the RBOD methodology, uses this two-pronged approach: 1) measuring ATP levels and 2) assessing metabolic activity of bacteria. These two complimentary techniques can provide a clear indication of a wastewater sample's level of active (i.e. metabolizing) bacteria in a short amount of time. This, in turn, is indicative of wastewater effluent quality. That is, higher levels of ATP and metabolic activity due to the presence of bacteria signify poorer wastewater quality that may require additional treatment prior to release into the environment.

3.0 MATERIALS AND METHODS

The methods that were being investigated are comprised of an initial test called E-tATP and a secondary confirmatory test, called %CBR. All of these tests were performed at Droycon Bioconcepts Inc. (DBI) on primary influent (PI) and final effluent (FE) samples. However, to demonstrate that the FE sample results obtained through the proposed test method were valid against industry standards, an identical set of FE samples were analyzed by Saskatchewan Research Council (Saskatoon) for select days. They independently performed the traditional $CBOD_5$ test and supplied the results.

The E-tATP method reduces testing time by 99.8%, while %CBR contributes to 76.7 to 90.8% reduction in required testing time relative to the $CBOD_5$ test. As of December 2013, the quoted price for testing $CBOD_5$ by the SRC is $60, where as the prices by DBI are $32 and $35 for E-tATP and %CBR, respectively. In addition to cost savings in terms of time, the proposed methodology could potentially reduce the financial cost by up to 55%. The rationale for the cost saving is based on the high probability that currently compliant WWTPs would only require the initial E-tATP tests for routine FE monitoring, thereby significantly reducing the need to conduct the %CBR method as a confirmatory test (Cullimore, 2013).

3.1 E-tATP Methodology

The enhanced total adenosine triphosphate (E-tATP) process was developed by Droycon Bioconcepts Inc. The method commences with a calibration of the luminase. The calibration measures the relative light unit (RLU) value that is generated only by the luminase when it is mixed with a calibrating solution, UltraCheck. The calibration RLU is used to calculate the actual E-tATP. In the calibration phase, 100uL of luminase is mixed with 100uL of UltraCheck

in a 12x55mm polypropylene culture tube. The tube is immediately placed into the Kikkoman C-100 lumitester which, in ten seconds, generates a calibration RLU reading, RLU_{UCL}. In the next step, a 15ml wastewater sample is added into the enhanced ENH-BART tester (Droycon Bioconcepts Inc., Regina, SK) and is agitated through six inversions over a one minute period performed at room temperature (22^{o}C). A 1 ml midpoint sample is taken from the tester to be used for total ATP measurement. The process to measure the ATP of this enhanced sample utilizes one 17 x 100mm extraction tube with 1mL of UltraLyse7, and a second 17 x 100 mm tube with 9mL of UltraLute. An 12 x 55mm assay tube is also dispensed with 100uL of the luminase. The 1 ml midpoint sample from ENH-BART tester is added to the extraction tube with UltraLyse7. The tube is shaken to mix the solutions. After a five minute waiting period, 1 ml midpoint sample is taken from the tube, and added to and mixed in the second extraction tube which contains UltraLute. A 100 uL of the mixture is collected from the second extraction tube and added to the assay tube which contains the lunimase. Immediately, the assay tube is placed onto the Kikkoman C-100 lumitester which generates the RLU value for the agitated sample, RLU_s. The data is derived as relative light units (RLU). The calibration data and test sample data are converted into E-tATP using the standard equation (Cullimore, 2013):

$$\text{E-tATP} = (RLU_s/RLU_{UCL}) \times 20\,000 \text{ (pg/ml)}.$$

Specifically for final effluent (FE) samples, if the results are greater than 20 000 pg/ml, then a confirmatory method (%CBR) is conducted immediately following the E-tATP method on the same sample. As the assay tubes and ENH-BART contain active cultured bacterial samples, the proper disposal methodology was provided in order to ensure the safety of the users and environment (Cullimore, 2013).

3.2 Percent Confirmatory Bacterial Reduction (%CBR) method

The percent confirmatory bacterial reduction (%CBR) test was developed by Droycon Bioconcepts Inc. and is used following the E-tAPT method if the results indicate it is required. %CBR utilizes the visual BART reader (VBR) system which analyzes up to 18 BOD-testers (Figure 1) at once and records jpeg images of the testers every fifteen minutes (produced by Droycon Bioconcepts Inc., Regina, SK). The VBR system can include a temperature-controlled environment for the BOD-testers so that the bacteria metabolize at a pre-determined temperature. The BOD-tester requires 15ml of wastewater sample, and utilizes proprietary media and a reduction-oxidation indicator to determine the timing in which the oxygen is removed by bacterial respiratory processes in the sample (Cullimore, D.R. and Alford, G.A. 1990).

The BOD-tester uses methylene blue dye as an oxidation-reduction indicator and measures the time lapse for the oxidative condition, which is indicated by blue color, to turn into a reductive condition, which is indicated by a clear to pale yellow color of the sample (Cullimore, 2006). This change in color represents the bacterial metabolic activity and is indicative of the original bacterial concentration in the sample. The jpeg images taken at 15-minute intervals are analyzed by confirmatory bacterial reduction (CBR) software (Figure 2) to calculate the time lapse value for each oxidative to reduction reaction in each individual BOD-tester based on the color change (Cullimore, 2013). After calibrating the software, it can detect when sufficient color change has occurred to signify the completion of a positive test. As such, the %CBR process correlates the time lapse for a bacteriological reaction to take place within a BOD-tester to the activity level of microorganism in the sample; shorter time lapses represent higher bacterial activity levels and longer time lapses indicate less bacterial activity. One of the research goals was to determine the impact of varying incubation temperatures had on the time lapse and subsequent level of

bacterial activity. The three chosen temperatures were 20, 22, and 28°C. The latter temperature (i.e. 28°C) is a common incubation temperature for bacteria (Chifiriuc et al., 2007; Nguyen, 2006). However, it has been seen that bacteria can be enumerated at lower temperatures when given a longer incubation period (Reasoner and Geldreich, 1985). Thus, the ability to incubate at or near room temperature is advantageous as it would allow WWTP operators to test the quality of wastewater without an incubator. Hence, this research examined the results from a typical incubation temperature as well as two lower temperatures so that this methodology could be applied in a cost-effective manner whereby expensive laboratory equipment, such as an incubator, would not be required. In order to ensure the safety and reduce contamination risks, the disposal protocol of BOD-testers should be followed (Cullimore, 2013).

Figure 1. VBR II including an advanced temperature controlled unit.

Figure 2. Operating screen for the CBR system showing the software controls on the right hand side and the BOD-testers under observation on the left hand side.

3.3 Wastewater treatment plants overview

The samples used in this research were collected from the primary treatment cell (primary influent, PI), and the final discharge (final effluent, FE) from the Cities of Regina and Moose Jaw Waste Water Treatment Plants (WWTPs).

The Regina Waste Water Treatment Plant begins with the primary treatment cell, where wastewater is then discharged to six secondary lagoons. The oxidative conditions of these lagoons allow organic compounds to degrade for a period of 30 days after which it is discharged to the tertiary treatment process. In this stage, phosphorous, algae, and other suspended materials that have not been eliminated are removed, and wastewater is mixed with an anionic polymer and alum. The wastewater then remains static while the settlement of sludge takes place. Subsequently, the clarified water is treated by ultraviolet disinfection prior to discharge into the Wascana Creek receiving water body as final effluent (FE). As of 2006, 194 971 people are served by the Regina WWTP (Statistics Canada, 2006) with an average flow rate of 74 x 106 L collected per day (City of Regina, 2004).

The WWTP for the city of Moose Jaw is the first plant in Western Canada to incorporate the Biolac Wave Oxidation technology in 2010 for removing nutrients. After 25 years of utilizing an aerated lagoon system, the Biolac Wave Oxidation process was adopted with a specific aim to reduce BOD and nitrogen from the wastewater, as well as to address the adverse fluctuations in the wastewater due to industrial wastewater from meat and pork packing plants. The Moose Jaw WWTP underwent a $26 million upgrade between October 2007 and early 2010 to adopt the Biolac Wave Oxidation process, replacing an aerated lagoon system which had been utilized by the plan for the previous 25 years. The upgraded process has been effective in reducing ammonia

nitrogen levels, and the clarified effluent is treated by UV prior to being discharged to the Moose Jaw River as final effluent (FE).

The WWTP model used in this study is described in Figure 3, and defines the PI as the wastewater coming out of pumping stations into both the Moose Jaw and Regina WWTPs, and FE as the discharged effluent into the receiving waters.

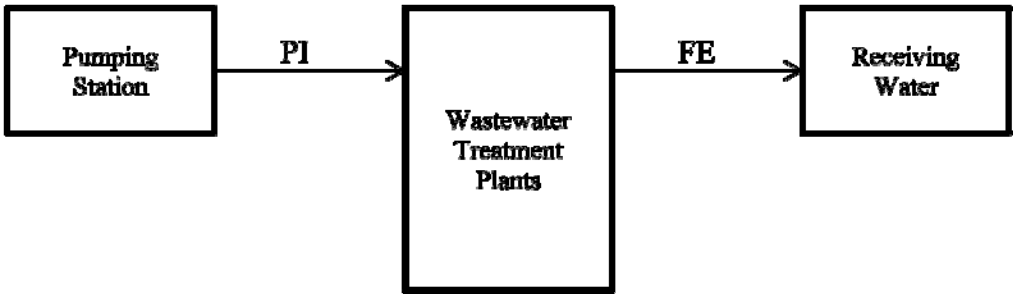

Figure 3. WWTP model used in this research.

