Evaluation of the adenosine triphosphate (ATP) bioluminescence assay for monitoring effluent quality and disinfection performance

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ABSTRACT

This study investigated the use of the adenosine triphosphate (ATP) bioluminescence assay as a tool for monitoring water and wastewater quality and disinfection performance subsequent to ultraviolet (UV) irradiation and chlorine disinfection. Two different commercially available ATP assays were used in the study and controlled experiments were carried out using a pure *Escherichia coli* culture to determine how the ATP content of samples change after they are exposed to UV and chlorine. Finally, a selected assay was used with samples collected from drinking water and wastewater treatment plants to assess its potential use by treatment plants for process and effluent monitoring. The ATP assay could detect the chlorine damage to cells but the detection limit of the assay was not sensitive enough to determine the level of chlorine disinfection performance. No clear trend was observed between UV irradiation and ATP content of the cells. Samples were also collected from water and wastewater treatment plants and a good correlation was observed between the culture-based methods and the ATP assay results, which indicate the potential use of the ATP assay as a process and effluent quality monitoring tool at treatment plants.

Key words | ATP, bioluminescence, chlorine, disinfection, monitoring, ultraviolet

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INTRODUCTION

Effective monitoring of water and wastewater effluent quality and disinfection performance is critical to assure required treatment levels and for the protection of public health and environment. Traditionally, determining the microbial quality of effluent water and wastewater relies on the detection of indicator organisms using culture-based methods that require a minimum of 24 h to obtain results. There exists a real need for quicker and robust monitoring tools and use of the adenosine triphosphate (ATP) bioluminescence assay may offer a potential solution.

The ATP assay measures light produced from a luminescent reaction between ATP and a mixture of luciferin, luciferase and magnesium (Lundin & Thore 1975). Measured light is proportional to the quantity of ATP of a sample; one photon is produced for one molecule of ATP consumed (McElroy & Deluca 1983). The light output is measured by a luminometer and results can be obtained within minutes. ATP is involved in the energy transfer of all living cells and is considered a measure of cell viability. ATP is a short-lived molecule and if a cell dies ATP concentration quickly declines (Neidhart *et al.* 1990). Contrary to DNA-based assays, the ATP assay is able to provide a measure of the amount of viable micro-organisms in a water sample (Shimomura 2006). This is the main advantage of the ATP assay compared to other assays used for water monitoring. The assay is also very simple to use and requires only a small sample volume to produce results within minutes. Most tests can be performed on-site and require no sample preparation. However, the ATP assay also has limitations. The assay provides an overall assessment of the microbial quality

doi: 10.2166/wqrjc.2013.110

of a sample but it cannot distinguish between different microorganisms unless an isolation step is first performed (Lee & Deininger 2004; Bushon *et al*. 2009; Cheng *et al*. 2009). In addition, the concentration of ATP in microorganisms is variable and depends on the species, strain, and environmental and metabolic factors (Hames *et al*. 2010). Thus, the ATP assay can only be used as an approximation of the amount of live cells in a solution. To identify changes in microbial quality, baseline conditions should be established and monitored regularly. Any fluctuations from the baseline may indicate a change of microbial concentration due to possible contamination or operational problems.

The ATP assay has been investigated for use in drinking water, recreational water, and wastewater as well as for use with recreational water monitoring. Deininger & Lee (2001) used samples primarily from water utilities from across the United States to show a strong correlation between the ATP concentration of the mixed microbial population to that of a culture method, heterotrophic plate count (HPC) and two molecular methods, direct viable count and acridine orange direct count. Immunomagnetic separation in combination with the ATP bioluminescence assay has also been suggested as a method to isolate a specific microorganism in untreated wastewater (Bushon *et al*. 2009) and beach water (Lee & Deininger 2004). The assay has also shown promise for use within a water distribution network (Delahaye *et al*. 2003) and for use as part of a contaminant warning system for drinking water (Ghazali *et al*. 2010).

