



## Monitoring of drinking water quality using automated ATP quantification

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### ABSTRACT

A microfluidic based system was developed for automated online method for the rapid detection and monitoring of drinking water contamination utilising microbial Adenosine-5'-Triphosphate (ATP) as a bacterial indicator. The system comprises a polymethyl methacrylate based microfluidic cartridge inserted into an enclosure incorporating the functions of fluid storage and delivery, lysis steps and real-time detection. Design, integration and operation of the resulting automated system are reported, including the lysis method, the design of the mixing circuit, the choices of flow rate, temperature and reagent amount. Calibration curves of both total and free ATP were demonstrated to be highly linear over a range from 2.5–5000 pg/mL with the limit of detection being lower than 2.5 pg/mL of total ATP. The system was trialled in a lab study with different types of water, with lysis efficiency being found to be strongly dependent upon water type. Further development is required before online implementation.

### 1. Introduction

Monitoring the quality of drinking water confirms the success of treatment systems, and the integrity of distribution networks, in the delivery of pathogen-free water. Most of the current testing of faecal indicators undertaken by water utilities is lab-based, time consuming and requires culturing, preventing therefore real-time warning of presence of pathogens. To combat these limitations, development of online methods has accelerated, with commercially available systems (Storey et al., 2011) as well as trial set-ups being adopted in water systems worldwide for source water surveillance (Tryland et al., 2015) and distribution network quality control (Delahaye et al., 2003). Microbial water quality monitoring systems based on optical, electrical or molecular methods of detection have all been developed, though not all have suitability for online or early-warning approaches (Bridle et al., 2014). For early-warning systems a variety of approaches have been tested ranging from the utilisation of online measurements of conductivity and/or turbidity as a proxy (Banna et al., 2014) to automated culture based systems, online flow cytometry platforms (Buysschaert et al., 2018), particulate imaging using 3D scanning (Hojris et al., 2016), microbiome analysis (Pinto et al., 2014) and use of indicators such as

tryptophan (Simões and Dong, 2018) or adenosine-5'-triphosphate (ATP) (Vang et al., 2014). ATP was recently found to be the most accurate approach in a comparison of three different sensor types (Sherchan et al., 2018).

ATP measurements are widely used in the analysis of microbial contamination in a variety of industries (Fukuba et al., 2011), including the water industry (Delahaye et al., 2003). Total ATP in drinking water is the sum of microbial ATP (in active and viable cells) and free ATP. An ATP assay will be able to detect either total ATP if an extraction reagent is used, or free ATP only without this reagent. The microbial ATP content can therefore be calculated as the difference between these two measurements. Recent work has highlighted the utility of ATP in detecting microbial ingress in drinking water by demonstrating that the approach was more sensitive than total direct counts and more rapid than culture based methods such as heterotrophic plate counts and Colilert-18 (Vang et al., 2014). However, the system was used in a benchtop batch processing setup which is bulky and laboratory based. As a proven fast and reliable technique, online integration of ATP quantification for drinking water quality would be a very useful tool, (e.g. Applitek (<https://www.applitek.com/products/ez-atp>, n.d.)).

Microfluidics refers to the design, manufacture and testing of

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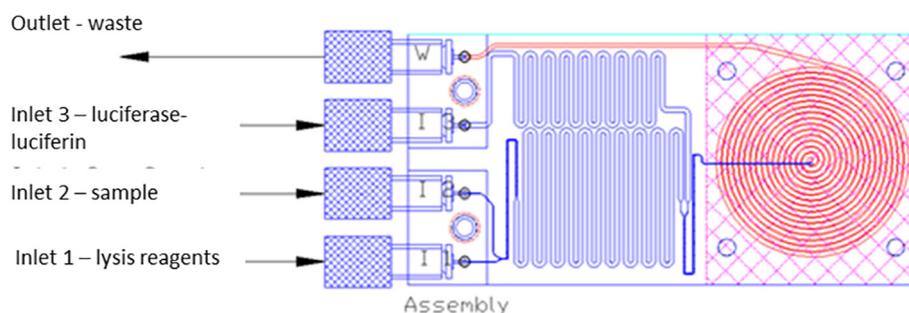