3.4 Sample Management

All samples were collected at 8:00 AM on the day of testing by the staff of DBI, from the PI and FE stages of the WWTP. Samples were collected on four separate testing days and each sample had a volume of 20 L, which was divided into aliquots at the lab for testing purposes. Prior to dividing each 20 L sample, it was well mixed to ensure that the bacteria, nutrients and other contaminants would be evenly distributed among the aliquots. From the City of Moose Jaw, the PI and FE sample was taken on Sept. 19, 2012, and from the City of Regina, samples were collected on Sept. 12, 2012, Nov. 7, 2012, and Dec. 4, 2012. The samples were brought back to the lab directly from the WWTP for immediate testing. On the first two test days (i.e. Sept. 12 and Sept. 19), an identical set of FE samples were couriered to the Saskatchewan Research Council (SRC, Saskatoon, SK) laboratory for their independent analysis of the $CBOD_5$ test. These results proved to be sufficient for comparison purposes, and due to high testing costs, this

was not performed for the third and fourth testing days (i.e. Nov. 7 and Dec. 4). A description of the samples collected on each of the four testing days from the two WWTPs, as well as the tests performed for each sample is described below, and is summarized in Table 1.

3.4.1 Moose Jaw WWTP – Sept. 19, 2012

The PI and FE samples were collected from the Moose Jaw WWTP on one day only. The 20 L samples of PI and of FE were each divided into aliquots after being thoroughly mixed. The PI sample was divided into 108 aliquots to be tested as follows: 27 E-tATP tests at 22°C, 27 %CBR tests at 20°C, 27 %CBR tests at 22°C and 27 %CBR tests at 28°C. The FE sample was divided into 135 aliquots. The same tests were performed on the FE samples as those performed on the PI samples, with the exception of the additional test performed by SRC on 27 FE samples to determine the results of the $CBOD_5$ test. In total, 243 tests were performed on PI and FE samples collected from the Moose Jaw WWTP.

3.4.2 Regina WWTP – Sept. 12, 2012

The first day of testing PI and FE samples from the Regina WWTP included an identical set of tests to those performed on Sept. 19 for the Moose Jaw WWTP samples. The PI sample was divided into 108 aliquots with 27 tests performed using each of: E-tATP tests at 22°C, %CBR tests at 20°C, %CBR tests at 22°C and %CBR tests at 28°C. The FE sample was divided into 135 aliquots with 27 of the aliquots sent to SRC for the $CBOD_5$ test. The remaining 108 aliquots were tested using each of: E-tATP tests at 22°C, %CBR tests at 20°C, %CBR tests at 22°C and %CBR tests at 28°C. Again, the total number of tests was 243.

3.4.3 Regina WWTP – Nov. 7, 2012

The second day of testing samples from the Regina WWTP did not include any $CBOD_5$ tests performed by SRC. Rather, there were 108 aliquots of PI and another 108 aliquots of FE, where 27 aliquots each (for both PI and FE) were tested as follows: E-tATP tests at 22°C, %CBR tests

at 20°C, %CBR tests at 22°C and %CBR tests at 28°C. In this case, 216 tests in total were performed in this batch of testing.

3.4.4 Regina WWTP – Dec. 4, 2012

Finally, on the third day of testing samples from Regina WWTP included a reduced amount of aliquots compared to the previous days. Again, no samples were sent to SRC for $CBOD_5$ tests. The PI and FE parent samples were each divided into 54 aliquots. For both PI and FE aliquots, 9 E-tATP tests were performed at 22°C, 18 %CBR tests were performed at 20°C, 9 %CBR tests were performed at 22°C, and 18 %CBR tests were performed at 28°C. In total, 108 experiments were performed on December PI and FE samples.

Table 1. A summary of samples collected from WWTPs and number of E-tATP and %CBR tests conducted on respective samples.

Type	Plant Location	Date	Num. of E-tATP tests (T_1)	Num. of %CBR tests (T_2)	Num. of %CBR Tests (T_1)	Num. of %CBR tests (T_3)
PI	Moose Jaw	09-19-12	27	27	27	27
FE*	Moose Jaw	09-19-12	27	27	27	27
PI	Regina	09-12-12	27	27	27	27
FE*	Regina	09-12-12	27	27	27	27
PI	Regina	11-07-12	27	27	27	27
FE	Regina	11-07-12	27	27	27	27
PI	Regina	12-04-12	9	18	9	18
FE	Regina	12-04-12	9	18	9	18

Note: * 27 $CBOD_5$ tests were performed at SRC. T_1 = 22°C, T_2 = 20°C, and T_3 = 28°C

3.5 Statistical methods

The correlation between the E-tATP and %CBR methods was determined from the PI and FE samples collected from the Regina WWTP since samples were collected on three dates, namely Sept. 12, Nov. 7, and Dec. 4. This was due to a large number of data points available for determining a statistical relationship. For each data set, mean values were used for comparison.

The average values were then plotted and the coefficient of determination was used as the indicator for the level of correlation using the exponential regression function provided by Microsoft Excel Professional Plus 2007. Unless otherwise indicated, Graphpad Prism 6 for Windows version 6.01 was used for all statistical analysis in this research.

The precision of the integrated analytical method, RBOD, which consists of the E-tATP and %CBR methods, was measured using relative standard deviation ratios to compare the relative precision of data groups. For assessing the accuracy of the E-tATP method, the relative standard deviations were calculated for each test, which consisted of 27 replicates. The exception was Dec. 4 which consisted of 9 only replicates each. Additionally, 95% confidence intervals were calculated and plotted the E-tATP method.

A similar approach was applied to assessing the precision of the %CBR method. There were 27 replicates of the BOD-tester, which houses the 15ml sample and is placed in the incubator at the three different temperatures as part of the %CBR method, with the exception of Dec. 4 where fewer aliquots were prepared. The relative standard deviation of each BOD-tester test at 20, 22, and 28°C, was determined. Also, the 95% confidence intervals were used to assess the accuracy of each test in order to determine the general accuracy level of the %CBR method.

The statistical significance of the E-tATP and %CBR methods was assessed with respect to varying temperature, seasons, and WWTP operations. This was done through performing parametric and non-parametric versions of analysis of variance (ANOVA) tests. Parametric tests are more statistically more powerful than their non-parametric counterparts. However, in order to use parametric tests, the data must meet certain requirements. This is determined from additional

statistical tests described below. Specifically, the data within a given data set must be normally distributed and the variances among the data sets must be homogeneous.

Each data set, which was comprised of a set of replicates for any one given test, was tested for normal distribution using D'Agostino-Pearson test. The critical value of 0.05, and the null hypothesis that a data set has a normal distribution were used. As such, if the calculated p value was less than 0.05, the null hypothesis was rejected. The statistical significance of the homogeneity of variances between two or more data groups were analyzed using Bartlett's test. This was conducted to determine whether or not the variances among the data groups are homogeneous with a critical value of 0.05 and null hypothesis that variances are homogeneous among the data sets. If the p values calculated were smaller then 0.05, the null hypothesis was rejected. If the data sets met the above two requirements through their calculated p values, the ANOVA test was used. If not, the non-parametric version of ANOVA, Kruskall-Wallis test was conducted.

The tests for statistical significances among selected data groups were conducted in order to assess the effects of varying temperatures, seasons and WWTP treatment processes. The resulting rationale is that if there are statistically significant differences among data sets taken at varying temperatures, at different months, and from different WWTPs, it would infer that the results in each scenario provide a unique indicator of the quality of wastewater for each particular temperature, month of the year, or WWTP.

The %CBR methodology was conducted at three different temperatures, 20, 22, and 28°C. If the results from the three different temperatures were statistically different from each other, it implies that temperature is one of the parameters that influence the outcome of the %CBR

process. If this is indeed true, which would be demonstrated through ANOVA results, then the %CBR methodology could be optimized for rapidness, cost and precision. The statistical significance tests using ANOVA have been utilized to evaluate the effect of temperature in various applications, such as evaluating temperature effects on biological reactions (Yasuda et al., 2002), production of biohydrogen (Saeed et al., 2012), and polymerization of resin-composites (Price et al., 2011).

The effects of seasonal variation and dynamics can be assessed by evaluating statistical significances through ANOVA methods. The testing months, comprised of September, November and December, have varying outdoor temperatures and precipitation in Saskatchewan, as well as differing anthropological activities that can affect wastewater content. This, in turn, can affect the level of bacterial activities in WWTP PI and FE samples. As such, E-tATP and %CBR testing methods must be able to detect these differences. ANOVA-related tests have been utilized to assess the seasonal effects on growth and population of microorganisms, such as Epischura baicalensis Sars (Ermakov, 2011), algal biomass and assemblage composition (Yang et al., 2009), and seasonal effects on biological parameters in dairy cows (Casella et al., 2012).

Statistical tests were used to determine if there were any significant differences between the performances of the Regina and Moose Jaw WWTPs. If there were significant differences among the data sets from the two WWTPs, it could be inferred that the differences are not by chance, but due to unique characteristics or treatment processes occurring at the WWTP that affects the biological quality of the effluent. For example, ANOVA methods have been used to examine the effects of two different composting bio-reactors with varying characteristics on variables, such as oxygen concentration and moisture content, as well as overall process performance of each reactor (Schloss et al., 2000).

The repeatability of the E-tATP and %CBR methodologies was assessed by either the either parametric unpaired t-test or the non-parametric Mann-Whitney test. As required by the statistical protocol, each data group was randomly split into two sets to determine if there was any statistical significant difference between the two subsets. If there was no statistically significant difference, then it suggests that the data represent reproducible results, confirming the repeatability of the test methodology. The t-tests have been applied for assessing repeatability of processes and methodologies, such as digital panoramic radiographs in the dental industry (Alkurt et al., 2007).

4.0 RESULTS

4.1 E-tATP Results

The values for mean, standard deviation, and relative standard deviation are tabulated for E-tATP (Table 2). The mean values for E-tATP ranged from 24 094 pg/ml to78 694 pg/ml for PI, and 2 932 pg/ml to 32 420 pg/ml for FE. The relative standard deviation of the means ranged from 8.5% to 10.3% for PI and 8.1% to 27.1% for FE. E-tATP values with 95% confidence intervals presented in Figure 4. The mean E-tATP values for FE samples were below 20 000 pg/ml, except for the FE sample from Regina WWTP collected on Sept. 12. It was subsequently determined that the Regina WWTP had problems in their treatment train over this time period, which caused increased levels of contamination to reach the final effluent. It is significant to note that the test methods presented in this research identified this problem at the WWTP, which they confirmed.