The main objective of this study was to evaluate the use of the ATP assay for monitoring water and wastewater effluent quality and to determine whether the assay can provide information on disinfection performance after ultraviolet (UV) and chlorine disinfection. Two different commercially available ATP assays were used in the study and controlled experiments were carried out using a pure *Escherichia coli* (*E. coli*) culture to determine how the ATP contents of samples change after they are exposed to UV irradiation and chlorine. Finally, the assay was used with samples collected from drinking water and wastewater treatment plants to assess its potential use by treatment plants for process and effluent monitoring.

MATERIALS AND METHODS

Escherichia coli culture

Escherichia coli strain ATCC®23631™ (American Type Culture Collection) obtained from Cedarlane Laboratories (Burlington, ON) was used for the experiments. Each new batch of culture was made by reviving a frozen *E. coli* stock overnight at 37 °C in 100 mL of Bacto™ Tryptic Soy Broth (BD Biosciences, Mississauga, ON). The culture was subsequently incubated for 4 h at 37 °C in a ratio of 1:100. This method assured that the *E. coli* culture was between late-exponential and early-stationary growth phases.

Laboratory prepared samples containing pure-culture *E. coli*

Phosphate-buffered solution (PBS) or 0.1% (w/v) peptone water were used to prepare samples with desired number of *E. coli* cells. Chlorine and UV disinfection experiments performed in the lab were done using PBS and peptone water to which pure *E. coli* culture was added to provide a uniform and consistent experimental solution. This solution allowed for controlled experiments and to reproduce the changes observed in ATP concentrations. PBS was chosen to simulate water samples and 0.1% peptone was chosen to simulate wastewater samples due to its higher chlorine demand.

Water samples

Water samples were obtained from the Britannia Water Treatment Plant (Ottawa, ON), plant capacity of 360 ML/day. The water treatment train of the plant is as follows: coagulation with alum and sulfuric acid, flocculation with activated silica, sedimentation, sand filtration, anthracite filtration, primary disinfection with sodium hypochlorite, pH correction, secondary disinfection with chloramine, and fluoridation. Water samples were taken from four points along the treatment train including from sampling ports that are regularly used for compliance testing. These sampling points were (1) river water intake, (2) settling effluent, (3) filter effluent, and (4) plant effluent.

Wastewater samples

Wastewater samples were collected before and after chlorination at the Robert O. Pickard Environmental Centre (Ottawa, ON). The annual daily average flow rate of raw sewage treated is 400 ML/day and treatment follows a conventional train, which includes: coarse screening, fine screening, grit removal, primary sedimentation, activated sludge, secondary sedimentation and disinfection with sodium hypochlorite. Samples were taken before and after disinfection.

ATP bioluminescence assays

Two commercially available ATP bioluminescence test kits were used to quantify cellular and extracellular ATP. The Quench-Gone Aqueous (QGA) test kit (LuminUltra Technologies, Fredericton, NB, Canada) was employed with the Lumitester C-110 luminometer (Kikkoman, Japan). For the duration of testing, manufacturer's protocols were followed (LuminUltra 2010). In brief, a sample was filtered through a 0.7 μm syringe filter to collect cells and eliminate extracellular ATP. A lysing solution was then added to the syringe, ATP was subsequently released from cells and the filtrate containing ATP collected. Equal volumes of filtrate and a luciferase enzyme mixture called Luminase were then gently mixed and light emitted by this reaction was measured with a luminometer. In some experiments, extra strength XXL Luminase was used to improve the sensitivity of the assay as explained in the 'Results and discussion' section. Reagents used to complete the tests were all supplied as part of the QGA assay test kit. According to the information provided by the LuminUltra Technologies personnel, the detection limit of the assay is 0.1 pg ATP/mL or 100 active cells/mL based on a conversion factor of 1 fg ATP/cell.