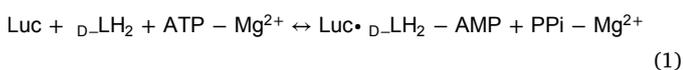
Fig. 1. Overview of the design and operation of the microfluidic system.

systems capable of manipulating and transporting tiny volumes of water with channel dimensions in the order of micrometres. Microfluidic technologies have been applied to numerous biological and biomedical challenges, including water quality monitoring (Bridle et al., 2012). The advantages of microfluidics are precise, automated control over reagent addition and mixing, an on-chip analysis and measurement approach avoiding human error or contamination and a small, portable device using small quantities of expensive reagents per measurement. Microfluidics has been utilised for ATP detection; specifically, microfluidics-based ATP devices have been reported for sub-metabolomic analysis (Liu et al., 2005), detecting airborne microorganisms (Lee et al., 2007) and for sea water monitoring (Fukuba et al., 2011; Fukuba et al., 2018). For water quality applications *E. coli* O157:H7 detection was reported by integration of the ATP assay with immunomagnetic separation (Ngamsom et al., 2017) and a paper based microfluidic system was used with *Salmonella* (Jin et al., 2015). Here, we report on the development and optimisation of a microfluidic device, integrated into a detection system, for ATP quantification for drinking water quality applications. The focus of the paper is on the design and operation of the system and the impact of lysis protocol on device performance in lab studies. Additionally, unlike in the previous systems, detection of both total and free ATP has been investigated.

## 2. Materials and methods

### 2.1. ATP detection assay

ATP is most commonly measured by a chemiluminescence assay (Luc assay). The first step of the assay is the extraction of ATP from the cells by lysis reagents (typically cold extractants) (Delahaye et al., 2003; Hammes et al., 2010; Vang et al., 2014). The extracted ATP is then quantified by a two-step reaction that uses the firefly luciferase enzyme (Luc) and its substrate luciferin ( $D\text{-LH}_2$ ). The bioluminescence is also dependent on the oxygen ( $O_2$ ) concentration and the presence of  $Mg^{2+}$  that leads to the formation of the ATP- $Mg^{2+}$  complexes as illustrated in reactions 1 and 2 (Vang, 2013).



where  $\text{Luc} \cdot D\text{-LH}_2 - \text{AMP}$  ( $D$ -luciferil-adenylate) is the enzyme-bound intermediate generated after the hydrolysis of ATP to AMP (adenosine monophosphate), and  $\text{PPi} - \text{Mg}^{2+}$  is the inorganic pyrophosphate ( $P_2O_7^{4-}$ ) bound to  $Mg^{2+}$ .

In the second reaction, the intermediate  $\text{Luc} \cdot D\text{-LH}_2 - \text{AMP}$  is oxidized and decarboxylized, emitting light whose intensity is proportional to the initial ATP content (Vang, 2013):



where  $\text{OxyLH}_2$  (oxyluciferin) is the light emitting molecule and  $h\nu$  is the energy of the emitted photons whose wavelength lies in the yellow-green spectrum. The light emission reaches its maximum intensity within a second, after which it decreases depending on the assay

conditions and the luciferase concentration (Guardigli et al., n.d.). Low luciferase concentrations give a relatively stable light emission, while high luciferase concentrations, also known as flash reagents, give light emission that lasts for a very short time. The bioluminescence reaction is particularly sensitive to pH and temperature. The optimal reaction temperature is 25 °C (Ford & Leach, n.d.) whilst the optimal pH during the assay is between 6 and 8 (Guardigli et al., n.d.) and is controlled by buffer addition. The luciferin-luciferase reagents were the LuminATE kit (92,687, Celsis).