Table 2. E-tATP mean, standard deviation, relative std. dev. and selected $CBOD_5$ values.

Type	Plant Location	Date	Num. of Samples	Mean (pg/ml)	Std. dev.	Relative Std. dev.	Mean $CBOD_5$ (mg/L) by SRC
PI	Moose Jaw	09-19-12	27	24 094	2 058	8.5%	
FE	Moose Jaw	09-19-12	27	2 932	795.6	27.1%	3.517
PI	Regina	09-12-12	27	33 449	3 079	9.2%	
FE	Regina	09-12-12	27	32 420	2 633	8.1%	7.074
PI	Regina	11-07-12	27	60 225	5 541	9.2%	
FE	Regina	11-07-12	27	14 758	1 451	9.8%	
PI	Regina	12-04-12	9	78 694	8 113	10.3%	
FE	Regina	12-04-12	9	18 865	3 023	16%	

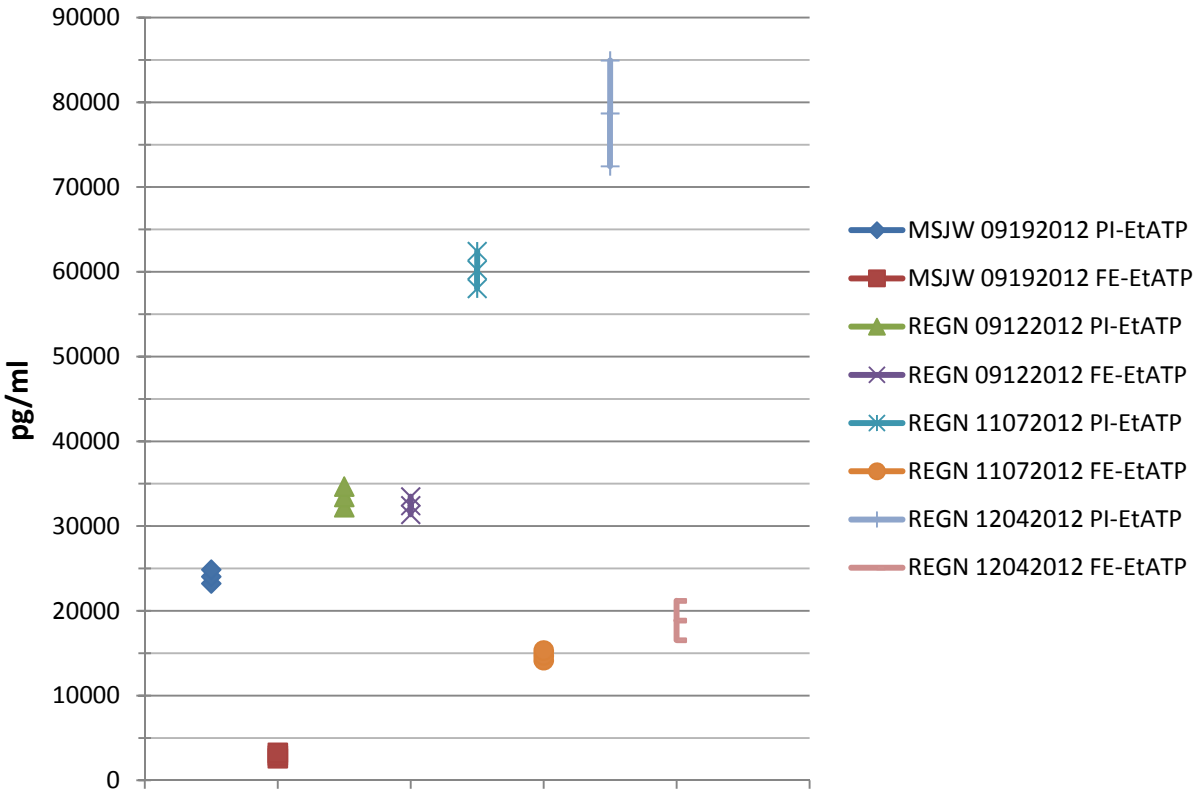

Figure 4. The 95% confidence interval for E-tATP test results, for corresponding dates. REGN: Regina WWTP; MSJW: Moose Jaw WWTP.

4.2 %CBR Results

The %CBR time lapse values represent the time required for microbiological metabolic reactions to be completed in the BOD-testers. Time lapse values are inversely correlated to the activity levels of the HAB community and are thus indicative of the quality of water. That is, longer time lapses indicate lower concentrations of bacteria, which in turn indicate a higher quality of water compared to those containing higher levels of microorganisms.

The mean values of time lapse for PI samples ranged from 6.102 to 9.491 hours and the relative standard deviations (RSD) ranged from 3.3% to 7.1% for the Moose Jaw WWTP. For PI samples from the Regina WWTP, the mean and RSD values ranged from 2.567 to 9.065 hours, and 3.1% to 16.0% respectively, (Table 3). The mean and RSD values for FE samples collected from the Moose Jaw WWTP ranged from 10.88 to 17.94 hours, and 3.3% to 7.1% respectively; where as in the Regina WWTP, they ranged from 13.22 to 25.29 hours, and 1.6% to 15.5%, respectively (Table 4). The 95% confidence intervals were calculated for time lapse data obtained from the %CBR method with FE samples at 20, 22, and 28°C, shown in Figure 5, for the Regina and Moose Jaw WWTPs based on the date of the tests.

Table 3. Mean, standard deviations, relative standard deviation of the time lapse values of PI samples from two municipal WWTPs at various dates and temperatures for %CBR method.

Type	Plant Location	Date	Num. of Samples	temp. (°C)	Mean (hour)	Std. dev.	Relative Std. dev.
PI	Moose Jaw	09-19-12	27	20	8.806	0.622	7.1%
PI	Moose Jaw	09-19-12	27	22	9.491	0.430	4.5%
PI	Moose Jaw	09-19-12	27	28	6.102	0.199	3.3%
PI	Regina	09-12-12	27	20	7.971	1.277	16.0%
PI	Regina	09-12-12	27	22	9.065	0.916	10.1%
PI	Regina	09-12-12	27	28	4.944	0.641	13.0%
PI	Regina	11-07-12	27	20	3.192	0.465	14.6%
PI	Regina	11-07-12	27	22	3.491	0.407	11.7%
PI	Regina	11-07-12	27	28	2.567	0.219	8.5%
PI	Regina	12-04-12	18	20	4.528	0.256	5.6%
PI	Regina	12-04-12	9	22	5.861	0.253	4.3%
PI	Regina	12-04-12	18	28	3.444	0.107	3.1%

Table 4. Mean, standard deviations, relative standard deviation of the time lapse values of FE samples from two municipal WWTPs at various dates and temperatures for %CBR method.

Type	Plant location	Date	Num. of Samples	temp. (°C)	Mean (hour)	Std. dev.	Relative Std. dev.
FE	Moose Jaw	09-19-12	27	20	17.43	1.170	6.7%
FE	Moose Jaw	09-19-12	27	22	17.94	0.305	1.7%
FE	Moose Jaw	09-19-12	27	28	10.88	0.272	2.5%
FE	Regina	09-12-12	27	20	21.57	1.174	5.4%
FE	Regina	09-12-12	27	22	22.47	0.467	2.1%
FE	Regina	09-12-12	27	28	13.22	0.429	3.2%
FE	Regina	11-07-12	27	20	24.35	3.785	15.5%
FE	Regina	11-07-12	27	22	25.29	3.089	12.2%
FE	Regina	11-07-12	27	28	15.3	2.15	14.1%
FE	Regina	12-04-12	18	20	20.6	0.772	3.7%
FE	Regina	12-04-12	9	22	22.08	0.354	1.6%
FE	Regina	12-04-12	18	28	13.9	0.348	2.5%