ENLITEN ATP Assay System (Promega, Madison, WI, USA) was used with the GlowMax 20/20 Luminometer (Promega 2009). The assay procedures were customized for the application based on the literature (Webster *et al*. 1980; Nichols *et al*. 1981; Schram & Weyens-van Witzenburg 1989; Luo & Luo 2006), suggestions received from the manufacturer and using the QGA test procedures as a template to maintain similar procedures. This test kit was designed to measure the total ATP of a sample. To measure cellular ATP, the extracellular ATP first needed to be isolated. To do so, a syringe filter was used to capture cells and eliminate extracellular ATP. A solution of 0.5% trichloroacetic acid (TCA) (Fisher Chemicals, Fair Lawn, NJ, USA) was added to lyse collected cells and 1X tris-acetate buffer (TAE) (Promega, Madison, WI, USA) was added to maintain neutral pH. Equal volumes of filtrate and luciferase enzyme mixture were gently mixed and light produced was measured by a luminometer. The detection limits reported by Promega for a similar ATP assay (BacTiter-Glo) for *E. coli*, *S. aureus*, *P. aeruginosa* and *B. cereus* are approximately 40, 150, 70 and 10 cells, respectively, for a sample size of 100 μL (Promega 2011).

Luminescence produced with the ATP assay is measured in relative light units (RLU). To standardize measurements, solutions of known ATP concentrations were used to determine corresponding quantities of light produced with a given test kit and luminometer. Reagents that contain the enzyme luciferase degrade with time, and calibration was performed daily to assure accurate measurement. Each ATP assay producer recommends a different method for standardization, which was followed for the duration of testing. A one-point calibration was recommended for the QGA assay while a minimum of a three point calibration curve was recommended for the ENLITEN assay. Due to the differences in the calibration methods and non-linear relationship between the results of the ATP assay at lower bacteria densities, it was found that calculating the ATP concentrations (e.g., fg/mL) may sometimes skew the data. Therefore, reporting results in RLU values provided the most useful information and ATP test results were reported in RLU in this study.

Culture-based enumeration methods

Membrane filtration was selected as the culture-based enumeration technique for *E. coli*, heterotrophic, and coliform bacteria, which offered a basis of comparison with the ATP assay. Wastewater samples were collected from the Robert O. Pickard Environmental Centre (Ottawa, ON) and total coliform bacteria were quantified using the *Standard Total Coliform Membrane Filter Procedure* (Protocol

9222 B) and m-Endo agar-LES (American Public Health Association (APHA) *et al.* 2005). Water samples were collected from the Britannia Water Treatment Plant (Ottawa, ON), and heterotrophic bacteria were quantified using the HPC (Protocol 9215) method (APHA *et al.* 2005) with R2A agar.

Chlorine and UV disinfection

Chlorination experiments employed laboratory grade 5.65–6% (w/v) SS290 hypochlorite solution (Fisher Scientific, New Jersey, NJ). A stock solution of 1 g/L Cl_2 was prepared and administered to achieve desired concentrations. Total chlorine was measured with a Hach DR2800 Spectrophotometer (Mississauga, ON) following Method 8167 using DPD (*N*,*N*-diethyl-*p*-phenyldiamine) total chlorine reagent. Chlorinated solutions were quenched with sodium bisulphate (Sigma Aldrich, MO).

UV irradiation experiments followed procedures outlined by Bolton & Linden (2003). In brief, a bench-scale collimated beam apparatus was used with four low-pressure mercury lamps (Phillips UV-C germicidal lamps, TUV 15W/G15 T8) emitting monochromatic UV light at 253.7 nm. The collimation of light was achieved by successively placed baffles along the UV light path. The irradiation stage was a magnetic stir plate (200 Mini Stirrer by VWR Scientific Products, West Chester, PA) and 20 mL samples were irradiated in a petri dish while being stirred on the plate. The incident UV intensity was measured by a radiometer (IL 1400, International Light, MA) before samples were exposed to UV light.

Statistical analysis

Microsoft Excel (San Leandro, CA) was used to perform simple statistical analysis. One-way analysis of variance (ANOVA) and post-hoc Tukey tests were performed with XLSTAT (Addinsoft, New York, NY). All statistical analysis maintained a significance level (α) of 0.05. If three or more replicates were performed, data points represent the average and error bars the corresponding standard deviation. If two replicates were performed, data points represent the average and vertical bars represent the positive and negative deviations from the mean.