### 2.2. Microfluidic design, microfabrication and operation

In similarity to ATP detecting microfluidic systems created by Lee (Lee et al., 2007) and Fukuba (Fukuba et al., 2011) the device is composed of a mixing zone for the various liquids, a detection area and several inlets to introduce the sample, lysis agent and detection reagents. More specifically, from left to right in Fig. 1, the sample and reagents are introduced from their storage flasks to inlet channels in the microfluidic system via semi-rigid tubing (Cheminert®). Two sequential mixing sections made of serpentine channels ensure the efficient combination of the sample with the lysis reagent (liquids from inlets 1 and 2), and of the lysed sample with the chemiluminescence reagents (output of first mixing section with liquid from inlet 3). The reagents require storage at 4 °C degrees to preserve their reactivity and shelf life. The long serpentine channels with the microfluidic device have therefore also been designed to allow time for the liquids to reach the optimal reaction temperature for the Luc reaction. Finally, the sample enters a detection zone in which the channel forms a 32 mm diameter spiral. The spiral shape ensures maximum capture of the luminescent signal from the samples. To ensure the produced light is reflected towards the detector a metallic layer of foil was placed externally below the device and opposite the spiral. Two different mixing designs were trialled and assessed via imaging (Dinolite) of the mixing of food dye.

The microfluidic system was manufactured by Epigem Ltd. using acrylic (PMMA) and epoxy. The choice of materials was based on the requirement for long term robustness, good compatibility with wet conditions in the device and good light transmission in 550–650 nm wavelength produced by the Luc reaction.

Sample and reagents were supplied to the microfluidic device inlets using a peristaltic pump (ISMATEC Reglo ICC). After calibration, the pump displayed a maximum 5% deviation from the nominal flow rate. The 0.51 mm inner diameter pumping tube used for the peristaltic pumps was made from a silicone based polymer and was linked to a 0.2 mm inner diameter and 1.59 mm outer diameter small bore analytical semi-rigid tubing (Cheminert) to minimize the volume of expensive reagents used (31  $\mu\text{L}$  per meter of tubing). Further reduction of volume was carried out using short length connections from the pumping tube to the reagents and sampling reservoirs next to the pump as well as the microfluidic inlets. Two-way pinch valves with an internal volume of 14  $\mu\text{L}$  (NI USB 6525 from National Instruments), were selected to control the flow of reagents and solution, and are electrically actuated using controllable solid state relays.

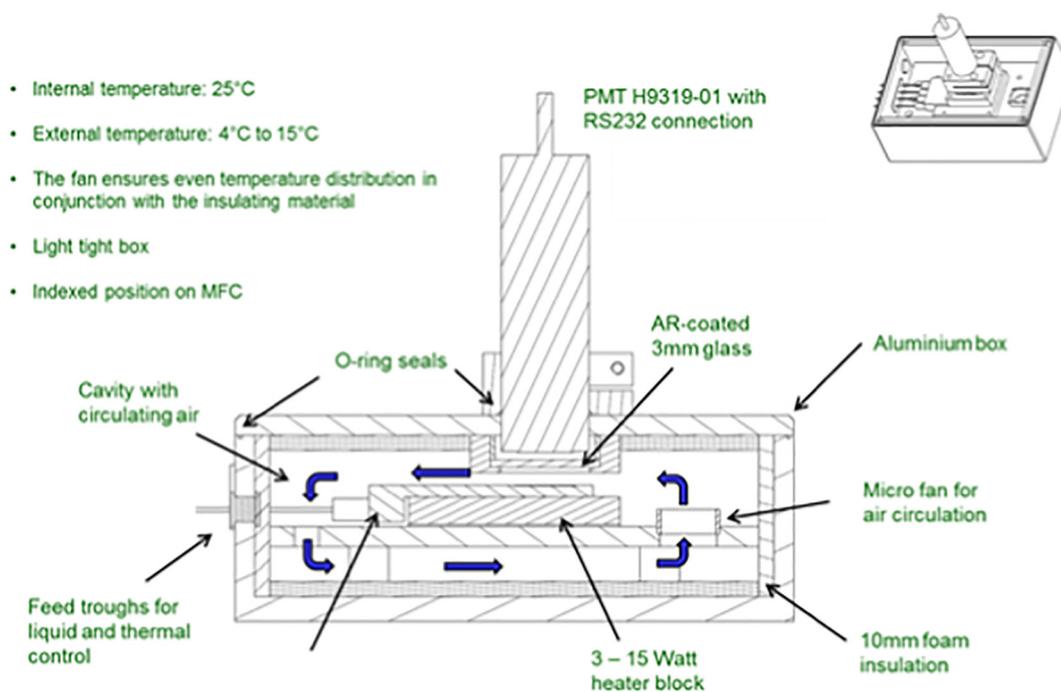


Fig. 2. Schematic of the design of the whole mixing and detection system.