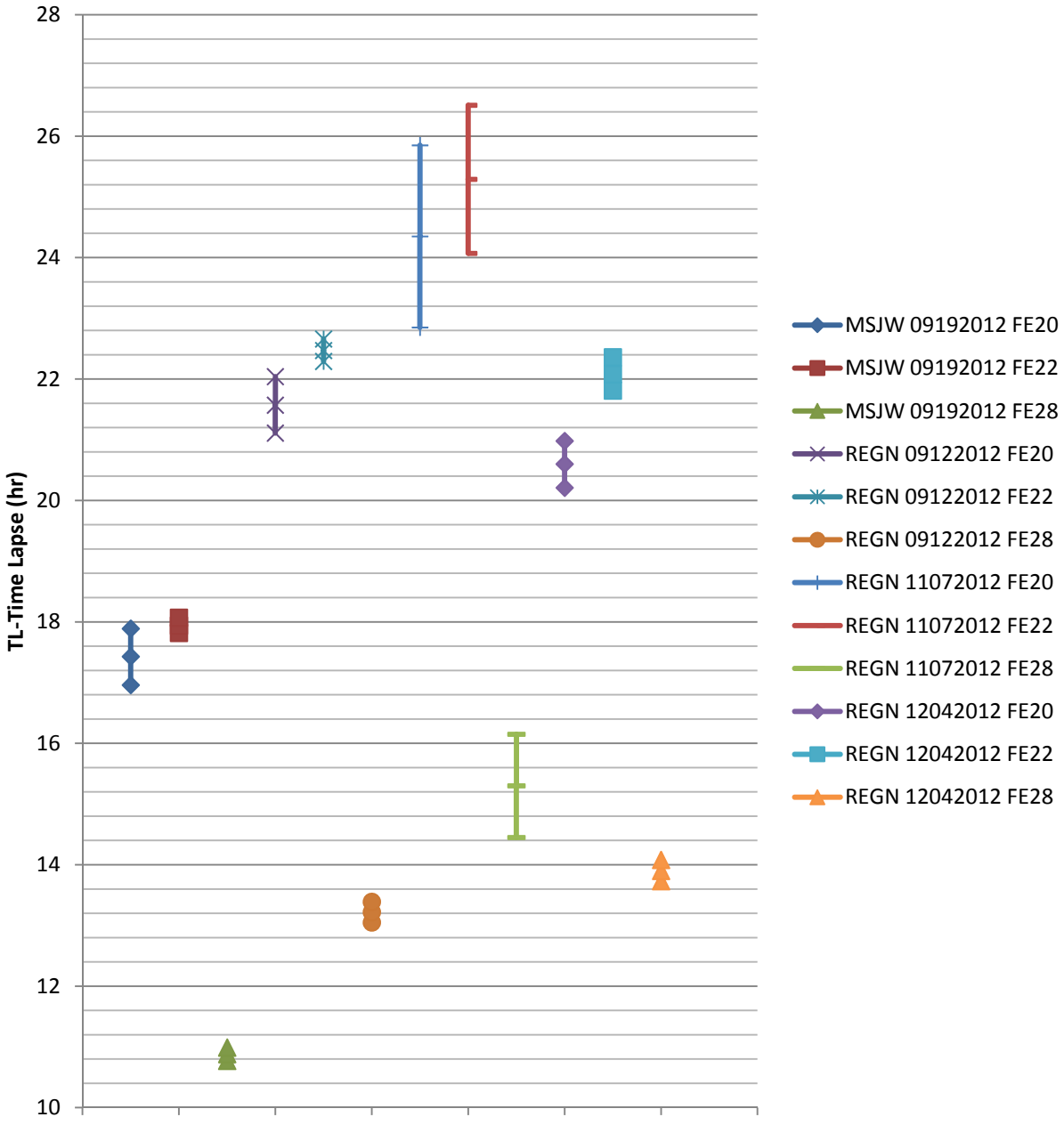

Figure 5. The 95% confidence interval of time lapse values for both WWTPs.

The FE samples collected in September from each of the Moose Jaw and Regina WWTPs on one date were sent to an independent research lab to determine $CBOD_5$ levels. The results are presented in Table 5, which includes the mean TL, E-tATP and $CBOD_5$ values for these FE samples. It is interesting to note that the lower $CBOD_5$ value measured by SRC corresponds to the lower TL and E-tATP numbers generated in this research. Likewise, the higher $CBOD_5$ value corresponds to the higher TL and E-tATP data. This initial correlation demonstrates the ability of the proposed methodologies to detect differing levels of water quality, as indicated by the $CBOD_5$ values. Additional $CBOD_5$ values were not measured for subsequent dates.

Table 5. Mean time lapse, E-tATP, and $CBOD_5$ values of FE samples from Moose Jaw and Regina WWTPs collected in September 2012.

Type	Plant Location	date	Mean time-lapse (hr)	Mean E-tATP (pg/ml)	Mean $CBOD_5$ (mg/L)
FE	Moose Jaw	09-19-12	17.94	2 932	3.519
FE	Regina	09-12-12	22.47	32 420.06	7.074

Statistical analyses were performed on the data to determine the effect, if any, the varying incubation temperature, seasonal changes and unique treatment processes specific to each WWTP had on the water quality level. In order to perform these statistical tests, the data had to be tested against certain parameters, to determine whether parametric or non-parametric statistical tests could be applied. Specifically, the data must have a normal distribution and their variances must be homogeneous among compared groups in order to be considered for parametric analysis. The following statistical analysis was conducted on 24 data groups of time lapse values from the %CBR test results on PI and FE samples from each WWTP.

The normality of the data groups was determined by using the D'Agostino-Pearson normality test with a critical value of 0.05. According to the D'Agostino-Pearson test, if the calculated p value is less than the critical value (0.05), then the null hypothesis that the particular data set is normally distributed is rejected. According to Table 6, five data groups were identified as demonstrating non-normal distributions. These groups were:

- Sept. 19 Moose Jaw WWTP FE sample at 20°C (p=0.0096)
- Sept. 12 Regina WWTP FE at 20°C (p=0.0174),
- Nov. 7 Regina WWTP FE sample at 20°C (p=0.0112),
- Nov. 7 Regina WWTP FE sample at 22°C (p=0.0248), and
- Dec. 4 Regina WWTP PI sample at 28°C (p=0.0358).

Table 6. D'Agostino-Pearson omnibus normality test of %CBR reaction time lapse values of PI and FE samples from two municipal WWTPs at various dates and temperatures.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p
PI	Moose Jaw	09-19-12	27	20	0.2357
FE	Moose Jaw	09-19-12	27	20	0.0096
PI	Moose Jaw	09-19-12	27	22	0.0644
FE	Moose Jaw	09-19-12	27	22	0.3709
PI	Moose Jaw	09-19-12	27	28	0.9787
FE	Moose Jaw	09-19-12	27	28	0.0707
PI	Regina	09-12-12	27	20	0.0931
FE	Regina	09-12-12	27	20	0.0174
PI	Regina	09-12-12	27	22	0.2496
FE	Regina	09-12-12	27	22	0.3537
PI	Regina	09-12-12	27	28	0.5318
FE	Regina	09-12-12	27	28	0.1062
PI	Regina	11-07-12	27	20	0.4407
FE	Regina	11-07-12	27	20	0.0112
PI	Regina	11-07-12	27	22	0.5116
FE	Regina	11-07-12	27	22	0.0248
PI	Regina	11-07-12	27	28	0.3034
FE	Regina	11-07-12	27	28	0.1807
PI	Regina	12-04-12	18	20	0.8759
FE	Regina	12-04-12	18	20	0.4916
PI	Regina	12-04-12	9	22	0.8356
FE	Regina	12-04-12	9	22	0.6301
PI	Regina	12-04-12	18	28	0.0358
FE	Regina	12-04-12	18	28	0.0877

Bartlett's test was conducted on the data sets to determine if variances among compared data groups do not significantly differ from each other. This step is critical for further analysis of variance studies. In Bartlett's test, if the p value calculated is smaller than the critical value of 0.05, then it indicates that variances among the data sets are not homogeneous. Hence, the null hypothesis that variances are equal among data groups is rejected. Subsequently, ANOVA tests

were performed on data that met the prerequisite parametric criteria, and the Kruskal-Wallis tests were applied to data that are non-parametric.

4.3 Effects of varying the incubation temperature on %CBR method for FE samples

The incubation temperature effects on the %CBR time lapse values for final effluent were investigated using either the one-way ANOVA or the Kruskal-Wallis test, based on whether or not the data groups represented normal distributions and homogeneous variances. The results are presented in Table 7. Noting the differences due to incubating at different temperatures allows for the future optimization of the testing protocol to use room temperatures, thereby reducing the overhead cost of the test.

Data generated from the Moose Jaw WWTP on Sept. 19 did not meet the required parameters for ANOVA analysis. The FE sample results at 20°C generated an alpha value smaller than the critical value (p=0.05) from the D'Agostino-Pearson test, and Bartlett's test indicated that variances among the data groups for different temperatures were not homogeneous. Hence, the non-parametric Kruskal-Wallis test was performed. This test assessed whether or not there were statistically significant differences among data groups based on the three different incubation temperatures of 20, 22, and 28°C. The results produced small p values (i.e. less than 0.0001). Thus, $p < 0.05$ indicated that there was a statistically significant difference among these groups. This demonstrates that this test protocol can clearly identify and produce distinctive results at different temperatures. Thus, the protocol can be optimized based on temperature; elevated temperature levels are not mandatory for the detection of bacteria metabolism of wastewater contaminants.

Table 7. Statistically significant test results of time lapse values of FE samples from two municipal WWTPs for varying incubation temperatures for %CBR method.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p value D'Agostino -Pearson	p value Bartlett's test	p value Kruskal -Wallis
FE	Moose Jaw	09-19-12	27	20	0.0096		
FE	Moose Jaw	09-19-12	27	22	0.3709		
FE	Moose Jaw	09-19-12	27	28	0.0707		
						< 0.0001	<0.0001
FE	Regina	09-12-12	27	20	0.0174		
FE	Regina	09-12-12	27	22	0.3537		
FE	Regina	09-12-12	27	28	0.1062		
						< 0.0001	<0.0001
FE	Regina	11-07-12	27	20	0.0112		
FE	Regina	11-07-12	27	22	0.0248		
FE	Regina	11-07-12	27	28	0.1807		
						0.0204	< 0.0001
FE	Regina	12-04-12	18	20	0.4916		
FE	Regina	12-04-12	9	22	0.6301		
FE	Regina	12-04-12	18	28	0.0877		
						0.0022	< 0.0001

Similar tests were performed on FE samples obtained from the Regina WWTP on Sept. 12. Again, the non-parametric Kruskal-Wallis test was used due to the data group at 20°C was identified as having a non-normal distribution through the D'Agostino-Pearson test, and Bartlett's test revealed a lack of homogeneous variances among data groups at 20, 22, and 28°C. The Kruskal-Wallis test resulted in $p < 0.05$, thus indicating, again, that there are statistically significant differences in time lapse values between the different data groups based on temperature. Not only did the testing protocol uniquely identify this, but this result was consistent with that obtained from the previous FE data set from the Moose Jaw WWTP.

The same statistical analysis procedure was applied for the two remaining test dates where FE samples were analyzed from the Regina WWTP (for Nov. 7 and Dec. 4). In both sets of analyses, the Kruskal-Wallis test was required based on the results from the D'Agostino-Pearson test and Bartlett's test for homogeneity of variances. In both cases, there were statistically significant differences among the three incubation temperatures as indicated by the measured differences in time lapse values for the %CBR method. Overall, incubation temperature had a statistically significant impact on time lapse readings, thus demonstrating both the ability to successfully use lower incubation temperatures to generate data related to wastewater quality and to identify this difference repeatedly, among four different instances, from two different wastewater streams.