RESULTS AND DISCUSSION

Comparison of ATP and membrane filtration

Using a serial dilution of an initial PBS sample containing 10^{10} CFU/mL *E. coli* bacteria, several samples with low to high *E. coli* densities were prepared. *E. coli* densities were quantified using the Quench-Gone Aqueous (QGA) and ENLITEN ATP assays as well as membrane filtration. CFU results obtained from membrane filtration were compared to RLU results obtained from ATP assays (Figure 1). A linear relationship between ATP and membrane filtration results was observed when the initial bacteria densities were above 10^6 CFU/mL. At lower bacteria densities, however, a deviation from the linear relationship was visible due to the lower detection limits of the tests.

For the ENLITEN assay, statistical analysis confirmed that there was not a significant difference between the light measured for $10^{5.5}$, $10^{4.5}$ and $10^{3.5}$ CFU/mL ($p = 1.00$, $p = 0.64$ and $p = 0.99$, respectively). Therefore, the detection limit for the ENLITEN assay was considered to be at or above $10^{5.5}$ CFU/mL under the conditions in which the test was performed. For the QGA assay, sample size was increased from 5 to 10 mL to increase the sensitivity of the assay. While all data points were significantly different from water, data points of 10^3 CFU/mL and below were not significantly different from each other. This indicated that the

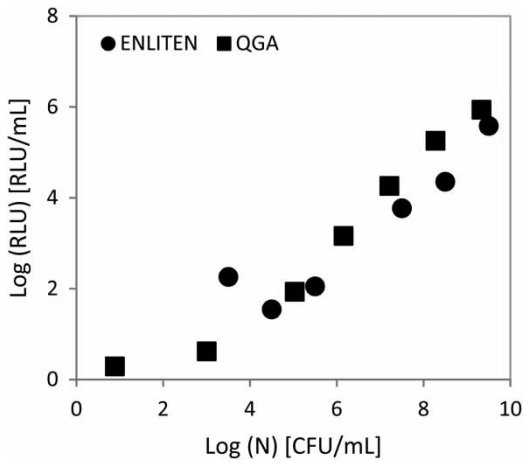

Figure 1 | Measured luminescence, using ENLITEN and QGA ATP assays, of a sample dilution series containing *E. coli* and corresponding cell densities measured with membrane filtration. Sample size used for ENLITEN was 50 mL and for QGA 10 mL.

detection limit for the QGA assay was at or above 10^3 CFU/mL when the sample size was doubled and under the conditions in which the test was performed.

Disinfection with chlorine

Chlorine is highly reactive and oxidizes cellular structures which can ultimately lead to cell death. If the cell membrane is ruptured, ATP would be oxidized. Therefore, ATP of the live cells can be measured and potentially used as a monitoring tool for chlorine disinfection.

For the chlorination experiments, initial samples consisted of *E. coli* bacteria suspended in 0.1% peptone. Peptone has a significant chlorine demand and was chosen to simulate wastewater (Allen & Brooks 1949). Disinfection experiments were performed using a stock solution of 1 g/L of sodium hypochlorite. Three initial chlorine concentrations of 1, 6 and 11 mg/L Cl_2 were investigated at five contact times of 0, 5, 15, 30 and 45 min. The QGA ATP assay was used with 5.0 mL samples and regular strength Luminase. Figure 2 shows the results of the membrane filtration and QGA assay for the chlorine concentration of 11 mg/L Cl_2 (similar results were obtained from other chlorine concentrations and are not shown due to space limitation). As expected, *E. coli* densities decreased with increased contact time and a 5-log disinfection was achieved after 45 min (Figure 2(a)). A decrease in the ATP content was also observed with increased contact time; however, the decrease in ATP was not as sharp as the decrease in membrane filtration results (Figure 2(b)). This was likely caused by the detection limit of the ATP assay, and the assay was not sensitive enough to detect *E. coli* densities less than 10^3–10^4 CFU/mL.