### 2.3. System setup

This mixing and detection system, whose schematic is displayed in Fig. 2, is contained within a light-tight box. The box is thermally insulated and includes a temperature control system for constant temperature of 25 °C to ensure stable and optimal temperature condition for the Luc reaction. A small fan is also employed to avoid any gradient of temperature inside the box. The temperature of an aluminium plate is regulated using an electrical heater fitted on the underside of the plate. The temperature of the plate is measured by a K-type thermocouple sensor on the side of the plate and the signal is used by a PID controller to maintain the desired temperature. The precision of the regulating loop is within  $\pm 0.1$  °C, and the temperature can be set in the interval from the surrounding temperature of the detection box up to 36 °C. A 3 mm thick, 98% total light transmission, anti-reflection coated glass was inserted between the head of the Photo-Multiplier Tube (PMT) and the microfluidic device to avoid the heating up of the light sensitive coating of the detector.

A H9319-01 Hamamatsu PMT detector was selected as it offers very effective in-built amplification of low light intensity signals for easier detection, the right spectral response (550 nm–650 nm) of the photosensitive material inside the detector as well as a low level of thermal noise. The spiral readout area on the microfluidic device was made slightly larger, with a diameter of  $\varnothing$  32 mm, to ensure full use of the  $\varnothing$  22 mm diameter detector area rather than trying to minimize the volume of the spiral.

The PMT detector and the centre of the spiral are placed on the same longitudinal axis, with the PMT mounted in the lid of the assay box as shown in Fig. 2. The tubing and the electrical wires enter the box through tight rubber gaskets that prevent light from entering the detection box.

### 2.4. System evaluation

ATP standard salt (92,638, Celsis) was reconstituted in Lumin (PM) buffer (92,678, Celsis), MilliQ-water or sterile filtrated autoclaved drinking water at a concentration of  $1 \times 10^6$  pg ATP/mL and stored at 1 °C. Dilutions were then made using autoclaved, sterile filtered tap

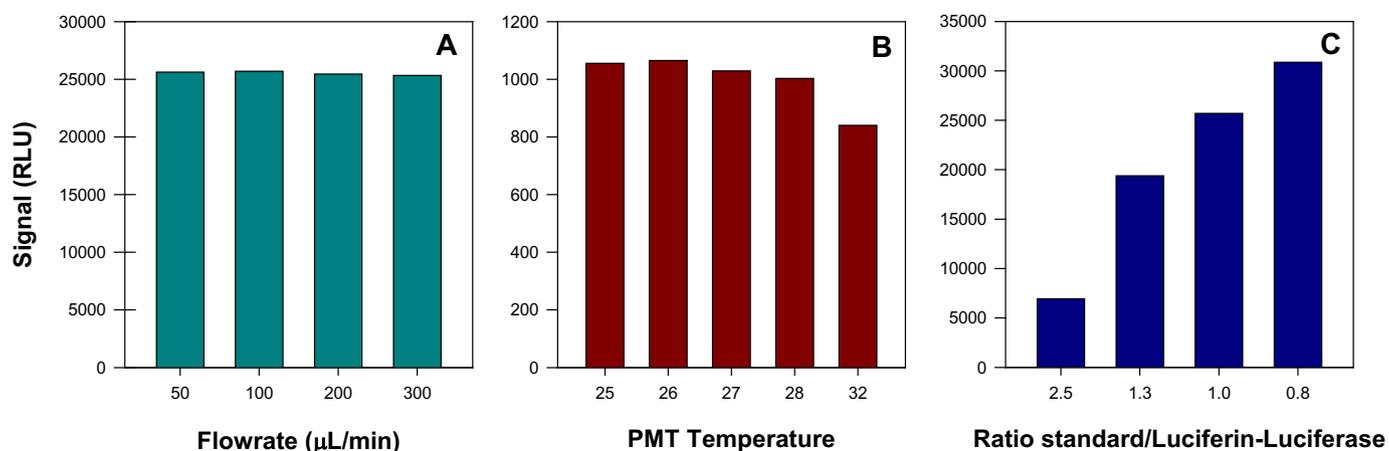
water (i.e. ATP free) for standards experiments in the range 2.5–500 pg/mL. Subsequently, the system was tested with different water types (collected at DTU). The wastewater utilised was diluted 100 times before use. The rainwater sample was collected after storage in a tank for 3 weeks. The tap water sample was taken from the lab tap during the middle of the day, and after a preliminary measurement where low levels of ATP were measured the ATP standard salt was used to spike the sample for the study evaluating different lysis approaches.