4.4 Effects of varying climates on E-tATP and %CBR methods on FE samples

The results from E-tATP tests on FE samples collected in September, November, and December from the Regina WWTP were analyzed to determine if there were any statistically significant differences based on the month of sample collection. In Saskatchewan, outdoor temperatures change significantly from the autumn season in September to the winter season of December. These temperatures can range from over +20°C down to less than -20°C. Based on results from the Kruskal-Wallis test which examined E-tATP values of microbial ATP levels, the calculated p values were less than the critical value of 0.05 (Table 8). As such, the null hypothesis, which states that the mean values are the same among these data groups, was rejected. This indicates that there are statistically significant differences among these data sets, and that varying seasons, as represented by a different calendar month, have significant effects on the microbial activity levels of wastewater effluent. These data can indicate whether the proposed testing methodology for wastewater quality is adequately sensitive to determine if outdoor temperature levels affect

biological degradation of contaminants. In the future, detecting changes in wastewater quality due to climate change will become increasingly significant.

Table 8. Statistically significant test results, based on Kruskal-Wallis p values, of E-tATP values of FE samples from Regina WWTP collected in September, November, and December, 2012.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p D'Agostino &Pearson	p Bartlett's test	p Kruskal -Wallis
FE	Regina	09-12-12	27	22	0.572		
FE	Regina	11-07-12	27	22	0.902		
FE	Regina	12-04-12	9	22	0.445		
						< 0.0001	< 0.0001

A similar statistical procedure was applied to %CBR reaction time lapse values to assess the effects of varying seasons using the %CBR methodology. In each instance, the Kruskal-Wallis test was used due to the groups of data failing at least one of the required parameters (i.e. normality or homogeneous variances among data sets) for ANOVA tests. The Kruskal-Wallis test was applied to Regina WWTP FE samples from September, November, and December at each temperature, i.e. 20, 22, and 28°C. The results, shown in Table 9, reveal that for each of the three incubation temperatures, the calculated p value was less than 0.0001. Because $p < 0.05$, there exists a statistically significant difference in time lapse values generated by the %CBR method among September, November, and December. This indicates that the wastewater quality testing protocol described in this research can detect differences in contamination levels of wastewater due to climate changes.

Table 9. Statistically significant test results of time lapse values of FE samples from Regina WWTP collected in different seasons and incubated at different temperatures for %CBR method.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p value D'Agostino &Pearson	p value Bartlett's test	p value Kruskal -Wallis
FE	Regina	09-12-12	27	20	0.0174		
FE	Regina	11-07-12	27	20	0.0112		
FE	Regina	12-04-12	18	20	0.4916		
						< 0.0001	< 0.0001
FE	Regina	09-12-12	27	22	0.3537		
FE	Regina	11-07-12	27	22	0.0248		
FE	Regina	12-04-12	9	22	0.6301		
						< 0.0001	< 0.0001
FE	Regina	09-12-12	27	28	0.1062		
FE	Regina	11-07-12	27	28	0.1807		
FE	Regina	12-04-12	18	28	0.0877		
						< 0.0001	< 0.0001

4.5 Effects of WWTP processes on E-tATP and %CBR methods on FE samples

There are many treatment options (unit processes) available to engineers when they design wastewater treatment plants. Each unit process has its own advantages and disadvantages, where cost and treatment effectiveness are generally two of the most important factors. The contamination levels that the methodology described in this research is limited to biological-related contaminants that could potentially be released into the environment, including bacteria and organic debris that may serve as a nutrient source for organisms. Thus, it would be expected that different WWTPs would have different levels of removal for biological contaminants based on their operational processes. This was investigated through comparing the E-tATP levels in FE samples between the two WWTPs studied in this research. Samples were collected from Moose

Jaw only during the month of September, hence the analysis to compare the two WWTPs is limited to only September samples.

The non-parametric Mann-Whitney test was conducted on the E-tATP results from the FE samples collected from the Moose Jaw and Regina WWTPs in September, 2012. This test was used due to the data failing one of the required parametric pre-tests of normality or homogeneous variances among data sets. The calculated p value was less than the critical value of 0.05, as shown in Table 10. As such, the null hypothesis that the mean values between these two data sets are equal was rejected. This indicates that there are statistically significant differences in E-tATP values from the two different WWTPs.

Table 10. Statistically significant test results of E-tATP values for FE samples from two different municipal WWTPs.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p D'Agostino &Pearson	p Bartlett's test	p Mann-Whitney
FE	Moose Jaw	09-19-12	27	22	0.001		
FE	Regina	09-12-12	27	22	0.572		
						< 0.0001	< 0.0001

Further statistical analysis was conducted to assess the impact of different WWTP processes on %CBR reaction time lapse values. Either the parametric unpaired t-test or the non-parametric Mann-Whitney test was conducted using a pair of data groups from each WWTP for September at each incubation temperature of 20, 22, and 28°C.

At 20°C, comparisons between the Moose Jaw WWTP and Regina WWTP FE samples collected in September required the use of the non-parametric Mann-Whitney test because the data did not meet the normal distribution requirement at this temperature. The Mann-Whitney test generated

a very small p that is less than 0.0001 (Table 11), leading to the conclusion that there are statistically significant differences between data groups between these WWTPs when incubated at 20°C.

Table 11. Statistically significant test results for time lapse values of FE samples from two different municipal WWTPs for %CBR method for varying incubation temperatures.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p value D'Agostino -Pearson	p value Mann -Whitney	p value Unpaired t-test
FE	Moose Jaw	09-19-12	27	20	0.0096		
FE	Regina	09-12-12	27	20	0.0174		
						< 0.0001	
FE	Moose Jaw	09-19-12	27	22	0.3709		
FE	Regina	09-12-12	27	22	0.3537		
							< 0.0001
FE	Moose Jaw	09-19-12	27	28	0.0707		
FE	Regina	09-12-12	27	28	0.1062		
							< 0.0001

The FE samples that were incubated at 22°C and at 28°C for both WWTPs met the parametric requirements. As such, the unpaired T-test was used to measure the differences, if any, between time lapse values for the two WWTPs. At both incubation temperatures, the generated p value was less than 0.0001. This indicates that there are statistically significant differences between these data groups, that is, the final effluent of the two plants have differing levels of active bacteria, as would be expected due to the different unit processes used in each plant.

4.6 Repeatability of E-tATP and %CBR methods

All data groups were each randomly sorted and divided into two sets, in order to conduct parametric or non-parametric t-tests, depending on whether or not each divided data set followed

a normal distribution, based on the D'Agostino-Pearson normality test. When the calculated p values of normality test were smaller than the critical value of 0.05 for both groups, parametric unpaired t-tests were applied. Otherwise, the non-parametric Mann-Whitney test was used. P values that exceeded the critical value of 0.05 for both the unpaired t-test and the Mann-Whitney indicate that the null hypothesis is supported. That is, the means of the two data groups are equal and thus, there are no significant differences among two data sets. Without any statistical difference within any given data set, it can be concluded that the generated data were produced in a repeatable manner, i.e. the methodology consistently produces reproducible results. As shown in Tables 12 and 13, all sets of data, based on WWTP location and incubation temperature, exhibited no significant differences among randomly divided data sets. Hence, it can be concluded that all E-tATP and %CBR tests follow a statistically repeatable methodology.

Table 12. Statistical significance tests of each E-tATP results.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p D'Agostino -Pearson	p Mann -Whitney	p Unpaired t-test
PIa	Moose Jaw	09-19-12	13	22	0.5470		
PIb	Moose Jaw	09-19-12	14	22	0.3947		
							0.5056
FEa	Moose Jaw	09-19-12	13	22	0.2704		
FEb	Moose Jaw	09-19-12	14	22	0.7819		
							0.1316
PIa	Regina	09-12-12	13	22	0.0523		
PIb	Regina	09-12-12	14	22	0.3980		
							0.9115
FEa	Regina	09-12-12	13	22	0.6290		
FEb	Regina	09-12-12	14	22	0.8971		
							0.4667
PIa	Regina	11-07-12	13	22	0.3260		
PIb	Regina	11-07-12	14	22	0.8994		
							0.7635
FEa	Regina	11-07-12	13	22	0.4438		
FEb	Regina	11-07-12	14	22	0.3916		
							0.6851
PIa	Regina	12-04-12	4	22	n too small		
PIb	Regina	12-04-12	5	22	n too small		
						0.6825	
FEa	Regina	12-04-12	4	22	n too small		
FEb	Regina	12-04-12	5	22	n too small		
						0.1905	

Table 13. Statistical significance tests of time lapse results for %CBR method.

Type	Plant location	Date	Num. of Samples	temp. (°C)	p value D'Agostino -Pearson	p value Mann -Whitney	p value Unpaired t-test
PIa	Moose Jaw	09-19-12	13	20	0.8056		
PIb	Moose Jaw	09-19-12	14	20	0.2008		
							0.9867
FEa	Moose Jaw	09-19-12	13	20	0.4443		
FEb	Moose Jaw	09-19-12	14	20	0.0722		
							0.6225
PIa	Moose Jaw	09-19-12	13	22	0.4324		
PIb	Moose Jaw	09-19-12	14	22	0.1690		
							0.9103
FEa	Moose Jaw	09-19-12	13	22	0.1604		
FEb	Moose Jaw	09-19-12	14	22	0.7443		
							0.7330
PIa	Moose Jaw	09-19-12	13	28	0.8855		
PIb	Moose Jaw	09-19-12	14	28	0.7391		
							0.7410
FEa	Moose Jaw	09-19-12	13	28	0.4454		
FEb	Moose Jaw	09-19-12	14	28	0.3413		
							0.6644
PIa	Regina	09-12-12	13	20	0.1375		
PIb	Regina	09-12-12	14	20	0.8217		
							0.8523
FEa	Regina	09-12-12	13	20	0.5577		
FEb	Regina	09-12-12	14	20	0.2384		
							0.2641
PIa	Regina	09-12-12	13	22	0.0731		
PIb	Regina	09-12-12	14	22	0.3192		
							0.7883
FEa	Regina	09-12-12	13	22	0.0560		
FEb	Regina	09-12-12	14	22	0.0479		
							0.4430