Chlorination experiments were repeated using PBS to simulate water samples. Based on the knowledge obtained from Figure 2, it was necessary to increase the sensitivity of the assay. The QGA assay was selected as the ATP testing assay and the sample volume filtered was increased to 100 mL. An extra strength ATP reagent, called XXL Luminase, was also used. In comparison to Luminase, XXL Luminase contained concentrated luciferase-luciferin. Lower concentrations of chlorine and shorter contact times were chosen this time in order to be able to observe a gradual drop in *E. coli* counts. Initial chlorine concentrations were 0.9, 0.6 and 0.4 mg/L Cl_2 with contact times of 0, 1, 5, 10 and 15 min. Figure 3 shows the results of membrane filtration and ATP assay for 0.4 mg/L Cl_2 (similar results were obtained from other chlorine concentrations and are not shown due to space limitation). The quantities of ATP and *E. coli* decreased with increased contact time. A 4-log reduction in *E. coli* was quickly achieved even at 0.4 mg/L Cl_2 since PBS did not exert any chlorine demand (Figure 3(a)). Steps taken to increase the sensitivity of the ATP assay appeared to have worked since lower quantities of ATP could be measured (<2 log RLU) and a gradual decrease in ATP results was observed (Figure 3(b)). However, the total decrease in the measured ATP quantities was approximately 1 log, which did not correspond well with the 4–5 log decrease in *E. coli* counts. The results indicated that the ATP assay could detect the chlorine damage to cells but the detection limit of the assay was not sensitive enough for a reliable evaluation of disinfection

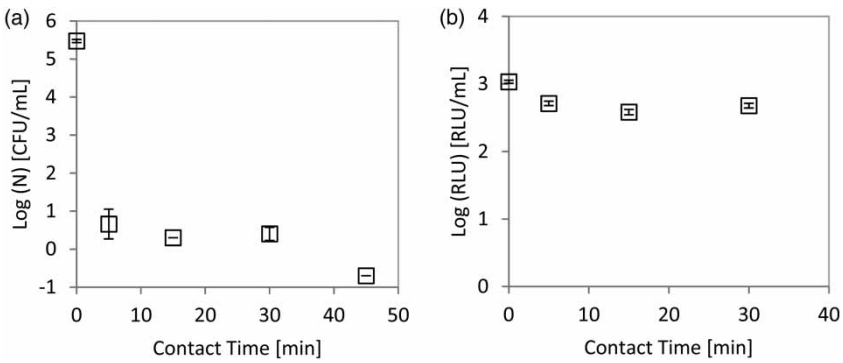

Figure 2 | (a) Results of membrane filtration of a sample with initial *E. coli* density of 3.0×10^5 CFU/mL and exposed to an initial chlorine concentration of 11.00 mg/L Cl_2 for up to 45 min. (b) Corresponding ATP assay results. Data points are the average of three replicates.

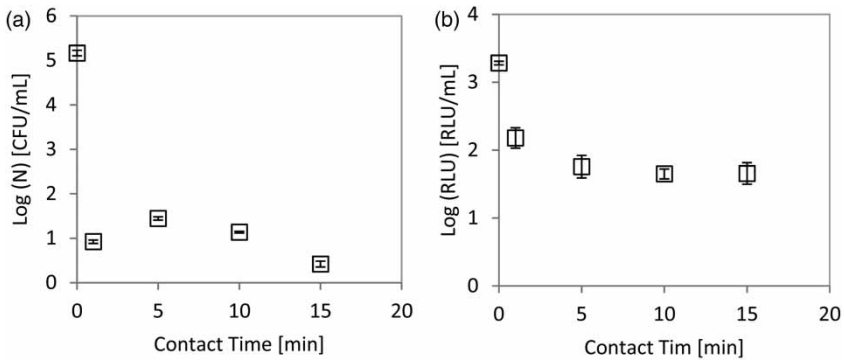

Figure 3 | (a) Results of membrane filtration of a sample with initial bacteria concentration of 1.5×10^5 CFU/mL and exposed to an initial chlorine concentration of 0.4 mg/L Cl_2 for 15 min. (b) Corresponding ATP assay results using XXL Luminase and 100 mL volumes of a sample. Data points are the average of three replicates.

performance. Higher chlorine concentrations and longer contact times were also tested and similar drops and final values in RLU were observed, which suggested that the viable but non-culturable (VBNC) bacteria did not significantly change the RLU values.