## 3. Results and discussion

### 3.1. ATP Detection and calibration

Using a 1000 pg/mL ATP standard, the impact of operating conditions on the obtained optical signal was investigated with flow rates ranging from 50  $\mu$ L/min to 300  $\mu$ L/min and temperatures between 25 °C and 32 °C. As shown in Fig. 3A, flow rates within the studied range have no impact upon the output signal whereas temperatures above 28 °C result in a small decrease in the signal with results being similar between 25 °C and 27 °C (Fig. 3B). The optimal temperature of 25 °C for the Luc reaction is also confirmed. Different ratios of sample to luciferin-luciferase reagents were also tested, as shown in Fig. 3C, to explore the possibility of reducing the amount of enzyme required for detection. Unsurprisingly, Fig. 3C shows that the greater amount of reagent is employed, the higher the obtained signal. From these three results, operating conditions of 100  $\mu$ L/min, 25 °C and a 1:1 ratio of sample/reagent were selected.

Calibration curves for the PMT detector were obtained using the optimised detection parameters described above for both total and free ATP detection as well as determination of the limit of detection. These curves are essential to convert the signals expressed in Relative Light Units (RLU) into pg/mL concentrations and to account for any possible inhibition or quenching caused by the lysis reagent. Two series of nine standards in the 2.5–500 pg/mL ATP concentration range were run to obtain the calibration curves for total and free ATP as shown in Fig. 4. Indicative peak responses of the standards and peak decrease back to the background levels after the injection of the standards are shown in the Supplementary Information (Fig. S2). The two calibration curves



**Fig. 3.** Characterisation of operational parameters for detection expressed as Relative Light Units (RLU). A) Impact of varying flow rates. B) Effect of varying temperatures in the PMT stage. C) Influence on the output signal of the luciferin-luciferase reagent for detection.

fitted well into a linear model ( $R^2 = 0.997$  for total and  $R^2 = 1$  for free ATP). The lowest standard, 2.5 pg/mL, is within the noise for the total ATP calibration curve and provides a signal to noise ratio (SNR) of 1.5:1 for the free ATP calibration curve. This result indicates that the limit of detection is better than 2.5 pg/mL, a slight improvement over the previously reported microfluidic ATP analyser (Fukuba et al., 2011). This is also significantly lower than the paper based device reported in 2015 although the paper system did not use a PMT detector (Jin et al., 2015).