PIa	Regina	09-12-12	13	28	0.3320	
PIb	Regina	09-12-12	14	28	0.9481	
						0.8968
FEa	Regina	09-12-12	13	28	0.0334	
FEb	Regina	09-12-12	14	28	0.5518	
						0.2825
PIa	Regina	11-07-12	13	20	0.1568	
PIb	Regina	11-07-12	14	20	0.0029	
						0.3102
FEa	Regina	11-07-12	13	20	0.0766	
FEb	Regina	11-07-12	14	20	0.1718	
						0.5238
PIa	Regina	11-07-12	13	22	0.3974	
PIb	Regina	11-07-12	14	22	0.9535	
						0.9052
FEa	Regina	11-07-12	13	22	0.0042	
FEb	Regina	11-07-12	14	22	0.0317	
						0.4633
PIa	Regina	11-07-12	13	28	0.6266	
PIb	Regina	11-07-12	14	28	0.1003	
						0.9211
FEa	Regina	11-07-12	13	28	0.3265	
FEb	Regina	11-07-12	14	28	0.6069	
						0.1752
PIa	Regina	12-04-12	9	20	0.7614	
PIb	Regina	12-04-12	9	20	0.8356	
						0.1736
FEa	Regina	12-04-12	9	20	0.6432	
FEb	Regina	12-04-12	9	20	0.4818	
						0.8268
PIa	Regina	12-04-12	4	22	n too small	
PIb	Regina	12-04-12	5	22	n too small	
						0.1111
FEa	Regina	12-04-12	4	22	n too small	
FEb	Regina	12-04-12	5	22	n too small	

						0.7063
PIa	Regina	12-04-12	9	28	0.1459	
PIb	Regina	12-04-12	9	28	< 0.0001	
						0.5765
FEa	Regina	12-04-12	9	28	0.0204	
FEb	Regina	12-04-12	9	28	0.5869	
						0.2500

5.0 DISCUSSION

The RBOD integrated analytical protocol that was assessed in this research essentially consists of two parts: the enhanced ATP assessment, or E-tATP, to determine microbiological activity levels through cellular ATP production, and the %CBR test that uses BOD-testers to determine the time lapse required for microbial metabolic reactions to occur in the sample effluent.

E-tATP tests are designed to be conducted at a nominal room temperature of 22°C. In the analysis, it was identified that for PI samples, the E-tATP values ranged from 24 094 pg/ml to 78 694 pg/ml. As for FE samples, the range of E-tATP values was between 2 932 pg/ml and 32 420 pg/ml, which is significantly lower than the values from PI samples. This is to be expected due to the treatment activity performed by the WWTP.

On Sept. 12, however, an anomaly occurred at the Regina WWTP in which the generated E-tATP values was notably higher than the range of other E-tATP values from other tests (under 20 000 pg/ml). During this time period, the plant was undergoing a major plant failure when the sample was collected. This was likely due to certain chemical characteristics that could have affected the outcome of E-tATP tests, as indicated by an announcement by the city major ("Regina mayor apologizes", 2012). During this time period, it was reported that chemicals had been added to reduce the odor ("Smell from sewage," 2012). The unusual chemical composition within the plant during this period could have caused abnormal levels detected by the E-tATP process. The E-tATP values for FE samples collected on the subsequent dates of Nov. 7, and Dec. 4 were found to be below 20 000 pg/ml. Thus, it was concluded that the anomaly seen in test data was due to the addition of chemicals required by the plant failure.

5.1 Relationship between E-tATP and %CBR methods

The relationship between E-tATP and %CBR processes was assessed by examining mean E-tATP values and time lapse values from tests using PI and FE samples collected on Sept. 12, Nov. 7 and Dec. 4 from the Regina WWTP (Table 14). As the Regina WWTP was undergoing a treatment failure, and it was publicly identified that the operators were modifying the chemical characteristics of the final effluent, the following comparison of average TL and E-tATP values, and the subsequent exponential regression, excludes data collected on Sept. 12. The exponential regression produced a R^2 value of 0.898 (Figure 6) with the following equation:

$y = 83905e^{-0.069x}$

x: TL (hr)

y: E-tATP (pg/ml)

Table 14. Average time lapse values for %CBR method, and E-tATP values from PI and FE sample from Regina WWTP, excluding FE data from Sept. 12, 2012.

Type	Plant location	Date	Time-lapse (hr)	E-tATP (pg/ml)
PI	Regina	09-12-12	9.07	33 448.942
PI	Regina	11-07-12	3.49	60 224.819
PI	Regina	12-04-12	5.86	78 693.943
FE	Regina	11-07-12	25.29	14 758.195
FE	Regina	12-04-12	22.08	18 865.182

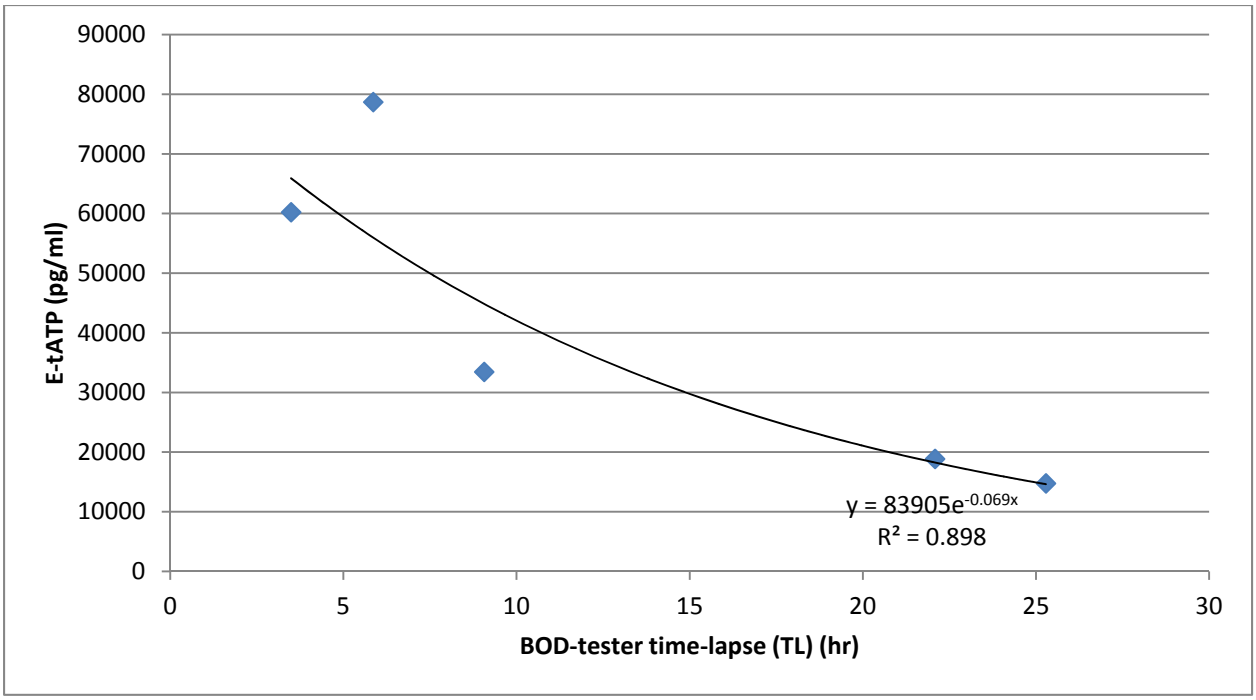

Figure 6. Exponential regression between average E-tATP and time lapse values for %CBR from Regina WWTP.

The September FE samples from the Moose Jaw WWTP and the Regina WWTP were analyzed for $CBOD_5$ at the Saskatchewan Research Council in Saskatoon, SK. The mean $CBOD_5$ values were 3.519 mg/L and 7.074 mg/L for the Moose Jaw WWTP and the Regina WWTP, respectively. They correspond to long time lapse values, indicating low levels of bacterial activity in the final effluent, as would be expected due to the treatment the wastewater underwent in the plant. These data confirm the detection ability of the %CBR method. As previously discussed, the E-tATP data from Sept. 12, where the Regina WWTP experienced a technical failure, exceed the threshold value, thus indicating the value of a secondary testing protocol that provides a more in-depth analysis of the sample when it is flagged by the E-tATP test method.

Table 15. Mean time lapse values for %CBR method, E-tATP and $CBOD_5$ values between Regina and Moose Jaw WWTPs for FE sample collected in September 2012.

Type	Plant location	Date	Mean time lapse (hr)	Mean E-tATP (pg/ml)	Mean $CBOD_5$ (mg/L)
FE	Moose Jaw	09-19-12	17.94	2 932.004	3.519
FE	Regina	09-12-12	22.47	32 420.062	7.074

5.2 Accuracy of E-tATP and %CBR methods

The second objective of this research was to assess the accuracy and precision of the E-tATP and %CBR methodologies. This was statistically evaluated through relative standard deviation values. For the E-tATP method, the average relative standard deviation of the results from FE samples from both WWTPs was 15.25%, and 9.3% for PI samples. The analysis of the %CBR results of FE samples from the Regina WWTP revealed that samples incubated at 20, 22, and 28°C resulted in average relative standard deviations of 8.2%, 5.3% and 6.6%, respectively. The results indicate that certain advantages and disadvantages exist from the perspective of plant operators. The %CBR methodology at 22°C definitely required less operational overhead and costs since no incubator or sophisticated temperature controlled units were required. However, the experiments revealed that the average mean time lapse values at 28°C was 14.14 hours compared to 23.28 hours at 22°C. This means that the %CBR method at 28°C required 39.3% less time than those at 22°C. Although the increased rapidness comes with the additional cost of incubation and temperature control requirements, the operators could benefit from time savings incurred by conducting the tests at 28°C and thus see results and respond to potential problems in less than 24 hours.