Disinfection with UV light

UV inactivation works by damaging the nucleic acid of cells and preventing their replication. If a cell is unable to replicate, it is unable to cause infection (Environmental Protection Agency (EPA) 2006). Unlike chlorine, UV light does not oxidize cell components such as cell membrane or cellular ATP; however, it may affect the ATP activity in cells. There is very little research available on the effect of UV light on ATP, and one study reported a continuous increase of both the level and rate of ATP production in a $RecA^+$ strain of *E. coli* after exposure to doses of UV irradiation up to 40 Jm^{-2} (Villaverde *et al.* 1986). The goal of these experiments was to determine whether UV irradiation causes significant changes in ATP content and if the ATP assay may be used as a monitoring tool for UV disinfection of water.

Figure 4 presents the results from UV disinfection experiments where the UV dose was increased in increments of 20 mJ/cm^2 in the dose range of 0–100 mJ/cm^2. The data show the UV-dose response curves established with membrane filtration and QGA ATP assay using XXL Luminase. The initial *E. coli* density of the samples was 1.5×10^5 CFU/mL. As expected, *E. coli* densities decreased with increasing UV dose, and a 4-log reduction was achieved in the first 20 mJ/cm^2 (Figure 4(a)). However, no clear trend was observed in corresponding ATP results. An ANOVA test determined a significant difference between the means of the samples measured at different UV doses ($p = 0.039$). A post-ANOVA Tukey test found that ATP quantities from doses of 20 and 60 mJ/cm^2 were significantly different from

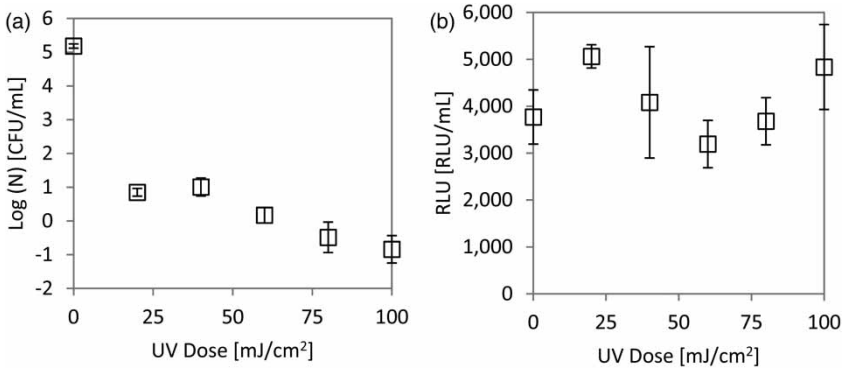

Figure 4 | (a) UV dose-response curve of initial solution of 1.5×10^5 CFU/mL as measured by membrane filtration. (b) Corresponding ATP results using QGA ATP assay with XXL Luminase. Data points are the average of three replicates.

each other ($p = 0.047$). All other pairwise comparisons of data points were not significantly different. The results and the statistical analysis suggested that UV irradiation does not cause a consistent increase or decrease in the ATP content of the cells to allow its use as a monitoring tool. No clear trend was observed. At 40 mJ/cm^2, which is the most commonly used UV dose for disinfection, the ATP quantity of the sample was in the same range as the ATP quantity of the initial sample that was not exposed to UV.