### 3.2. Optimisation of the sample preparation procedure

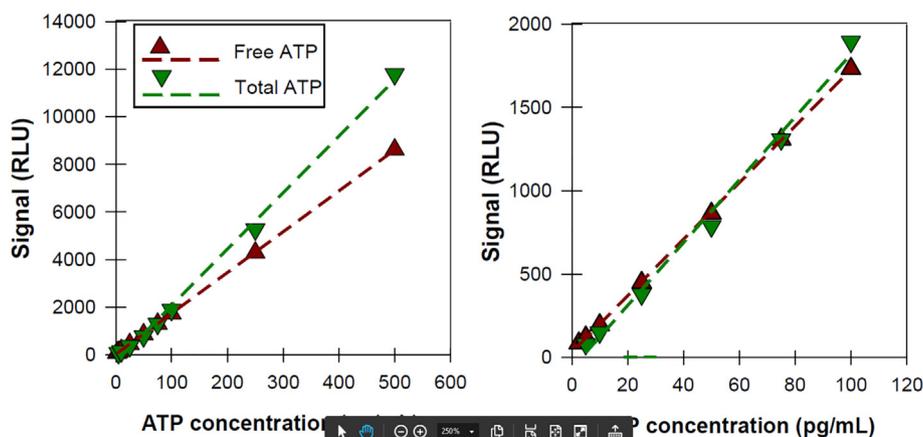
Sample processing is often the most challenging part of a monitoring solution. For our device, two critical tasks were considered: the efficacy of the mixing of the reagents with the sample and the efficiency of the lysis process.

Fluid flows are generally laminar in microfluidic devices and mixing occurs primarily by diffusion, which tends to occur over long distances. Therefore mixing can be enhanced by splitting and laminating the flow or by introducing recirculating flows, thereby effectively reducing the diffusion length (Lee et al., 2011). Two configurations of mixers were investigated as shown in Fig. 5: the serpentine mixer consisting of 161 meandering turns and the Tesla mixer (Hong et al., 2004). The mixing efficiency was investigated in the serpentine mixer by observing under a microscope the mixing of a dark liquid dye and a clear liquid as shown in Fig. 5B. From this figure, the distances  $L_1$  and  $L_2$  are defined as the

width of each of the two liquids within the channel, when it is possible to discern the boundary from imaging. The mixing efficiency can therefore be calculated using the equation  $\eta = (L_1 - L_2)/L_1$  with  $L_1$  and  $L_2$  indicated in Fig. 5B. The mixing efficiency was plotted as a function of distance from the mixer inlet for 5 different flow rates in the 10–50  $\mu\text{L}/\text{min}$  range (Fig. 5C and D). As it was not possible to distinguish the mixed and unmixed zones above 70% only the first part of the mixer structure was examined (35 mm of length for the serpentine design). The Tesla mixer design displayed an increased mixing efficiency compared with the serpentine, reaching a 70% mixing efficiency after just a 15 mm distance from the inlet at high flow rates. An additional advantage is the lower fluidic resistance in the channel (Fig. 5C and D). Note that the mixing efficiency increases for higher flow rates for both mixing designs. The mixing distance required is shorter than that reported for the seawater ATP analyser, which utilises a serpentine channel length of 133 mm for the mixing of lysis reagent and sample at the end of which the luminescence reagents are added and then allows a straight channel distance of 350 mm until the bioluminescence is measured. The mixing in a straight channel is ascribed to the asynchronous operation of their peristaltic pumps causing pulsation (Fukuba et al., 2018). Shorter mixing reduces device footprint.

### 3.3. Device performance with varying types of water

Further work was undertaken to quantify the total and free ATP in a variety of water types. The performance of two different lysis



**Fig. 4.** Calibration curves for both total and free ATP. Figure on the left shows the entire investigated range whereas the figure on the right is a magnified view of the lower end of the concentration range.