The analysis of the %CBR method on FE samples from the Moose Jaw WWTP indicated that the relative standard deviation of mean time lapse values were 6.7%, 1.7% and 2.5% at 20, 22, and 28°C respectively. Again, the greatest accuracy was achieved at the 22°C incubation temperature. The mean time lapse values were 17.43 hours, 17.93 hours, and 10.88 hours at 20, 22, and 28°C, respectively. Similar to data from the Regina WWTP, the mean time lapse at 28°C was 39.32% shorter than the average time lapse at 22°C. These data were tabulated in Table 16, and demonstrate consistency in the results between the two treatment plants. That is, the most accurate results came from incubating at 22°C while the shortest time lapse occurred at 28°C – where they were roughly 39% shorter in time for both plants, compared to 22°C. One reason why there was less relative standard deviation of mean time lapse values at 22°C could be due to the bacterial community present in the wastewater had optimal metabolic activity at that temperature, i.e. they metabolized nutrients and grow in a more consistent manner at that temperature due to their inherent mesophilic characteristics.

Table 16. Mean, standard deviations, relative standard deviation of time lapse values of FE samples from two municipal WWTPs organized by temperatures for %CBR method.

Type	Plant Location	Date	Num. of Samples	temp. (°C)	Mean (hour)	Std. dev.	Relative Std. dev.
FE	Moose Jaw	09-19-12	27	20	17.43	1.170	6.7%
FE	Moose Jaw	09-19-12	27	22	17.94	0.305	1.7%
FE	Moose Jaw	09-19-12	27	28	10.88	0.272	2.5%
FE	Regina	09-12-12	27	20	21.57	1.174	5.4%
FE	Regina	11-07-12	27	20	24.35	3.785	15.5%
FE	Regina	12-04-12	18	20	20.6	0.772	3.7%
FE	Regina	09-12-12	27	22	22.47	0.467	2.1%
FE	Regina	11-07-12	27	22	25.29	3.089	12.2%
FE	Regina	12-04-12	9	22	22.08	0.354	1.6%
FE	Regina	09-12-12	27	28	13.22	0.429	3.2%
FE	Regina	11-07-12	27	28	15.3	2.15	14.1%
FE	Regina	12-04-12	18	28	13.9	0.348	2.5%

5.3 Statistical significance of E-tATP and %CBR methods with varying incubation temperatures

The third objective of this research was to determine the effects, if any, of the three different incubation temperatures of 20, 22, and 28°C on the %CBR method. Specifically, it was hypothesized that the time lapse values of the BOD-testers would generate positive results at different incubation temperatures since temperature affects bacterial metabolic activity. The time lapse values were analyzed for statistically significant differences. The results demonstrated that there were statistically significant differences in time lapse values among the three different incubation temperatures in every single case. This confirms that the %CBR method is temperature dependent, and the operators can choose optimal temperature, such as 22°C or 28°C, based on the trade-off between the advantages and disadvantages of conducting the tests at each

temperature. Namely, to avoid higher capital and operation costs of investing in incubators and heating them to elevated temperature levels (i.e. 28°C), WWTP operators may choose the run the tests at 22°C with the foreknowledge that the lapse in time for a positive result would be expected to be longer. This may be suitable for smaller plants or where the receiving waters are located in less populated areas and have fewer anthropological and environmental uses. In this case, the time to respond to problematic effluent would be slightly increased compared to those using a 28°C incubation temperature.

5.4 Statistical significance of E-tATP and %CBR methods with varying climates

The fourth objective of this research was to assess the validity of the RBOD integrated protocol as a wastewater effluent quality indicator for varying climate effects on E-tATP values and %CBR method time lapse values. This was done through statistically analyzing the FE samples collected from the Regina WWTP in from September through December. The statistically significant differences among these data groups indicated that different times of the year do indeed have distinguishable effects on E-tATP and %CBR methods. As such, the RBOD protocol can produce unique indicator values for September, November, and December and thus could be used as a tool to monitor the wastewater effluent quality in different climates. As climate change becomes of greater concern around the world, noting the wastewater effluent quality and mitigating any potentially serious contamination levels would prove to be a valuable tool in not only protecting the environment, but in protecting human health as well.

5.5 Statistical significance of E-tATP and %CBR methods with varying WWTP treatment trains

The fifth objective of this research was to assess the methodology's ability to detect differences among different wastewater treatment plants. Due to the varying unit processes employed by the plants, it is to be expected that different plants would have differing levels of wastewater quality.

A sensitive detection method is valuable to WWTP operators as this allows them to closely monitor and compare results in order to optimize their treatment train and assists them in choosing, developing, or enhancing specific treatment steps. Statistical analysis was conducted to determine whether or not there were any significant differences in E-tATP levels and %CBR method time lapse values between the Moose Jaw and Regina WWTP FE samples in the month of September, 2012. Results showed significant differences among E-tATP values between two WWTPs. For the time lapse values in the %CBR method, the comparisons were made at the three different incubation temperatures. The statistical results showed significant differences in the time lapse values for the two WWTPs at each of the three temperatures. This indicates that unique operational attributes and treatment processes for different WWTPs have distinct effects on the quality of wastewater effluents. As such, both the E-tATP and %CBR methods can be used as a wastewater effluent quality monitoring tool that can produce unique indicators for each WWTP.

5.6 Repeatability of E-tATP and %CBR methods

The repeatability and reliability of the RBOD analytical protocol comprised of the two sub-methodologies was assessed. Statistical analysis was performed on each data group based on the test methodology: six E-tATP tests with 27 replicates each, two E-tATP tests with 9 replicates each, eighteen %CBR tests with 27 replicates each, four %CBR tests with 18 replicates each, and two %CBR tests with 9 replicates each. The results confirmed that these tests were repeatable in all cases. Thus, it is concluded that the RBOD integrated analytical protocol with E-tATP and %CBR methods is indeed a reliable and repeatable approach to assessing wastewater quality and is potentially viable for commercial application.

6.0 CONCLUSION

The RBOD integrated analytical protocol examined in this research was shown to be a robust, accurate and reliable alternative for providing indicators for wastewater effluent quality monitoring. The RBOD approach is designed with a confirmatory test for the 15-minute E-tATP test as means to provide rigor and reliability in the generated data. This supplemental test, i.e. the %CBR test, is used in cases where the E-tATP results exceed the 20 000 pg/ml threshold. No other technique uses a two-step in-depth and integrated approach such as this.

The validity of the analytical protocol was statistically confirmed. The results verified that this protocol is temperature dependent as there were statistically significant differences in time lapse values among different temperatures at 20, 22, and 28°C for %CBR method. Accordingly, the %CBR method could be used to monitor the temperature effects on wastewater effluent quality, and also be optimized using temperature as the optimizing parameter.

The effects of varying climates on wastewater quality can be detected using the RBOD method presented herein. The E-tATP and %CBR results on FE samples from the Regina WWTP collected in September, November and December, were shown to be statistically significantly different among the three different months. As such, the characteristics of the wastewater effluent changed in each month, as was expected. The protocol detected these changes and could be used to subsequently monitor the changes in the effluent based on climate effects in order to optimize the treatment and improve the discharged effluent.

Different wastewater treatment plants are expected to achieve different levels of treatment based on their unit processes, operation protocols, volume of incoming wastewater, and other anthropological and environmental activities. As such, the proposed testing method should be

able to detect these different levels of treatment through detecting the corresponding varying levels of contamination. In this research, the statistically significant differences in E-tATP values and %CBR time lapse values of the FE samples for two different municipal WWTPs indicated that the RBOD testing methodology identified the differences in treatment abilities and contamination levels. This, in turn, can provide a unique wastewater effluent quality indicator for any individual WWTP and allows detailed monitoring of the effluent as may be required.

This research validated that the RBOD testing protocol can indeed produce useful wastewater effluent indicators for rigorous monitoring within 15 minutes using the E-tATP method and within 28 hours using the %CBR methodology if high levels of contamination exceed the threshold level in the E-tATP method. Moreover, the requirement that E-tATP values exceeding 20 000 pg/ml requires the confirmatory %CBR protocol was validated on Sept. 12 from the Regina WWTP when the average E-tATP value was 32 420 pg/ml. Yet, the confirmatory %CBR tests yielded an average time lapse of 22.47 hours, which indicates low levels of active bacterial concentrations, and the $CBOD_5$ result produced an average of 7.07 mg/L. This $CBOD_5$ value meets environmental standards and is acceptable for discharge, which corroborates the %CBR findings of low bacterial contamination levels. Thus, the E-tATP method was able to flag the sample (that the $CBOD_5$ result indicated as being acceptable) as having unusual characteristics that may require further investigation, as indicated by the need for chemical addition to combat the elevated odor levels. This further investigation, i.e. the confirmatory process using the %CBR method, identified the sample as being safe to enter the receiving waters. This two-pronged approach makes this method more robust and reliable than other alternatives which rely on only one technique.

7.0 RECOMMENDATIONS FOR FUTURE RESEARCH

Analysis of the results from Sept. 12 indicated that high E-tATP values sometimes produced longer time lapse values by the %CBR method with acceptable $CBOD_5$ levels. It was speculated that differing and unusual chemical compositions found in the wastewater might be affecting the results of the E-tATP values. An examination of relationships between various chemical composition and E-tATP process would be an important advancement in the field of exploring alternative wastewater quality monitoring techniques. Additionally, the impact of unique chemical compositions on time lapse values should be further explored as industrial effluent effects on the environment are becoming of great concern.

Future research should also include a rigorous analysis of the impacts of dry and hot climates have on data generated by E-tATP and %CBR test procedures. This research clearly suggests that the results would be repeatable and reliable, but it has not been confirmed for temperatures experienced by tropical or arid regions. As well, further analysis should be done in the area of unique wastewater streams that may arise from accidental contamination. For example, the City of Moose Jaw frequently deals with increased nutrient loading when the waste stream from a meat packing plant enters the wastewater treatment plant. In this scenario, elevated levels of blood and other organic material that are rich in nutrients for bacteria will significantly impact the ability of the plant to treat the wastewater and the possible subsequent levels of bacteria and organics remaining in the final effluent. While there are an endless number of scenarios that could be researched in terms of the application of E-tATP and %CBR testing methods, examining various levels of contamination and wastewater streams would strengthen the testing

methods through applying an iterative and optimization approach in refining the methodology test procedure.