To confirm these results, experiments were repeated using the ENLITEN ATP assay (Figure 5). The starting *E. coli* densities were increased to 5.4×10^{12} CFU/mL to minimize the concerns about the detection limit of the assay and to be able to measure the remaining *E. coli* densities after disinfection. In addition, the UV dose was increased in 10 mJ/cm^2 increments up to 50 mJ/cm^2 to focus on the UV doses that are most relevant for disinfection applications. Figure 5(a) presents the results of the membrane filtrations where a more gradual decrease in *E. coli* densities can be observed due to the higher initial *E. coli* content of the samples. Figure 5(b) presents the results of the ATP assay and again no clear trend was observed between the cellular ATP and UV dose. An ANOVA test determined there was a significant difference between the means of the samples measured at different UV doses ($p = 0.012$). To further investigate where the differences in the data existed, a post-ANOVA Tukey test was performed and determined that results from Dose 10 and Dose 20 and results from Dose 10 and Dose 40 were the only points that were significantly different from each other ($p = 0.013$ and $p = 0.017$, respectively). All other pairwise comparisons of data points revealed no significant differences. The results from the second ATP assay and statistical analysis also indicated that UV irradiation does not cause a consistent change in the ATP content of the cells to allow its use as a monitoring tool. The ATP trends observed in Figures 4(b) and 5(b) appeared to be similar and an initial increase in ATP quantities were observed supporting the results reported by Villaverde *et al.* (1986). A future study is suggested to better understand the effects of UV irradiation on cellular activity and ATP.

Experimental work presented above utilized monobacterial solutions at a consistent physiological state to assess the performance and limitations of the ATP assay in controlled conditions. However, natural water and wastewater are complex matrices of microorganisms (e.g., bacteria, protozoa, fungus, and algae) that can contribute to the overall ATP content of the samples and would be detected by the ATP assay. In addition, water and wastewater samples include various organic and inorganic compounds, suspended particles, and ions, as well as residual chemicals added during treatment which may interfere with the luminescent reaction of the ATP assay. The final stage of this study aimed to determine whether the ATP assay could be used at water and wastewater treatment plants for the monitoring of treatment processes and effluent quality.

Use of the ATP assay within water and wastewater treatment plants

Samples were collected throughout a drinking water treatment plant (river water, after settling, after filtration and final plant effluent). Heterotrophic bacteria were quantified

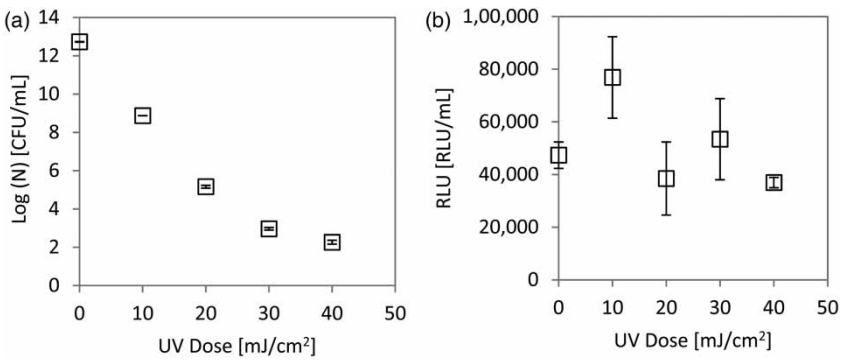

Figure 5 | (a) UV-dose response curve of initial solution of 5.4×10^{12} CFU/mL as measured by membrane filtration. (b) Corresponding ATP results using ENLITEN ATP assay. Data points are the average of three replicates.

using the heterotrophic plate count method and ATP contents of the samples were measured using the QGA assay (Figure 6). With increasing level of treatment, a step-wise decrease was observed both in heterotrophic bacteria counts and in ATP quantities, measured with the QGA assay (Figures 6(a) and 6(b)).

For the final experiments, secondary effluent (before chlorination) and plant effluent (after chlorination) wastewater samples were collected from a wastewater treatment plant and total coliform bacteria and ATP quantities were measured. Results of the membrane filtration and the QGA ATP assay are presented in Figure 7 and show a similar trend demonstrating a reduction of coliform density and sample luminescence after disinfection. A decrease in coliform densities of 99.99% CFU/mL and a reduction of luminescence of 83.99% RLU/mL were observed. Due to the mixed microbial population and other constituents in wastewater, wastewater samples that contained no coliform colonies still maintained a luminescence.