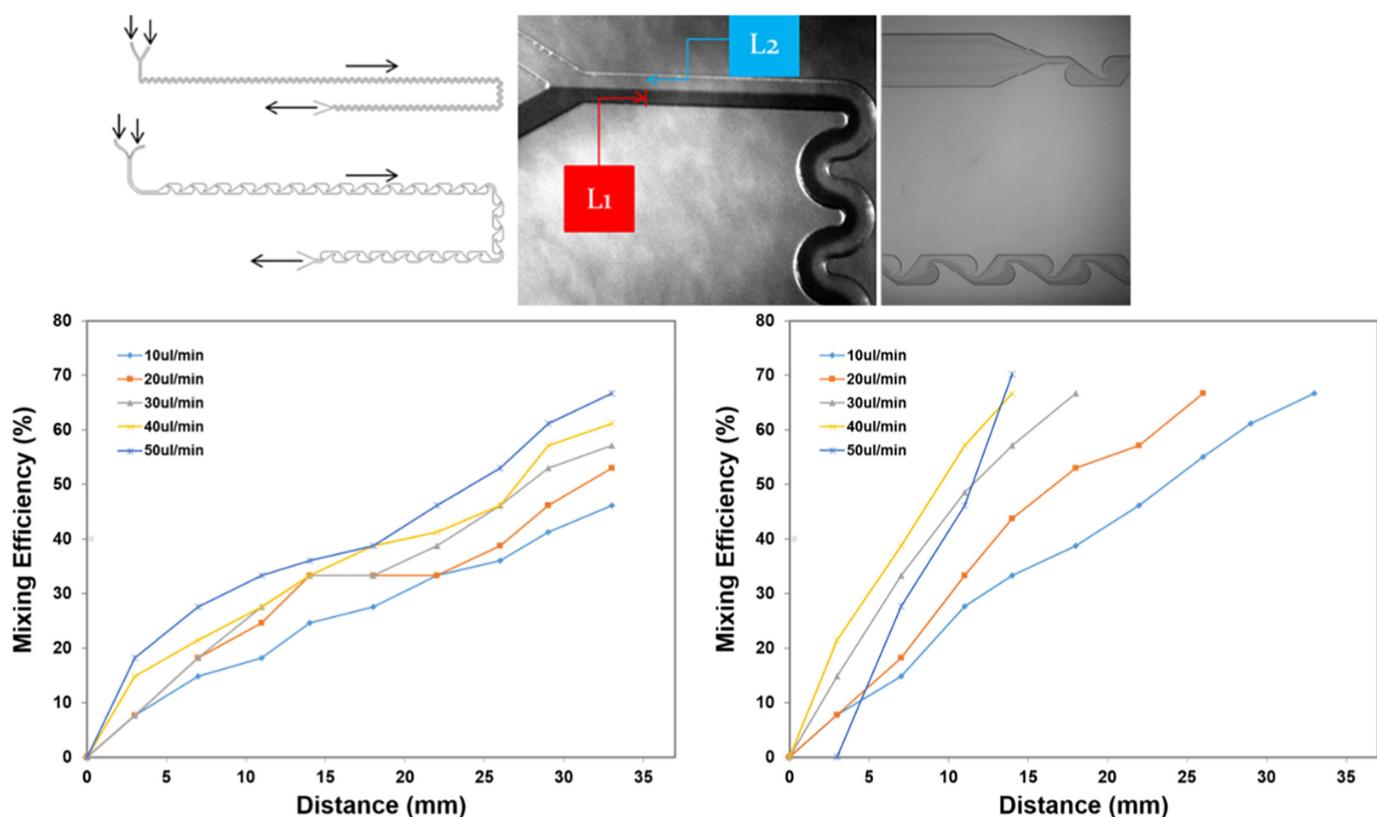


Fig. 5. A) Top left - Design of the two different mixing channel structures (top –serpentine; bottom – Tesla). B) Top right - Images from the mixing experiments. C) Bottom left - Mixing efficiency for the serpentine design. D) Bottom right - Mixing efficiency for the Tesla layout.

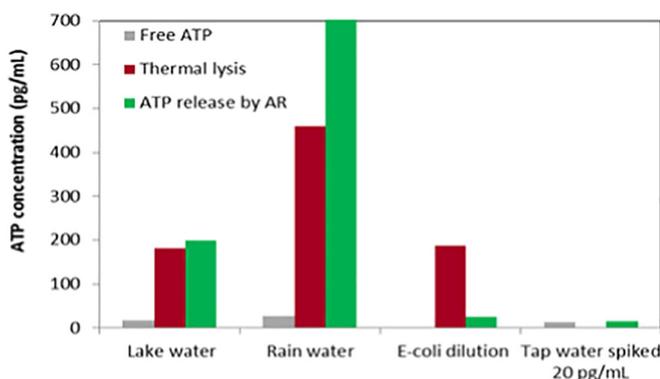


Fig. 6. ATP released from the cells by chemical and thermal lysis methods in different types of water. Free ATP is the ATP measured without any releasing step.