8.0 REFERENCES

APHA 1992. Standard Methods for the Examination of Water and Wastewater, 18th Ed. Amer. Public Health Assoc. Washington, D.C.

Aiello, A.E., Larson, M.S., and Sedlak, R. 2008. Hidden heroes of the health revolution. Sanitation and personal hygiene. Am J Infect Control, 36:128–151.

Alkurt, M.T., Peker, I., and Sanal, O. 2007. Assessment of repeatability and reproducibility of mental and panoramic mandibular indices on digital panoramic images. International Dental Journal. 57:433-438.

Brault, J.M., Whalen, P., and Stuart, P. 2011. Early warning signs of bulking in an activated sludge system through interpretation of ATP data in a systems analysis context. Environ. Technol. 32:1649-1660.

Canadian Council of the Ministers of the Environment. 2006. Coordinated science and research on municipal wastewater effluent (MWWE). Canadian Council of the Ministers of the Environment. http://www.ccme.ca/assets/pdf/mwwe_science_research_smry_e.pdf Accessed online 12/21/2012

Casella, S., Sciano, S., Zumbo, A., Monteverde, V., Fazio, F., and Piccione, G. 2013. Effects of seasonal variations in Mediterranean area on haematological profile in dairy cow. Comp. Clin. Pathol. 22:691-695.

Chapelle, E.W., and Levin, G.V. 1968. Use of the firefly bioluminescent reaction for rapid detection and counting of bacteria. Biochemical Medicine 2:41-52

Chifiriuc, M.C., Israil, A.M., Larion, C., Alexandru, I., and Dobre, G. 2007. Virulence and resistance markers in clinical and environmental aeromonas strains isolated in Romania. Roum. Arch. Microbiol. Immunol. 66:69-79.

City of Regina. 2004. Wastewater Treatment Plant Annual Report. Regina, SK.

City of Regina. 2013. Appendix B – Permit Limits. http://www.regina.ca/opencms/export/sites/regina.ca/residents/water-sewer/.media/pdf/appendix-a-d.pdf Accessed online 07/30/2013

Cullimore, D.R., and Alford, G.A. 1990. Method and Apparatus Producing Analytic Culture. U.S. Patent number 4,906,566

Cullimore, D.R. 2006. Preliminary Comparison of the HAB-BART system and Agar Spreadplate Methods for the Quantification of Bacterial Populations in Dilutions of Three Bacterial species Cultures. Journal of Environmental Micropaleontology, Microbiology, and Meiobenthology. 3:31-44.

Cullimore, D.R. 2013. Standard methods for the application of BART testers in environmental investigations of microbiological activities. 3rd edition. http://www.dbi.ca/books/PDFs/Standard%20Methods%20v3.pdf Accessed online on 01/03/2013

Dalzell, D.J.B., and Christofi, N. 2002. An atp luminescence method for direct toxicity assessment of pollutants impacting on the activated sewage sludge process. Water Res. 36:1493-1502.

Deininger R.A., and Lee J. 2001. Rapid determination of bacteria in drinking water using an ATP assay. Field Anal. Chem. Technol. 5:185-189.

De Anda, J., and Shear, H. 2008. Challenges facing municipal wastewater treatment in Mexico. Public Works Management & Policy. 12:590-598.

Environment Canada. 2012. Wastewater Systems Effluent Regulations. SOR/2012-139. Minister of Justice, Ottawa, ON. http://laws-lois.justice.gc.ca/PDF/SOR-2012-139.pdf. Accessed online 15/08/2013.

Ermakov, E.L. 2011. Estimation of seasonal dynamics of number and age structure of south Baikal natural population of epischura baicalensis sars using anova. Contemporary Problems of Ecology. 4:35-41.

Ghafourian, T., and Cronin, M.T.D. 2004. The endocrine disrupting activity of pesticides. Outlooks on Pest Management. 15:211-214.

Gutierrez, M., Etxebarria, J., and Fuentes, L. 2002. Evaluation of wastewater toxicity: comparative study between microtox and activated sludge oxygen uptake inhibition. Water Resour. 36:919-924.

Holeton, C., Chambers, P.A. and Grace, L. 2011. Wastewater release and its impact on Canadian waters. Can. J. Fish. Aquat.Sci. 68:1836-1859

Jia. J., Tang, M., Chen, X., Qi, L., and Dong, S. 2003. Co-immobilized microbial biosensor for bod estimation based on sol-gel derived composite material. Biosens. Bioelectron. 18:1023-1029.

Johnston L., Cullimore R, and Singh K.. 1990, Field Trials of the BOD-BART system™ for the Rapid Determination of Biochemical Oxygen Demand in Secondary and Tertiary Effluents. Droycon Bioconcepts Inc, Regina, Saskatchewan, and University of New Brunswick, Fredricton, New Brunswick.

Kale, M.M., and Mehrota, I. 2009. Rapid determination of biochemical oxygen demand. International Journal of Civil and Environmental Engineering. 1:15-22.

Kobori, H., Ham, Y.S., and Saito, T. 2009. Influence of treated sewage effluent on organic pollution assessment in the Sakai River basin in Central Japan. Environ. Monit. Assess. 151:243-249.

Larsen, O. 2008.The history of public health in the Ancient World
Int Encycl Public Health (2008), pp. 404–409

Lee, S.J., Park, J.S., Im, H.T., and Jung, H. 2008. A microfluidic ATP-bioluminescence sensor for the detection of airborne microbes. Sens. Actuators, B. 132:443-448.

Luo, J., Liu, X., Tian, Q., Yue, W., Zeng, J., Chen, G., and Cai, X. 2009. Disposable bioluminescence-based biosensor for detection of bacterial count in food. Anal. Biochem. 394:1-6.

Nguyen, M.T. 2006. The effect of temperature on the growth of the bacteria Escherichia coli dh5a. http://homepages.stmartin.edu/fac_staff/molney/website/SMU%20Bio%20Journal/Nguyen%202006.pdf Accessed online 11/04/2013.

Price, R.B., Whalen, J.M., Price, T.B., Felix, C.M., and Fahey, J. 2011. The effect of specimen temperature on the polymerization of a resin-composite. Dental Materials. 27:983-989.

Razban, B., Nelson, K.Y., Cullimore, D.R., Cullimore, J., and McMartin, D.W. 2012. Quantitative bacteriological assessment of aerobic wastewater treatment quality and plant performance. J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. 47:727-733.

Reasoner, D.J., and Geldreich, E.F. 1985. A new medium for the enumeration and subculture of bacteria from portable water. Appl. Environ. Microbiol. 49:1-7.

Regina mayor apologizes for foul smell. 2012. http://www2.macleans.ca/2012/09/13/regina-mayor-apologizes-for-foul-smell Accessed online 09/13/2012.

Saeed, F.F.M., and Ibrahim, M.A. 2012. Hydrogen production by green alga gaf99 in sea water bioreactor: II modeling the effect of temperature. Biotechnology. 11:258-262.

Sakaguchi, T., Morioka, Y., Yamasaki, M., Iwanaga, J., Beppu, K., Maeda, H., Morita, Y., and Tamiya, E. 2007. Rapid and onsite BOD sensing system using luminous bacterial cells-immobilized chip. Biosens. Bioelectron. 22:1345-1350.

Schloss, P.D., and Walker, L.P. 2000. Measurement of process performance and variability in inoculated composting reactors using anova and power analysis. Process Biochemistry. 35:931-942.

Smell from sewage lagoon lingers over Regina. 2012. http://www.cbc.ca/news/canada/saskatchewan/story/2012/09/12/sk-smelly-regina-pat-fiacco-120912.html Accessed online 09/12/2012.

Stoech, T., Duineveld, G.C.A., Kok, A., and Albers, B.P. 2000. Nucleic acids and ATP to assess microbial biomass and activity in a marine biosedimentary system. Mar. Biol. 137:1111-1123.

Tetreault, G.R., Bennett, C.J., Cheng, C, Servos, M.R., and McMaster, M.E. 2012. Reproductive and histopathological effects in wild fish inhabiting an effluent-dominated stream, Wascana Creek, SK, Canada. Aquat. Toxicol. 110:149-161.

Uludag-Demirer, S., Demirer, G.H., and Bowers, A.R. 2001. Comparison of Methods for Estimating Carbonaceous Bod Parameters. Environ. Technol. 22:915-926.

Van der Wielen, P.W., and van der Kooij, D. 2010. Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands. Water Res. 44:4860-4867.

Wiesmann, U., Choi, I.S., and Dombrowski, E. 2007. Fundamental of biological wastewater treatment. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. ISBN: 978-3-527-31219-1

World Health Organization. 2000. Global water supply and sanitation assessment 2000 report. Geneva, Switzerland: Author. http://www.who.int/docstore/water_sanitation_health/Globassessment/GlobalTOC.htm Accessed online 03/13/2008.

Yang., G.Y., Tang, T., and Dudgeon, D. 2009. Spatial and seasonal variations in benthic algal assemblages in streams in monsoonal hong kong. Hydrobiologia. 632:189-200.

Yasuda, T., Funakubo, A., and Fukui, Y. 2002. An investigation of blood damage induced by static pressure during shear-rate conditions. Artificial Organs. 26:27-31.

9.0 APPENDICES

Appendix A – List of materials required for E-tATP

1. UltraLute, ULU requires 9ml per test
2. Ultralyse 7, UL7 requires 1ml per test
3. Luminase, LU requires 100microL per test (plus 100microL for calibration)*
4. 12x55mm Polypropylene Culture Tubes requires 200microL per test
5. 17x100mm Polypropylene Culture Tubes
6. Ultracheck1, UC1 requires 100microL for calibration
7. 17mm Polypropylene Culture Tube Caps
8. Pipet tips 20 – 200µL PT1
9. Pipet tips 0.1 – 1.0ml PT1
10. Pipet tips 1.0 – 5ml PT5
11. Kikkoman C-110 Luminometer
12. 1-5mL Adjustable Micropipettor
13. 100-1000µL Adjustable Micropipettor
14. 100-200µL Adjustable Micropipettor
15. ENH- BART™ enhancer, beige flexible capped