In water and wastewater treatment, faecal indicator organisms are typically used to assure that treatment has been effective against pathogenic microorganisms. The ATP assay provides an aggregate measurement of the quantity of ATP and cannot provide information on a particular type of organism. To overcome this limitation, some researchers have combined the use of the assay with immunomagnetic beads coated with antibodies specific to an organism of interest (Lee & Deininger 2004; Bushon *et al*. 2009). The beads are able to bind to the selected organism, then removed from the sample, and then concentrated for measurement with the ATP assay. However, this approach reduces the two greatest advantages of the assay: (1) the ease with which it is performed; and (2) the speed in which results are obtained. Results from Figures 6 and 7 demonstrate a different application for the assay. When used at critical points along a water and wastewater treatment train the results of the ATP assay follow a similar trend to those of culture methods. If incorporated into the regular testing regime at a treatment plant, a spike in the quantity of ATP compared to the average or baseline ATP measured under similar conditions could point operators to a contamination event or to a treatment process that is malfunctioning. In either case, further investigations could be triggered and the treatment could be adjusted accordingly in near real-time (approximately 5–10 min). This would not be possible with culture-based

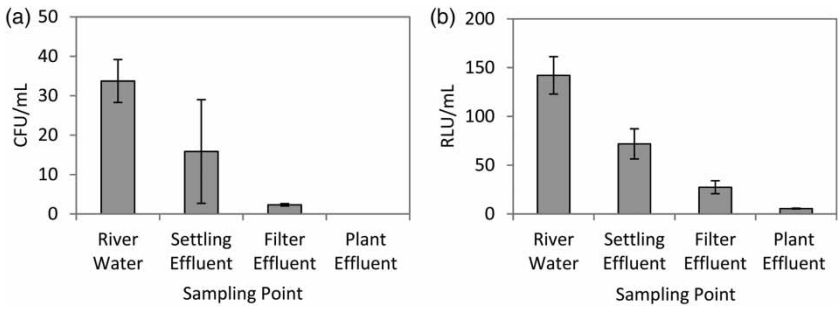

Figure 6 | (a) Quantity of heterotrophic bacteria. (b) Quantity of ATP measured using the QGA assay and regular strength Luminase. Data points are the average of three replicates.

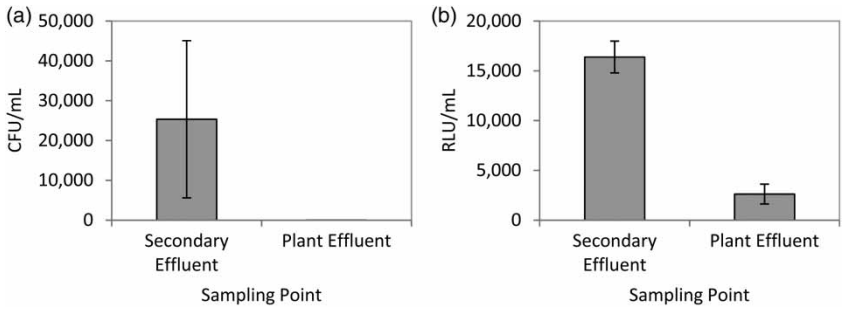

Figure 7 | (a) Quantity of total coliform bacteria. (b) Quantity of ATP measured using the QGA assay and regular strength Luminase. Data points are the average of three replicates.

methods. It should also be noted that the relatively high detection limit ($>10^3$–10^4/mL) and lack of specificity of the assay restricts the use of the assay from replacing culture methods to assure the safety of drinking water and wastewater.

CONCLUSIONS

This study evaluated the use of the ATP assay for monitoring water and wastewater effluent quality and disinfection performance after UV and chlorine disinfection. The ATP assay could detect the chlorine damage to cells but the detection limit of the assay was not sensitive enough to determine the level of chlorine disinfection performance. No clear trend was observed between UV irradiation and ATP content of the cells. Samples were also collected from water and wastewater treatment plants and a good correlation was observed between the culture-based methods and the ATP assay results, which indicated the potential use of the ATP assay as a process and effluent quality monitoring tool at water and wastewater treatment plants.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support received from the Walkerton Clean Water Centre (WCWC) in Ontario, Canada to carry out this work.

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First received 10 February 2013; accepted in revised form 25 October 2013. Available online 27 November 2013