approaches was also carried out with these water types as shown in Fig. 6. The previously generated calibration curve was utilised to convert signal into ATP concentration, although this does not account for any impact of the water type on ATP measurement performance. While chemical lysis was shown to provide good output signals, as shown in Fig. 6, problems were observed with channel clogging due to the lysis agent interacting with the luciferin-luciferase reagents (Fig. S3). Fukuba et al employed EDTA to avoid issues of precipitation (Fukuba et al., 2018). As an alternative, thermal lysis was incorporated into the system and trialled. Preliminary data for a calibration standard of 100 pg/mL suggests that no damage or destruction of the ATP has occurred during the thermal lysis step. Moreover similar RLU as the chemical lysis experiments is demonstrated, with measurements of free (without lysis) and total (with lysis) ATP almost identical (Fig. S4). This data also indicates that thermal lysis is successful since the signal for

total ATP was higher than the signal for free ATP in the *E. coli* suspension and the tap water, meaning that the thermal lysis extracted ATP from the cells in these samples.

Free and total ATP signals were however also similar for the diluted wastewater sample (Fig. S4). This result suggests that either thermal lysis did not successfully extract ATP from the cells, or that the extracted ATP was consumed before the analysis, due to either the different water matrix or the different types of microorganisms present in each sample.

For surface water, rainwater and tap water total ATP was consistently higher than free ATP as shown in Fig. 6, suggesting the thermal lysis efficiency problem was specific to wastewater (Fig. S4) and requires further investigation in relation to the components in the wastewater. For surface (lake) water, thermal and chemical lysis were equally effective whereas the tap water sample showed variation between Fig. 6 and Fig. S4 in terms of the effectiveness of thermal lysis. However, when analysing rainwater samples, chemical lysis outperformed the thermal approach in terms of the level of total ATP measured. The rainwater ATP were high though the sample was stored in a collection tank for 3 weeks before processing, and so this data could indicate possible contamination of the storage tank. An *E. coli* dilution, included as a control, showed the opposite trend whereby thermal lysis gave a much higher detection of total ATP. This data confirms that sample preparation steps are challenging to optimise for waterborne pathogen detection and that universal approaches, for all pathogens and all water types, can be difficult to develop. Other microfluidic approaches to ATP detection have used either chemical or thermal lysis and have not compared the performance. A paper based system for *Salmonella* detection utilised boiling of the sample as the sample processing steps were undertaken external to the device; this system is cheap and disposable but not suitable for online early warning applications (Jin et al., 2015). Otherwise, the microfluidics systems reported have utilised chemical lysis. Potentially problems relating to the

interaction of the lysis reagents and particle formation were avoided, even in wastewater samples, in iFAST system due to the use of magnetic beads for specific pathogen capture and washing (Ngamsom et al., 2017).

#### 4. Conclusions

This article presented the development of a microfluidic system acting as an online early warning system for measuring microbial contamination in drinking water samples through quantification of ATP. In terms of detection, system operation with regards to flow rate, temperature and reagent amount were optimised. Highly linear calibration curves for both total and free ATP were determined, estimating the limit of detection to be better than 2.5 pg/mL; a result which is comparable to benchtop studies and one previously reported microfluidic set-up.

For the sample preparation part of the platform, mixing design was optimised with a Tesla layout shown to provide a high mixing efficiency over a short channel length with low fluidic resistance. Different lysis protocols were trialled and efficiency was shown to depend upon the type of water sample.

Overall the developed prototype system demonstrates highly sensitive rapid detection of bacterial contamination in drinking water samples. Future work should concentrate on further investigating the impact of water type on lysis efficiency and consideration of the potential to link with immunomagnetic separation to provide micro-organism specific information as well as online testing of the system within drinking water networks.

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#### Appendix A. Supplementary data

See supplementary material for further images of the system (Fig. S1), the raw data time course for calibration (Fig. S2), the clogging observed with chemical lysis (Fig. S3) and the preliminary thermal lysis data (Fig. S4). Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2019.105713>.

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